PHYLOGEOGRAPHY OF THE INDONESIAN WATER PYTHON, LIASIS MACKLOTI SSP. (SQUAMATA: BOIDAE: PYTHONINAE): A COMPARATIVE APPROACH TOWARD RESOLVING PHYLOGENY

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by

Christopher Knight Carmichael

A Dissertation
Submitted to the Graduate Studies Office
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

Approved:

August 2007

by

Christopher Knight Carmichael

Abstract of a Dissertation Submitted to the Graduate Studies Office of The University of Southern Mississippi in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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ABSTRACT

PHYLOGEOGRAPHY OF THE INDONESIAN WATER PYTHON, LIASIS MACKLOTI SSP. (SQUAMATA: BOIDAE: PYTHONINAE), OF INDONESIA’S LESSER SUNDAS ARCHIPELAGO: A COMPARATIVE APPROACH TOWARD RESOLVING PHYLOGENY

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August 2007

_Liasis mackloti_ is currently recognized as three subspecies (_L. m. sawuensis_, _L. m. dunni_ and _L. m. mackloti_) inhabiting several islands of the Outer and Inner Banda Arc of islands of Indonesia’s Lesser Sundas Archipelago. We used partial mitochondrial cytochrome _b_ sequences and morphological character states to examine and resolve the phylogenetic relationship of these three subspecies. Maximum likelihood and parsimony analysis showed that _Liasis fuscus_ is the sister lineage to the _L. mackloti_ ssp. complex. There is strong support for the recognition of three clades that are delineated by _savuensis_, _dunni_ and _mackloti_. The current subspecies taxonomic scheme accurately delineates the evolutionary distinctiveness of the various insular populations based on congruent topologies revealed by both the molecular and morphological data sets. The lineage is monophyletic, and each of the three subspecies differs from the other two both morphologically and genetically. Given the morphological and genetic distinctiveness of each taxon, we believe there is substantial empirical evidence and justification for the elevation of the three species to full species status. Morphologically, there are several characteristics that differentiate the three subspecies including the location of the heat
sensing pit on the labial scales (L. m. mackloti has heat sensing pits on labial scales 9, 10, 11 and 12, L. m. savuensis on labial scales 10, 11, 12 and 13, and L. m. dunni on 9, 10, 11, 12, and 13), number of loreal scales (L. m. savuensis and L. m. dunni have 1 while L. m. mackloti have 2), midbody scale row count (L. m. savuensis has 46-49, L. m. dunni has 52-63, and L. m. mackloti has 59-65), and number of postanal scales (L. m. savuensis has 65-76, L. m. dunni has 85-89 and L. m. mackloti has 88-93). In terms of cytochrome b mitochondrial DNA sequence data, there is the greatest sequence divergence between L. m. savuensis and L. m. mackloti (5.6%), followed by L. m. savuensis and L. m. dunni (5.2%) and L. m. mackloti and L. m. dunni (2.6%). There was no sequence divergence between the three insular populations studied of L. m. mackloti. Both molecular and morphological data sets reveal similar patterns of phylogeny. Their distribution and evolution appears to have been shaped by combined effects of dispersal and vicariance.

We conducted pheromone trailing experiments to investigate the level of geographic variation present in this behavior in the macklot's python, Liasis mackloti (Serpentes: Boidae). Three subspecies (L. m. mackloti, L. m. savuensis, and L. m. dunni) are currently recognized and are found on several of the Lesser Sundas islands of Indonesia including Sawu, Wetar, Timor, Semau and Roti. Based on prior studies, three clades have been delineated (Wetar, Sawu, and a Timor-Semau-Roti clade). The three subspecies display remarkable interpopulational (morphological, genetic and behavioral) variation but only slight intrapopulational polymorphisms. A modified Y-maze was used to test homotypic and heterotypic and male and female preferences both within and between insular populations. The results of this study of the L. mackloti complex indicate that during the breeding season male pythons were able to discriminate between
homotypic and heterotypic odors within each of the clades (P<0.05). However, male pythons from Timor, Semau and Roti were unable to differentiate pheromone trails produced by females from these three islands (P>0.05). Male *L. m. dunni* from the island of Wetar are generally longer than females and also exhibited specificity toward homotypic male trails. This homotypic male trailing behavior, in addition to several male-male combat interactions observed during the study suggests that males from Wetar may attempt to displace males for access to females. Pheromone trailing discrimination is an important pre-zygotic reproductive isolating mechanism that may have played an important role during speciation. We also present the role of dispersal and vicariance in shaping current patterns of geographic variation.

Courtship behaviors were compared between three subspecies of the Indonesian water python, *L. m. mackloti, L. m. savuensis,* and *L. m. dunni,* to elucidate patterns of geographic variation in male courtship behavior in standard laboratory conditions. The three subspecies are found on a series of island that are part of the Outer Banda Arc and Inner Banda Arc of the Lesser Sundas Archipelago. The insular populations of *L. mackloti ssp.* within this archipelago indicate that separation between islands during the Pleistocene played a role in determining current assemblages and variation within species. The islands of eastern Indonesia form biogeographic subregions that have relatively high levels of endemism and evidence of incipient speciation as a consequence of changes in sea-levels and climate during the Pleistocene. Two predominant models to explain the biogeography of *L. mackloti ssp.* include vicariance and dispersion, both of which likely played an important role in shaping the distribution pattern of these pythons that we currently observe and are discussed in this chapter. *L. mackloti* generally adheres
to the triphasic schema including tactile-chase, tactile-alignment, and intromission and coitus. However, the use of the pelvic spurs during tactile-chase and tactile-alignment is a unique boid-typical motor pattern and likely plays an important premating reproductive isolating mechanism that prevents interbreeding between conspecific individuals. Behavioral sequence chains were derived from videotaped experimental tests and subjected to transition analysis. We observed variation in both the frequency of occurrence and the sequence of the principal courtship behaviors and, when compared statistically, most of these behaviors differed between populations. We observed geographically unique island patterns in the sequence in which male courtship behaviors are displayed. Our data yielded the following information: 1) the courtship sequence in all three subspecies is not random; 2) the sequence of *L. m. savuensis* is much less complex than that of *L. m. mackloti* and *L. m. dunni*; and 3) the tactile pelvic spurring target sites used by males on the females’ dorsum is different between the three subspecies. We also determined whether sexual isolation among selected populations existed. In the first study, we performed male-female reciprocal crosses of pythons between two different insular populations and measured mating success and whether eggs were produced. The results of this test reveal that sexual isolation occurs between the three defined subspecies. The behavioral differences in regards to courtship sufficiently delineate the subspecies and no significant difference was detected when comparing the three different island populations of *L. m. mackloti* (Timor, Semau and Roti), however, they were significantly different than *L. m. savuensis* and *L. m. dunni*. The historical isolation of Sawu and Wetar Islands, and connection of Timor, Semau and Roti Islands reveals interesting biogeographic patterns and the snake fauna of islands within the
Lesser Sunda group indicate that separation between islands during the Pleistocene played a role in determining current assemblages and variation within species detected in this study.
ACKNOWLEDGMENTS

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CHAPTER I

PHYLOGEOGRAPHY OF THE INDONESIAN WATER PYTHON (SQUAMATA: BOIDAE: PYTHONINAE), LIASIS MACKLOTI, OF INDONESIA'S LESSER SUNDAS ARCHIPELAGO: DNA AND MORPHOLOGY YIELD EQUIVALENT PATTERNS

ABSTRACT

Liassis mackloti is currently recognized as three subspecies (L. m. savuensis, L. m. dunni and L. m. mackloti) inhabiting several islands of the Outer and Inner Banda Arc of islands of Indonesia’s Lesser Sundas Archipelago. We used partial mitochondrial cytochrome b sequences and morphological character states to examine and resolve the phylogenetic relationship of these three subspecies. Maximum likelihood and parsimony analysis showed that Liassis fuscus is the sister lineage to the L. mackloti ssp. complex. There is strong support for the recognition of three clades that are delineated by savuensis, dunni and mackloti. The current subspecies taxonomic scheme accurately delineates the evolutionary distinctiveness of the various insular populations based on congruent topologies revealed by both the molecular and morphological data sets. The lineage is monophyletic, and each of the three subspecies differs from the other two both morphologically and genetically. Given the morphological and genetic distinctiveness of each taxon, we believe there is substantial empirical evidence and justification for the elevation of the three species to full species status. Morphologically, there are several characteristics that differentiate the three subspecies including the location of the heat sensing pit on the labial scales (L. m. mackloti has heat sensing pits on labial scales 9, 10, 11 and 12, L. m. savuensis on labial scales 10, 11, 12 and 13, and L. m. dunni on 9, 10,
11, 12, and 13), number of loreal scales (L. m. savuensis and L. m. dunni have 1 while L. m. mackloti have 2), midbody scale row count (L. m. savuensis has 46-49, L. m. dunni has 52-63, and L. m. mackloti has 59-65), and number of postanal scales (L. m. savuensis has 65-76, L. m. dunni has 85-89 and L. m. mackloti has 88-93). In terms of cytochrome b mitochondrial DNA sequence data, there is the greatest sequence divergence between L. m. savuensis and L. m. mackloti (5.6%), followed by L. m. savuensis and L. m. dunni (5.2%) and L. m. mackloti and L. m. dunni (2.6%). There was no sequence divergence between the three insular populations studied of L. m. mackloti. Both molecular and morphological data sets reveal similar patterns of phylogeny. Their distribution and evolution appears to have been shaped by combined effects of dispersal and vicariance.

INTRODUCTION

Since Darwin's time, insular populations have played an important role in our understanding of the nature of variation and the determinants of its patterns. Empirical studies of natural populations are benefited by a simplified population structure generally observed on islands with reduced or no migration between adjacent islands, spatiotemporal sequential relationships, and small population size. These natural coincidences have allowed critical insights into our understanding of the evolutionary processes (Carson and Kaneshiro, 1976; Gorman et al., 1975; Schmitt, 1978; Peterson and Heaney, 1993) that influence patterns of geographical variation. Phylogeography, the science that is concerned with the evolutionary processes that affect geographic distributions of genealogical lineages (of closely related species), is a tool that can be used effectively to determine these patterns and the amount of biodiversity currently present (Avise, 2000).
The geological history of the Indo-Australian archipelago has been reinterpreted in the last twenty years as a consequence of new data provided by major geological surveys in the region and a better understanding of plate tectonics in the region (Hamilton, 1979; Audley-Charles, 1987). It is now known that the islands that make up Indonesia have had a complex and varied past and it is generally accepted that all the major islands were, at some time in the past, part of the Gondwana supercontinent (Burrett et al., 1991).

The Pliocene collision between the Australian, Pacific and Asian Plates resulted in major geological upheaval and produced the contemporary geographical positioning of most of the islands in the region. The major changes in the ensuing Pleistocene epoch have been those of fluctuating sea levels, changed seasonality in precipitation and increased temperatures associated with alternating glacial periods (Hall, 1998; Heaney, 1991). Sea levels were as much as 120 m lower during the last glacial maximum around 18,000 years ago (Hall, 1998).

The extensive exposure of land resulting from lowered sea levels during the Pleistocene glacial maxima resulted in the connection of many of the islands of the Indonesian archipelago (Heaney, 1991). The Greater Sunda islands of Borneo, Sumatra and Java were all linked by land as were many of the smaller islands of the Lesser Sunda archipelago (Heaney, 1991). Kitchener and Suyanto (1996) have recently shown this area to have a higher degree of mammalian endemicty than previously recognized or suspected, with over twenty-seven new taxa of mammals being described. Additionally, recent systematic examination of reptile taxa from the islands of eastern Indonesia also indicate that there is greater endemism than previously recognized (Musters, 1993; How...
et al., 1996; Harvey et al., 2000). These local patterns of endemism provide conservation managers a critically needed tool to evaluate the biogeography of well-defined and incipient species.

Conservation management requires a thorough knowledge of the amount of biodiversity present and phylogeography provides an important framework to convey critical information about species distribution, genetic variation, and the mechanisms which have evolved species, communities and ecosystems. This type of information can also clarify the biogeographic basis for interspecific and intraspecific divergences (Morrone and Crisci, 1995; Keogh et al., 2000). Inference of biogeographic process from strictly distributional data is problematical. Even when the phylogenetic relationships of taxa are well supported, it may not be possible to precisely infer the biogeographic process that produced current distributions. Dispersal and vicariance hypotheses, therefore, are not easily distinguishable.

The pythons in the genus *Liasis* Gray, 1842 have had a dynamic taxonomic history since their description in the mid-1800s (McDowell, 1975; Underwood and Stimson, 1990; Kluge, 1993). McDowell (1975) considered the many *Liasis* species to be included into two groups, the *Liasis boa* and *L. olivaceus* groups. The *L. boa* group comprised *Bothrochilus boa*, *Leiopython albertisii*, and the single species of *Anatresia* recognized at the time, *L. childreni*, none of which are any longer included in *Liasis* sensu Kluge (1993). The *L. olivaceus* group, comprised the olive python, *L. olivaceus*, the Papuan python, *L. papuana* (*Apodora papuana*), and the water pythons, *L. fuscus* and *L. mackloti*. Whereas McDowell's *Liasis* boa group appears not to be a natural lineage, the taxa of the *L. olivaceus* group appear to have some phylogenetic affinities (Kluge,
Indonesian water pythons (*L. mackloti* ssp.) are the subject of this study as they have a widespread range throughout the eastern Lesser Sunda archipelago. Water pythons are found in the Lesser Sunda islands of Indonesia, the trans-fly River region of southern New Guinea and across northern Australia from the Kimberley district of Western Australia, through the tropical region of Northern Territory and Queensland as far south as Bowen on the east coast (Appendix A) (McDowell, 1975).

The water pythons are currently recognized as one or two species. *Liasis mackloti* Dumeril & Bibron, 1844, was originally described from Timor. The Australian water python, *L. fuscus* Peters, 1874, was described from Port Clinton in Queensland, but this name has been applied also to specimens from New Guinea (e.g. de Rooij, 1917). Boulenger (1893) presented a key to separate *L. fuscus* and *L. mackloti* based on the presence/absence of a groove or pit in the rostral scale, and ventral and subcaudal scale counts. This key was determined from published records of specimens from Timor and Semau and observations of specimens from Queensland and New Guinea available in the Natural History Museum, London (McDowell, 1975). McDowell (1975) used Boulenger's key to examine the status of *L. fuscus*/*L. mackloti* by comparing two specimens from Wetar and four from New Guinea, one from the Northern Territory and three from Queensland, but could not differentiate populations, which may have been due to the limited samples available from Indonesia. McDowell (1975) could distinguish specimens from Australia-New Guinea and Wetar Island on the basis of color variation, but attributed the difference to intra-specific geographical variation. Water pythons from Wetar and Sawu Island had been classified previously as the subspecies *L. mackloti* *dunni* by Stull (1935) and *L. mackloti* *savuensis* by Brongersma (1956), respectively. Barker

The *L. mackloti* complex is an insularized group that are robust moderately thick bodied snakes attaining lengths of up to 3-4 meters and demonstrate geographic variation with regard to morphological (size, coloration, markings, scalation), behavioral (courtship and premating distance reduction behaviors), and molecular (mtDNA) characters (Carmichael et al., unpubl. data; Rawlings et al. 2004). Two subspecies of *L. mackloti* are found on the outer Banda Arc of islands, *L. m. mackloti* (Dumeril and Bibron, 1844), from the islands of Timor, Semau and Roti (referred to as ‘Greater Timor’), and *L. m. savuensis* (Brongersma, 1956), from the island of Sawu. One subspecies, *L. m. dunni* (Stull, 1932) is found on one of the inner Banda Arc of islands, Wetar (see Appendix B). Additionally, How and Kitchener (1997) discovered *L. mackloti* to exist on the island of Alor located west of Wetar, also part of the inner Banda Arc. How and Kitchener (1997) found that the snake fauna on Alor and Wetar were more similar when compared to the outer Banda Arc islands as compared to the adjacent inner Banda Arc islands to the west, suggesting that a major barrier exists between Lembata and Alor. They also detected another barrier between Sumba and Sawu on the outer Banda Arc that delineates assemblages to the east and west. The Sawu snake fauna appear to be more closely allied to the ‘Greater Timor’, Alor and Wetar islands at the level of species based on principal component analysis when comparing species/genera assemblages on the different islands (Rawlings et al., 2004).
*Liavis mackloti* are sexually dimorphic (in size and color/pattern). In the Wetar population, males are typically larger in overall size than females, while females on the islands of Timor, Roti, Semau and Sawu are typically larger than males. This intersexual morphometric difference has interesting behavioral consequences. Typically male-male combat and territoriality (a behavior that has been difficult to identify in snakes) occurs more frequently in populations of animals where the males achieve greater size, and female choice for males in populations where females are larger than males. We have found that male *L. m. dunnii* from the island of Wetar do engage in male-male combat, which has not been observed in *L. m. mackloti* and *L. m. savuensis* (Carmichael et al., 2003; Carmichael et al., 2007).

*L. mackloti* ssp. are primarily creatures of the grassy upland habitat, and despite being commonly called the “Indonesian water python,” the species is not particularly aquatic in its habitat preference. This species occurs in many habitats on the five islands studied, but it is most strongly associated with the rolling hills and open grasslands found in its range and is rarely encountered off the ground or in water.

*Liavis mackloti* ssp. exhibit a tremendous amount of interpopulational polymorphism in several morphometric conditions including head scale counts and configurations, proportionate size of pelvic spurs, overall color and pattern, color of iris, post-anal scale count, squamation, and body proportions. Like many subspecies complexes, *L. mackloti* ssp. have been recognized based on differences in color, adult body size, meristic scale traits, and geographic distributions (Brongersma, 1947; Stimson, 1969). Given the broad and fragmented distribution of *L. mackloti* ssp., there is a high potential for existence of independent evolutionary lineages that could represent separate
species (Kluge, 1993). In the work presented here, we will take a conservative view of
species boundaries and attempt to identify such lineages using a “total evidence”
approach that incorporates morphological and molecular data plus biogeographic
information and consideration of the probability of gene flow. Kluge (1993) and others
have argued convincingly that species complexes are perhaps best investigated using
phylogenetic analyses in which demes or individuals are chosen as terminal taxa, and this
approach is taken herein. Reproductive isolation is not considered in our analysis,
because (1) the data are not available and (2) reproductive isolation and cladistic
hierarchy may not be perfectly correlated (Rawlings and Donnellan, 2002).

In this paper, the phylogenetic relationships within the Liasis mackloti complex
are estimated using DNA sequence and morphological data. As well as providing a basis
for conservation and management of these large tropical predators, information on the
phylogeny of L. mackloti ssp. is of considerable interest in terms of biogeography.
Recent advances in our understanding of the geological history of this important
biogeographic region – and especially, the timing and sequence of divisions and
connections between landmasses (Hall, 1998; Metcalfe, 1999) – offer the opportunity to
interpret pythonine phylogeny in terms of vicariance and dispersal events through
evolutionary time. With these issues in mind, we gathered genetic and morphological
data to clarify phylogenetic relationships and biogeography within the L. mackloti group.
MATERIALS AND METHODS

Specimens Examined

Fifty individuals representing three subspecies of L. mackloti (L. m. mackloti, L. m. savuensis, and L. m. dunni) were used for this study and were field collected from five insular populations/islands of the Indonesian Archipelago, including Timor (L. m. mackloti; Dumeril and Bibron, 1844), Roti (L. m. mackloti), Semau (L. m. mackloti), Sawu (L. m. savuensis; Brongersma, 1956), and Wetar (L. m. dunni; Stuhl, 1932) (see Appendix B). Sequence data for Liasis fuscus, Liasis olivaceus, Leiopython albertisii, Antheresia childreni and Apodora papuana were obtained from GenBank and used as outgroups for generating a molecular phylogenetic hypothesis. Liasis fuscus was used as an outgroup to polarize our phylogenetic analysis based on morphological character states.

Molecular Data

DNA was extracted from shed skins produced by snakes maintained in captivity. Shed skins were collected from the enclosure within 1-3 days after production and digested for three hours at 65°C with occasional agitation in 900 ul of lysis buffer (TRIS HCL 100 mM at pH 8.0, EDTA 50 mM at pH 8.0, NaCl 10 mM, SDS 0.5%) containing 15 ul of Proteinase K. Once digestion was completed, an extraction procedure was conducted consisting of 5M ammonium acetate.

DNA was subsequently precipitated from the aliquot with 5M ammonium acetate and then washed in 70% ethanol, dried, and redissolved in TE buffer (Tris 10mM, EDTA 1 mM, pH 8.0) (modified from Fetzner, 1999). Oligonucleotide primers for amplification and sequencing were taken from the literature (Burbrink et al., 2000; Harvey et al., 2000)
and personal correspondence (Chippendale, pers. comm.). A 415-750 bp segment of the cytochrome b gene was successfully amplified from the shed skin samples and subsequently sequenced using the primers GLUDG (5' - TGACTTTGAARAACCAYCGTTG-3) and CB2H (5' - CCCCTCAGAATGATATTGTCCTCA-3') (Chippindale pers. comm.). Reactions consisted of a volume of 25 ul using 25 mM KCl, 5 mM Tris-HCL (pH 8.3), 0.005% gelatin, 1 mM MgCl₂, 100 uM dNTP's, 0.75 ul Taq polymerase, 0.15 uM of each primer, 100-300 ng template DNA and water to the final volume. DNA was amplified in a thermal cycler with a 7-minute denaturing step at 94°C followed by 30 cycles of denaturing for 40 seconds at 94°C, primer annealing for 30 seconds at 46°C, and elongation for 1 minute at 72°C. PCR products were purified using the QIAquick PCR purification kit (QIAGEN Inc.; Santa Clarita, California).

Cycle sequencing was performed on both strands of the cleaned PCR products by using the ABI Big Dye ™ (Perkin Elmer) reaction premix including the original primers. Centrifugation of Sephadex columns (Princeton separations for Sephadex columns) was subsequently used to remove excess nucleotides prior to sequencing. The nucleotide base sequence was visualized using an automated sequencer (DNA Sequencing Facility at Iowa State University). The nucleotide sequence of gene segments were edited and aligned using the program SEQUENCHER ver. 3.0 (Gene Codes, Ann Arbor, MI).

In order to screen a large number of individual specimens (N=10 from each insular population) from each population for genetic polymorphism, restriction enzyme analysis was used to identify restriction fragment length polymorphisms (RFLPs) within each insular population to measure the level of inter and intrapopulational variation in L.
Restriction enzyme $Hae$III generated unique RFLP haplotypes for each of the three subspecies; however, resolution of haplotypes between populations inhabiting the islands of Roti, Semau and Timor were indistinguishable. The polymorphic sites were determined using gel electrophoresis of the ethidium bromide stained RFLPs and were identified as those generating different RFLP patterns for each species.

Restriction enzyme incubations were run in 14ul volume, consisting of 1.4 ul of the appropriate 10x buffer, 0.2-0.4 ul of restriction enzyme solution to give a final concentration of 2-4 U/assay, 5 ul of the PCR amplified product, and the remaining volume pure water. Incubations were performed for 4h at 37°C, followed by 2% agarose gel electrophoresis of 10 ul of the digestion products.

Morphological Data

The three currently recognized $Lialis mackloti$ taxa have been poorly described and diagnosed. We examined a total of 10 snakes from each of the five ($N = 50$) insular populations (islands of Sawu, Wetar, Timor, Semau and Roti) we studied, representing the three currently established subspecies ($L. m. savuensis$, $L. m. dunnii$ and $L. m. mackloti$). Subspecies status was assigned based on collection locality and geographical ranges of subspecies established in the literature (Rawlings et al., 2004). Our morphological analysis was not exhaustive and was only meant to detect the presence of diagnostic characteristics that could separate the three subspecies of $L. mackloti$ and to complement the molecular and behavioral data (Carmichael et al., 2007) in order to elucidate patterns of geographic variation. A taxonomic key was also created for the three subspecies based on the morphological characters examined (Table 1). A more detailed morphological analysis would be required to detect incipient species based on
the morphological data set especially between the various insular populations of *L. mackloti* from islands where they are known to occur (e.g. Alor Island, How and Kitchener, 1997; and Babar Island; Rawlings et al., 2004).

Table 1. Taxonomic key to the three subspecies.

| 1a. There are 280 or less ventral scales or 76 postanal scales or less or white iris | ......................................................................................... *L. m. savuensis* |
| 1b. There are 285 or more ventral scales or more than 85 postanal scales or gold iris | ......................................................................................... 2 |
| 2a. There is one loreal scale on each side of the head | ......................................................................................... *L. m. dunnii* |
| 2b. There are two loreal scales on each side of the head | ......................................................................................... *L. m. mackloti* |

**Data collection.** Morphological characters were measured using both preserved and live specimens. Live specimens were quantified as to their morphomeasurements by placing individual snakes into an appropriately sized clear plastic restraining tube. Once the snake was gently restrained, morphomeasurements were obtained and the scalation accurately counted. Length measurements (of head, snout-vent length or SVL, and total length or TL) were taken using the program *SnakeMeasurer* (2000) by digitizing digital photographs taken by the author of the snakes to be measured. This was shown to be a much more reliable method of gaining length measurements for distances exceeding our digital caliper length of 12 cm and for structures that were irregularly shaped.

Characteristics of scutellation and pattern were noted and morphological characters were subsequently scored by island population and coded in such a way that each frequency array of conditions for a given character represented a different state. Morphomeasurement scoring generally followed that of Harvey et al (2000), Kluge (1993), McDowell (1975), and Underwood and Stimson (1990). The morphological descriptions used throughout this paper often will either have a slash to separate counts from opposite sides of the same specimen’s body (e.g., 2/3 preocular scalces) or a dash.
which indicates ranges (e.g., 295-326 ventral scales). Eighteen lepidotic characters were examined as follows:

A. *Postanal scale divided:* Either present (1) or absent (0).

B. *Anal plate divided:* Either present (1) or absent (0).

C. *Labial scale contacts eye:* Either present (1) or absent (0).

D. *Number of suprocular scales contacting eye:* Two (0) or one (1) supraocular scales contacting the eye.

E. *Iris color:* White (0) or gold (1) iris present.

F. *Midbody scale row count:* Less than or equal to 48 scale rows (0) or greater than 48 scale rows (1).

G. *Number of postanal scales:* Less than 76 (0) or greater than 83 (1).

H. *Number of pairs of parietal/occipital scales:* One pair of scales (0) or two pairs (1) present.

I. *Number of scales along each side of mental groove:* Seven scales along each side (7/7) of the mental groove (0) or eight scales along each side (8/8) of the mental groove (1).

J. *Number of ventral scales:* Less than or equal to 280 (0) or greater than 285 (1) ventral scales.

K. *Number of lower labial scales:* Less than or equal to 18 lower labial scales (0) or greater than 18 (1).

L. *Pit count on labial scales:* When pits present on scales (left side/right side of head) 10/10, 11/11, 12/12, 13/13 (0), 9/9, 10/10, 11/11, 12/12, 13/13 (1) and 9/9, 10/10, 11/11, 12/12 (2).
M. Number of upper labial scales: Between 9-11 upper labial scales (0) or greater than 11 (1).

N. Presence of diminuitive labial scales: Either present (1) or not present (0).

O. Number of loreal scales: Either have 1 scale on each side (0) or two scales (1).

Authors have used a variety of techniques to characterize variation in the scales occupying the loreal region. In total number, the loreals are highly variable (McDowell, 1975). In the species accounts, we report the number of loreals lying between the preoculars and nasals at the level of the middle of the eye and nostril [i.e., Kluge’s (1993) characterization of scales in this region]. However, we also counted the number of rows of loreals just posterior to the nasal scale. Usually, a large scale we refer to as a loreal is bordered anteriorly, dorsally, and posteriorly by the nasal, anterior preocular, and posterior preocular, respectively. This scale may contact the supralabials (the specimen is characterized as having one row of loreals) or be separated from them by one (= two rows of loreals) or two (= three rows of loreals) scales counted in a vertical line.

P. Number of pairs of prefrontals/internasal scales. Either have 2 pairs (0) or 1 pair (1).

The color pattern of these pythons is among the most variable of all pythonine snakes and we found it difficult to quantify based on population specific differences. Subjective notes were taken on all examined specimens. Additional data were recorded from live and photographed specimens. Subjective notes were taken on all examined
specimens. Additional data (weight, robustness, proportions) were recorded from live specimens.

We recorded several measurements for each specimen. Using SnakeMeasurer (2000), we measured snout-vent length (SVL) and tail length to the nearest 1 mm (using digital pictures of the dorsal and ventral surface of each snake studied); with a dial caliper, we measured head length (the distance from the posterior tip of the last supralabial to the center of the rostral), head width (the distance between the corners of the mouth measured from the ventral aspect), eye diameter (the horizontal diameter of the externally visible portion of the eye), eye-nostril distance (the distance from the anterior border of the orbit to the center of the nostril), internarial distance (the distance between the upper borders of the narial aperture), and interocular distance (measured between the anterolateral corners of the supraoculars) to the nearest 0.01 mm. Institutional abbreviations are as listed in Leviton et al. (1985).

Like Kluge (1993), we sought a total evidence approach by using the best fitting cladogram obtained from the molecular and morphological data. We first developed a phylogenetic hypothesis from each of the two data sets (molecular and morphological), and then used the total evidence approach to obtain an overall phylogenetic representation from the two data sets. Synapomorphies were identified instead of homologies as synapomorphies do not presuppose common ancestry whereas homology does. Several putative outgroup taxon (e.g. *Apodora papuana, Leiopython albertisii, Liasis olivaceus, and Antaresia (Liasis) childreni*) were also measured for the 11 different morphological characters and morphometrics (e.g. SVL and TL) and used to
infer polarity to give the most parsimonious hypothesis of character evolution.

Institutional abbreviations are as listed in Leviton et al. (1985).

**Phylogenetic Analysis**

Following sequence alignment, the sequences were analyzed for maximum parsimony analysis using the exhaustive search option in the computer program PAUP* 4.0 (Swofford, 2000) in order to establish phylogenetic relationships within this subspecies complex. Maximum parsimony was calculated using the branch-and-bound algorithm. The ability of our sequence data set to reject alternative phylogenetic hypotheses was examined further with non-parametric Templeton Tests (Wilcoxin signed-rank test) (Templeton, 1983) in PAUP. This test examines if there is a significant difference between the most parsimonious tree and an alternative topology. *Leiopython albertisii, Antaresia childreni, Apodora papuana, L. fuscus,* and *L. olivaceus,* were used as outgroups in the phylogenetic analysis. In a separate MP analysis (1000 bootstrap replicates), morphological characters were weighted equally and treated as unordered. Molecular and morphological data sets were also combined into a single matrix for parsimony analysis. All characters were unweighted. The molecular and morphological partitions were first tested for heterogeneity in PAUP*, using the partition homogeneity test (100 replicates) and a phylogenetic tree was derived using WinClada. To infer approximate temporal boundaries for mtDNA divergence and to gain a better understanding of the biogeographic events that have led to the current distribution of *L. mackloti ssp.*, we used a molecular clock estimation of 2% per million years which has been used for several Australian snakes including the king brown snake, *Pseudechis australis* (Brown et al., 1979). Arbogast and Slowinski (1998) used Brown et al (1979)
to develop a much greater estimate of 5.18% MYA for a cytb divergence rate and therefore both rates have been used to generate a molecular clock.

RESULTS

Molecular Data

RFLP revealed three unique haplotypes that also distinguished the three subspecies, *L. m. mackloti, L. m. dunni* and *L. m. savuensis*, however there were no variant intrapopulational haplotypes detected within each insular population studied. Partial cytb sequences were obtained from two individuals from each of the five insular populations (Wetar, Sawu, Semau, Roti, and Timor). We compared a 415 bp sequence of cytb. Sequences will be deposited on GenBank prior to publication. Average Jukes-Cantor (1969) genetic distances between mitochondrial haplotypes are presented in Table 2. Within subspecies, sequence divergence ranged from low, all less than 1%: 0.2%-0.7% among savuensis, 0.2-0.6% among dunni, and 0% among mackloti (no sequence divergence was detected when comparing the three island populations of mackloti) haplotypes. Among subspecies sequence divergence ranged between 2.9-3.1% between mackloti and dunni, 5.6-5.8% between mackloti and savuensis, and 5.4-5.6% between dunni and savuensis. *Liasis mackloti ssp.* forms a monophyletic group, with *L. m. savuensis* being the sister taxon of this clade (96% bootstrap support). Differences between the outgroups and ingroup ranged from 4-6.3% for the outgroup *L. fuscus*, 7.9-9.2% for the outgroup *L. olivaceus*, 11.9-13.4 for the outgroup *Leiopython albertisii*, 9.6-11.8% for the outgroup *Apodora papuana*, and 10.8-14.1% for *Antaresia childreni.*
Table 2. Average Jukes-Cantor (1969) genetic distances between mitochondrial haplotypes (LA = *Leiopython albertisii*, AC = *Anteresia childreni*; AP = *Apodora papuanus*; LO = *Liasis olivaceus*; LF = *Liasis fuscus*; LMS = *Liasis m. savuensis*; LMD = *Liasis m. dunni*; LMMT = *Liasis m. mackloti* from Timor; LMMS = *Liasis m. mackloti* from Semau; LMMR = *Liasis m. mackloti* from Roti).

<table>
<thead>
<tr>
<th></th>
<th>LA</th>
<th>AC</th>
<th>AP</th>
<th>LO</th>
<th>LF</th>
<th>LMS</th>
<th>LMD</th>
<th>LMMT</th>
<th>LMMS</th>
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Of 415 sequence characters (considering all ingroup and outgroup OTUs), 289 were constant, 79 were parsimony-informative, and 27 were variable but uninformative under the parsimony criterion. Parsimony and maximum likelihood analyses of the sequence data alone yielded trees that were mostly consistent with respect to relationships of ingroup taxa (Fig. 3). Parsimony analysis of sequence data alone with all substitution types treated as equally probable yielded 2 equally parsimonious trees (CI = 0.601, RI = 0.755, length 208). Very high levels of support (82-100%) for three major mtDNA clades of *L. mackloti* subspecies was obtained from bootstrap analysis (see Fig. 3). There appears to be minimal geographic structuring within island populations and between the islands of Roti, Semau and Timor based on RFLPs and mtDNA sequences, and it appears that the highest level of variation corresponds with comparisons between *savuensis* and *mackloti* and *savuensis* and *dunni*, however, additional samples would be required to confirm the level of homogeneity on each of the islands studied. The most parsimonious tree suggests significant structure and differentiation between *L. m. savuensis*, *L. m. mackloti*, and *L. m. dunni*.

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The topology of the single MP tree strongly supports the monophyly of the three taxa with high bootstrap values (Hillis and Bull, 1993) (Fig. 3). The taxon *mackloti* is supported by a bootstrap value of 82%, *dunni* by a value of 100%, and *savuensis* by a value of 96%. In all three taxa there is evidence for phylogeographic substructure. Templeton tests of alternative topologies strongly support for the topology illustrated in Figure 3. When *L. m. dunni* snakes are made the sister group to *L. m. mackloti* the resulting tree is six steps longer than the most parsimonious tree using *savuensis* as the sister taxon (*z* = 2.4495, *P* < 0.015) therefore we can reject the hypothesis that *mackloti* is more closely related to *savuensis* as compared to *dunni*. The phylogenetic tree presented in Figure 1 shows that *dunni* and *mackloti* are more closely related to each other than *savuensis*. Interestingly, we also see that the outgroup *L. fuscus* is more closely related to *savuensis* than is *savuensis* to *mackloti* or *dunni*.

*Morphological Data and Systematic Description*

*Phylogenetic Inference.* MP analysis of the 16 morphological characters (Tables 3 and 4) led to two equally parsimonious trees, the strict consensus of which is shown in Appendix B. The tree gave a congruent phylogenetic pattern to the molecular-generated phylogenetic tree and also supported the monophyly of *L. mackloti* ssp. based on the high bootstrap values (81-100%). *Liasis fuscus* was the basal group to the *L. mackloti* ssp. complex.
**Figure 1.** Simplified cladogram of *L. mackloti* ssp. based on morphological character states presented in Table 4 (derived from WinClada). Each branch was represented with strong support (80-100%).

**Figure 2.** Current phylogeny of *Liasis mackloti* based on exhaustive search using maximum parsimony of 415 cytochrome *b* sequence positions. Bootstrap proportions are provided. Island populations for *Liasis mackloti* phylogroups are indicated to the right. Two sequences for *L. m. savuensis* were taken from GenBank (accession numbers provided) (CI = 0.601, RI = 0.755, length 208).

**General Description and Species Accounts.** *Liasis mackloti* ssp. is characterized as a medium- to large-sized python from the Inner and Outer Banda Arc of islands within Indonesia’s Lesser Sundas Archipelago. The head is generally wider than the neck, the
tail is relatively long, but not particularly prehensile. This species is sexually dimorphic with females generally being slightly larger than males on the islands of Sawu, Timor, Semau and Roti, however on the island of Wetar, males generally tend to be larger. Both sexes have prominent cloacal (pelvic) spurs. The spurs of adult males are more strongly hooked inward and thicker than those of females. Typically the spurs of adult males are more worn; they may be rounded and blunt and shorter than those of females. The head is distinctly wider than the neck and is longer than wide.

Geographic variation in color pattern among *L. mackloti* is both locality specific and on most islands to some extent polymorphic as well. Despite wide ranges of polymorphism, there does appear to be locality specific prototypes that typify each of the insular populations. At least five distinct phenotypes (one on each island, and in most cases, there are two general phenotypes on each island) in regards to color pattern are found on each of the five islands studied. *L. mackloti ssp.* can be differentiated from the other closely related species within the genus *Liasis* (e.g. *L. olivaceus* and *L. fuscus*) by having two pairs of prefrontal scales as opposed to having a pair (Table 3). Also, *L. mackloti* can be separated from *L. fuscus* by usually having 55 or less mid-body rows of scales, whereas *L. fuscus* generally has over 60. Several authors have historically placed both *L. fuscus* and *L. mackloti* into the same species (Kluge, 1993; McDowell, 1975), however additional analysis since then (both behaviorally, Carmichael et al., 2007; and genetically, Carmichael et al., 2002 and Rawlings et al., 2004) have confirmed that not only are *L. fuscus* and *L. mackloti* distinctly separate species, but the subspecies complex of *L. mackloti* may also represent various incipient species.
Table 3. Variation in selected morphological characteristics of *L. mackloti* ssp. For meristic characters, ranges are followed by means +/- standard deviations in parentheses.

<table>
<thead>
<tr>
<th></th>
<th><em>L. m. savuensis</em> (n = 18)</th>
<th><em>L. m. dunni</em> (n = 14)</th>
<th><em>L. m. mackloti</em> Timor Island (n = 18)</th>
<th><em>L. m. mackloti</em> Semau Island (n = 5)</th>
<th><em>L. m. mackloti</em> Roti Island (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iris Color</td>
<td>White</td>
<td>Gray-Gold</td>
<td>Gold</td>
<td>Gold</td>
<td>Gold</td>
</tr>
<tr>
<td>Midbody Scale</td>
<td>46-49 (48 ± 0.5)</td>
<td>52-63 (58 ± 2.7)</td>
<td>59-65 (62 ± 0.4)</td>
<td>57-64 (62.5 ± 1.1)</td>
<td>56-64 (61.9 ± 1.2)</td>
</tr>
<tr>
<td>Row Count</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postanal Scales</td>
<td>65-76 (68 ± 1.6)</td>
<td>85-89 (87 ± 2.4)</td>
<td>88-93 (90 ± 1.2)</td>
<td>87-93 (90 ± 1.6)</td>
<td>88-92 (90 ± 1.1)</td>
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<td>Postanal Scale Divided</td>
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<td>Yes</td>
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<tr>
<td>Anal Plate Divided</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<td>Yes</td>
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<tr>
<td>Pit Count on Labial Scales</td>
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</tr>
<tr>
<td>Upper Labial Scales</td>
<td>9-11 (10 ± 0.4)</td>
<td>9-11 (10 ± 0.6)</td>
<td>11-12 (11.5 ± 0.2)</td>
<td>11-12 (11.5 ± 0.2)</td>
<td>11-12 (11.5 ± 0.2)</td>
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<tr>
<td>Lower Labial Scales</td>
<td>14-18 (15 ± 0.6)</td>
<td>15-18 (16 ± 0.2)</td>
<td>18-19 (18 ± 1.2)</td>
<td>18-19 (18 ± 1.2)</td>
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<td>Yes</td>
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<td>Ventral Scales</td>
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<td>285-293 (288 ± 2.6)</td>
<td>292-302 (298 ± 1.5)</td>
<td>291-301 (296 ± 1.6)</td>
<td>292-301 (296 ± 1.7)</td>
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</table>

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Table 4. Matrix for cladistic analysis of *L. mackloti* ssp. Characters A-P are identified below and states are indicated present (1-2) or absent (0). The primitive (plesiomorphic) conditions are considered to be the states present in *L. fuscus* (the outgroup) and the other character states are considered derived (apomorphic). A, postanal scale divided (1 = yes); B, anal plate divided (1 = yes); C, labial scale contacts eye (0 = no, 1 = yes); D, number of supraocular scales contacting eye (0 = 2, 1 = 1 scale); E, iris color (0 = white, 1 = gold); F, midbody scale row count (0 = \leq 48, 1 = >48); G, postanal scales (0 = <76, 1 = >83); H, pairs of parietal/occipital scales (0 = 1 pair, 1 = 2 pairs); I, number of scales along each side of mental groove (0 = 7/7, 1 = 8/8); J, number of ventral scales (0 = \leq 280, 1 = >285); K, number of lower labial scales (0 = \leq 18, 1 = >18); L, pit count on labial scales (0 = 10/10, 11/11, 12/12, 13/13; 1 = 9/9, 10/10, 11/11, 12/12, 13/13; 2 = 9/9, 10/10, 11/11, 12/12); M, number of upper labial scales (0 = 9-11, 1 = >11); N, presence of diminutive labial scales (0 = no, 1 = yes); O, number of loreal scales (0 = 1 scale, 1 = 2 scales); P, number of pairs of prefrontals/intemasaal scales (0 = 2 pairs, 1 = 1 pair).

<table>
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<th>Characters</th>
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<th>C</th>
<th>D</th>
<th>E</th>
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**Synonymy of Liasis mackloti**

*Python amethystinus* (part) Schlegel, 1837: 419-421 (variety from New Ireland [erroneous], Timor and Samoa, described p. 420, suspected to be environment-induced abnormality).

*Liasis amethystinus* (not *Boa amethystina* Schneider) Gray, 1842: 44 (listed in genus *Liasis* Gray, “Inhabits India. Mus. Leyden”, rostral and upper labials [by generic diagnosis] flat [thus, based on Schlegel’s specimens later described as *Liasis mackloti*]).
*Liāsis mackloti* Dumeril and Bibron, 1844: 440-442 (original description; type [by designation of Brongersma, 1968: 56] M.H.N.P. 1625, Timor, S. Muller and H. C. Macklot, coll.; diagnosis in key, p. 433, under name *Liāsis* de Macklot; reference of [unexamined] Samao specimen to this form); Jan and Sordelli, 1860-66: livr. 7 (publ. Nov. 1864], pl. vi (figure of type; name spelled *Liāsis* Machloti on plate); Werner, 1897-261 (closely related to *L. tornieri* Werner [=*L. papuanus*]); Boulenger, 1897:505 (Savu [=Sawoe]; Bethencourt Ferreira, 1898: 154 (Timor).

*Liāsis (Simalia) mackloti* Gray, 1849:92 (compiled description).

*Liāsis (Lisalia) olivacea* (part) Gray, 1849:92-93 (specimen c, Sir Charles Hardy’s l.).

*Liāsis fuscus* W. Peters, 1874-607-608 (original description; Type, ZMU 7840, from Port Bowen, Queensland, Frau A. Dietrich coll.; nearest to *Liāsis olivaceus*);

Boulenger, 1893:77, 78-79 (diagnosis in key, description, synonymy; Sir Charles Hardy Is.; Cornwallis I.; Fly River, New Guinea; S. E. New Guinea); Zenneck, 1898:3, 23, fig. 29 (color pattern); Boulenger, 1898:702 (specimen with no locality, Loria coll., presumably S. E. New Guinea or d'Entrecasteaux Is.); Bethencourt Ferreira, 1898:154 (Dilly, Timor); Werner, 1899a:155 (in key);

Barbour, 1912:30, 189 (listing for Timor, British Papua, Queensland); de Rooij, 1917:16-17, 304, 312 (diagnosis in key, description, distribution); Werner, 1921:233 (references, counts, size, distribution); Thomson, 1935:725-726 (aquatic; tail nonprehensile; oviparous; nocturnal; docile; feeds chiefly on mammals, all based on Queensland specimens); Glauert, 1950:16, 18 (Kimberly Division, Western Australia; on p. 18, Key, “Brown Rock Python”); Worrell, 1976.
1951: 22, 25 (aquatic; feeds mainly on reptiles, based on Northern Territory material; diagnosis in key; ventrals 270 to almost 300); Worrell, 1956:205-206, 207, 208 (skull figured; cranial and dental differences from Leiopython albertisii); Worrell, 1963:99, 188, pl. 36 (description; color photograph; may grow to a little over 10 ft.; feeds mostly on reptiles; sometimes found in salt-water mangroves).

*Liasis mackloti* Peters and Doria, 1878:81 (closely related to *L. papuanus* Peters and Doria).

*Liasis cornwallisius* Gunther, 1879:85-86 (original description; type, BM 1946.1.10.41 from Cornwallis I.; Torres Strait, S. MacFarlane coll.; figure; allied to *Liasis childrenii*).

*Nardoa crassa* Macleay, 1886:66-67 (original description; syntypes Austr. Mus. (Sydney) 5940.

*Liasis mackloti* Boulenger, 1893:77, 79 (diagnosis in key, compiled description, references); Zenneck, 1898:3, 23 (color pattern); de Rooij, 1917: 16, 17-18, 303, 304 (diagnosis in key, description, figure, distribution); Werner, 1921:234 (references, size, distribution); Dunn, 1927:1, 6 (Uhak, north coast of Wetar I.; specimen, not preserved, measuring 2200 mm, and two others; distribution discussed); Mertens, 1930:174, 175 (significance of distribution).

*Liasis mackloti* Barbour, 1912:30, 189 (listing for Timor, Savu, Samao).

*Liasis mackloti dunni* Stull, 1932:25-26, pl. 1 (original description; type, AMNH 32263, Uhak, Wetar I., E. R. Dunn coll.; paratype, AMNH 32264, same data); Stull, 1935:390, 392 (listing); de Haas, 1950:521 (listing).
Liiasis mackloti mackloti Stull, 1932:25 (distinguished from *L. m. dunni* Stull); Stull, 1935:392 (listing); de Haas, 1950:521 (listing); Brongersma, 1951b:4, 7, 18, pl. i (lungs and pulmonary artery); Brongersma, 1956a:290-296 (detailed description, figures, complete references; *L. m. dunni* not recognized, but trinomial used to contrast with *L. m. savuensis* Brongersma); Stimson, 1969:25 (synonymy, lectotype, distribution).

Liiasis fuscus fuscus Stull, 1935:390, 392 (*L. fuscus* and *L. albertisii* considered conspecific, without argument given); Loveridge, 1948:320 (listing); de Haas, 1950:521 (listing);

Liiasis mackloti savuensis Brongersma, 1956a:290, 294, 296-297 (original description; type, BM 96.6.21.33, from Savu [=Sawoe] I.; A. Everett Coll.; figure; counts on type and four paratypes with same data); Stimson, 1969:25 (type specimen, distribution [number of Type given as BM 97.6.21.33]).

**Descriptions of Taxa.** Since the main objective of this investigation was to detect patterns of geographic variation, we have only described various diagnostic characteristics that are variable between the three different subspecies (see Tables 3 and 4).

Liiasis mackloti savuensis

**Holotype.** BM 97.6.21.33

**Type Locality.** Savu (=Sawoe) Island of the Outer Banda Arc of the Lesser Sundas Archipelaga, Indonesia.

**Etymology.** Named in honor of H. C. Macklot and in reference to distribution on Savu Island.
Diagnosis. *L. m. savuensis* has 273-280 ventral scales, while *L. m. mackloti* has 292-302 and *L. m. dunni* has 285-293. *L. m. savuensis* has 65-76 subcaudal scales as compared to 88-94 in *L. m. mackloti* and 83-92 in *L. m. dunni*. *L. m. savuensis* has an average of 49 scale rows, while *L. m. mackloti* and *L. m. dunni* have an average of 59. *L. m. savuensis* and *L. m. dunni* have one loreal scale on each side of the head, while *L. m. mackloti* and *L. m. dunni* have two loreal scales on each side of the head. *L. m. savuensis* has an average of 49 scale rows, while *L. m. mackloti* and *L. m. dunni* have an average of 59. *L. m. savuensis* and *L. m. dunni* have one loreal scale on each side of the head, while *L. m. mackloti* and *L. m. dunni* have two loreal scales on each side of the head. *L. m. savuensis* has one set of parietal/occipital scales whereas *L. m. mackloti* and *L. m. dunni* have two sets of these scales. The frontal scale in *L. m. savuensis* also is divided, while in *L. m. mackloti* and *L. m. dunni* the scale is entire. Pits were located on 3 to 4 of the lower labial scales in both *L. m. savuensis* and *L. m. dunni*, and 4-5 pits in *L. m. mackloti*. *L. m. savuensis* has a white iris which is lacking in both *L. m. mackloti* and *L. m. dunni*.

Body size. *L. m. savuensis* is sexually dimorphic with females generally larger than males. Maximum total length for the taxon approaches 150 cm total length. In our sample of wild-caught snakes from our colony (N=45), adult male snakes averaged 99.1 cm total length (SD = 11.95) and 45 adult females averaged 129.4 cm total length (SD = 11.82).

Pattern and color variation. This taxon displays tremendous variation in color and pattern. The dorsal aspect of the head is generally uniformly dark charcoal to charcoal gray with a creamy white color infusing throughout the upper labial scales and continuing throughout the lower jaw. No distinct patterning is present on the head. These pythons are generally not patterned as adults and are either uniform dark colored or a peppering of dark and light colored scales. Preliminary review of pythons in our collection suggests that color/pattern may be a sexually dimorphic feature as males are generally mottled.
color while females are uniformly dark. This needs further analysis. The dorsal part of the body is often dark and the ventral scales generally a creamy white (same color as chin). Interestingly, in some individuals the creamy white ventor will slowly turn into a pumpkin orange coloration toward the posterior third of the body starting well anterior of the cloaca and extending toward the tip of the tail. All of the adult snakes studied had a white iris. Although the pattern does not change once they reach sexual maturity, there is an ontogenetic color change. The dorsum is generally a reddish-orange color while the head is dark colored with a creamy white ventor. *Liasis m. savuensis* will gradually darken with age within about 12-18 months after hatching. By about 3-5 years of age, the pythons will achieve their adult coloration.

*Liasis mackloti dunni*

*Holotype.* AMNH 32263 (Paratype AMNH 32264)


*Etymology.* Named in honor of H. C. Macklot and in reference to distribution on Wetar Island.

*Diagnosis.* *L. m. dunni* has 285-293 ventral scales, while *L. m. mackloti* has 292-302 and *L. m. savuensis* has 273-280. *L. m. dunni* has 83-92 subcaudal scales as compared to 88-94 in *L. m. mackloti* and 65-76 in *L. m. savuensis*. *L. m. savuensis* has an average of 49 scale rows, while *L. m. mackloti* and *L. m. dunni* have an average of 59. *L. m. dunni* and *L. m. savuensis* have one loreal scale on each side of the head, while *L. m. mackloti* has two. Both *L. m. mackloti* and *L. m. dunni* have two sets of parietal occipital scales while *L. m. savuensis* has one set of parietal occipital scales. The frontal scale in *L. m. dunni*
and *L. m. mackloti* is not divided while in *L. m. savuensis* the frontal scale is divided. *L. m. dunni* also has an extra set of prefrontal and internasal scales which is not present in either *L. m. savuensis* nor *L. m. mackloti*.

**Description**

*Body size.* *L. m. dunni* is sexually dimorphic, with males generally larger than females. Maximum total length for the taxon approaches 190 cm. In the sample of wild-caught snakes from our colony (N=45), adult male snakes averaged 171.5 cm total length (SD = 11.95) and 45 adult females averaged 144.8 cm total length (SD = 11.82). This was the only subspecies of *L. mackloti* ssp. where males had a generally larger average total length than females and is also rare in most python species.

*Pattern variation.* This taxon displays highly variable pattern and color polymorphisms. *L. m. dunni* range from light to dark browns to light and dark grays. Generally the dorsum is darker than the ventor, which is creamy white. On the head, there are no obvious markings and the dorsal aspect of the head is generally darker than chin, with the upper and lower labial scales being a similar color to the chin color (creamy white). This was also observed in *L. m. mackloti* and *L. m. savuensis*. The ventor is also heavily mottled in *L. m. dunni* while in *L. m. mackloti* and *L. m. savuensis* the ventor is uniformly colored without any pattern. The dorsum is variable in *L. m. dunni*, with varying degrees of light colored flecks throughout the dorsum. These “flecks” are likely a result of a lack of pigmentation in the cells of this area (Harvey et al., 2000).

*Liásis mackloti* mackloti

**Holotype.** MHNP 1625

**Type Locality.** Timor Island
Etymology. Named in honor of H. C. Macklot and in reference to distribution on Timor Island.

Diagnosis. *L. m. mackloti* has 292-302 ventral scales, while *L. m. dunni* has 285-293 and *L. m. savuensis* has 273-280. *L. m. mackloti* from Timor, Roti and Semau Islands have 65-76 subcaudals (there was no consistent scale counts on each of these three islands), as compared to 83-92 subcaudal scales in *L. m. dunni* and 65-76 in *L. m. savuensis*. *L. m. savuensis* has an average of 49 scale rows, while *L. m. mackloti* and *L. m. dunni* have an average of 59. *L. m. dunni* and *L. m. savuensis* have one loreal scales on each side of the head, while *L. m. mackloti* has two. Both *L. m. mackloti* and *L. m. dunni* have two sets of parietal/occipital scales while *L. m. savuensis* has one set of parietal/occipital scales. The frontal scale in *L. m. dunni* and *L. m. mackloti* is not divided while in *L. m. savuensis* the frontal scale is divided. *L. m. dunni* also has an extra set of prefrontal and internasal scales which is not present in either *L. m. savuensis* nor *L. m. mackloti*. *L. m. mackloti* and *L. m. dunni* have between 4-5 pits on the lower labial scales, while *L. m. savuensis* has 3-4.

Description

Body size. *L. m. mackloti* is sexually dimorphic with females generally larger than males. Maximum length for the taxon approaches 3.1 m. In the sample of wild-caught snakes from our colony (N=45), adult male snakes averaged 170.5 cm total length (SD = 11.95) and 45 adult females averaged 243.9 cm total length (SD = 11.82).

Pattern variation. This taxon displays a variable pattern. Indonesian water pythons have generally pale heads with yellow to white chins and throats, and dark bodies with pale freckles. The undersides of their necks are pale yellow, and their ventral surface becomes
increasingly dark posteriorly. The underside of the tail may be uniformly dark. The dorsal surface ranges from light brown to a dark iridescent brown. The head is unremarkable in pattern, with the dorsal surface being dark colored, and the ventral surface ranging between white to cream to light yellow. By in large the amount of pattern variation is mainly in regards to the amount of freckling throughout the dorsal surface. \(L. m. mackloti\) from the island of Roti are overall very dark with few pale flecks on the dorsum. \(L. m. mackloti\) from the island of Timor tend to have an even flecking of pale scales on their bodies. \(L. m. mackloti\) from the island of Semau may have dark flecks on their heads and may have so many pale flecks on the body and particularly on the sides as to appear overall nearly as pale as the head coloration.

**Color variation.** The color of the heads of most Indonesian water pythons is brown and is very similar to the color of the pale freckles seen on the bodies. The color of the bodies varies among individuals from olive-browns to rich dark browns. Semau specimens tend to have the palest overall appearance, while Roti specimens are the darkest.

There are no records of albinism or other forms of hypomelanism for this subspecies. While Indonesian water pythons do exhibit considerable polymorphisms in regards to color, we know of no reports of dramatically unusual or anomalous conditions of color in this species.

Indonesian water pythons have generally pale heads with yellow to white chins and throats, and dark bodies with pale freckles. The undersides of their necks are pale yellow, and their ventral surface becomes increasingly dark posteriorly. The underside of the tail may be uniformly dark. The dorsal surface ranges from light brown to a dark
iridescent brown. The head is unremarkable in pattern, with the dorsal surface being dark colored, and the ventral surface ranging between white to cream to light yellow. By in large the amount of pattern variation is mainly in regards to the amount of freckling throughout the dorsal surface. *L. m. mackloti* from the island of Roti is generally dark dorsally and uniform with reduced freckling. *L. m. mackloti* from the island of Timor are heavily freckled with pale flecks on their bodies. The backs may appear darker than the sides in some pale Timor specimens. Semau specimens may have dark speckling on their heads; they may have so many pale freckles on the body and particularly on the sides as to appear overall nearly as pale as the head coloration.

**Combining Molecular and Morphological Data**

Maximum parsimony (MP) analysis was applied to the combined molecular and morphological data. Under parsimony, this led to a single tree (Fig. 3) with congruent nodes strongly supported by bootstrap values greater than 80. *Liasis m. savuensis* emerged as the sister taxa to *L. m. dunni* and *L. m. mackloti*, which was consistent with topologies generated when using either molecular or morphological data alone.
Figure 3. Phylogeny of *Liasis mackloti* derived from the combined analysis of molecular and morphological characters (MP analysis; bootstrap 1000 replicates) (CI = 0.694, RI = 0.572).

**Evolutionary Timescale**

The estimates for calibration of a molecular clock in *L. mackloti* ssp. using Brown et al. (1979) (2% and 5.18% divergence rate) suggest that the recent differentiation of this monophyletic clade could have occurred during the Mid- to Late Pliocene (<2.8 mya). A low end estimate (2% mya) dates the three clades to the Mid- to Late Pliocene. This estimate places a divergence of the following: *L. fuscus* (Australia)/Sawu (*L. m. savuensis*) 2.15 mya, Sawu (*L. m. savuensis*)/Wetar (*L. m. dunni*) 2.75 mya, Wetar (*L. m. dunni*)/Timor (*L. m. mackloti*) 1.45 mya, and Sawu (*L. m. savuensis*)/Timor (*L. m. mackloti*) 2.8 mya. Based on the 5.18% mya clock, lineage divergence is dated to Early to Mid-Pleistocene (0.56-1.08 MYA for the three clades).
DISCUSSION

The major results of our analysis show that three taxa are recognizable within this group, and are easily distinguished from each other genetically and morphologically. Our study contributes to the taxonomic debate in providing strong evidence for the recognition of several species from within the allopatric insular populations of *L. mackloti*: Sawu clade (*L. m. savuensis*), Wetar clade (*L. m. dunni*) and Greater Timor clade (*L. m. mackloti*). Although criteria for recognizing taxa as species versus subspecies are necessarily subjective, the patterns of divergence represented in our data clearly express the genetic distinctiveness of each taxon and show that each taxon is on its own unique evolutionary trajectory. Thus, under the phylogenetic and evolutionary species concept (Cracraft, 1983), we believe the species level rank is the most appropriate for each of these three taxa. Each of the new species (*L. savuensis*, *L. dunni* and *L. mackloti*) is readily diagnosable based on morphology and mitochondrial sequences. These facts, coupled with the restriction of each new species to an isolated island group, indicates that each represents a distinct lineage which is likely to maintain its identity from other such lineages until it goes extinct or experiences additional speciation.

*Liasis mackloti mackloti* from the islands of Timor, Semau and Roti (Greater Timor) are virtually indistinguishable based on the genetic data and are extremely polymorphic in regards to color and pattern. However, there appears to be some level of pattern/color divergence between the three insular populations of *L. m. mackloti* under study. The Roti *mackloti* are very dark dorsally with minimal flecks dorsally, while the Timor population is generally uniformly flecked and the Semau population heavily and unevenly flecked. This requires additional study to further differentiate the level of
intrapopulational variation in various morphomeasurements. We doubt that gene flow is currently occurring between Timor, Semau and Roti due to the barrier of the sea and preference for dry grassland habitat, and that these three insular populations are also experiencing unique evolutionary histories despite their similarity in mitochondrial sequences at this time. *Liasis m. dunni* appears to be more similar to *L. m. mackloti* while *L. m. savuensis* is much more distinctive based on molecular and morphological data sets. Phylogenetic analyses reveal that this latter taxon is the sister-group to the other two (Fig. 3 & 4). The maximum sequence divergence between *L. m. savuensis* and *L. m. mackloti* (5.6%) is slightly larger than the minimum sequence divergence (4.3%) between all three ingroup taxa and the outgroup species, *L. fuscus*.

Two aspects of our results are striking: the strong divergence between *L. m. savuensis* versus the other taxa, and the low divergence between *L. m. dunni* and *L. m. mackloti* (2.7-3.0%) or lack thereof between the three insular populations of *L. m. mackloti* (0%). There was a high level of congruence between the molecular and morphological data in yielding a similar phylogenetic pattern (Figs. 5). Within each subspecies there is tremendous polymorphism in coloration and pattern, yet despite this high degree of polymorphism there is an overall genetic similarity within each of the three subspecies.

Within the polymorphic *mackloti* there is no evidence to support the notion that the color morphs correspond to phylogenetic lineages and it is likely that these might be recently insularized populations that have yet to experience strong geographic divergence. The morphological characters proved useful for diagnosing individual subspecies and for resolving relationships. Use of morphological data alone yielded a tree
that is consistent and supported by the molecular tree. The addition of the morphological
data to the sequence data appeared to support the phylogenetic signal obtained by either
of these data sets alone.

**Biogeography and Geologic History of the Indonesian Islands**

Our phylogenetic hypothesis has strong implications for the biogeographic
processes involved in the evolutionary history of these animals. The Indonesian
Archipelago is host to a unique and diverse assemblage of reptiles, especially pythons,
and consists of many thousands of islands that encompass the Oriental-Australian faunal
interface. This region has long attracted the attention of biogeographers who have sought
to delineate the Australian-Oriental divide (Simpson, 1977). Nevertheless, little is
known, from a genetic perspective, of the within-species variability of the fauna in the
region. We have attempted to relate our data to the geologic history of this region and to
evaluate the roles of dispersal and vicariance in shaping current patterns of geographic
variation. This set of biogeographic parameters provides an excellent opportunity to
investigate basic questions about the nature, distribution, and correlates of genetic
diversity in the Indonesian pythons, specifically the species complex in question, the
macklot’s python, *Liasis mackloti* ssp. (Dumeril and Bibron, 1844; Type Locality, Timor,
Indonesia). *Liasis mackloti* and *L. fuscus* have historically been viewed as the same
species (McDowell, 1975) however Cogger (1992) identified the Australian form (*L.
fuscus*) as a distinctly different species. Barker and Barker (1994) recognize
intraspecific variation within *L. fuscus* and Rawlings et al (2004) identified two major
clades of *L. fuscus*, a western clade which includes *L. fuscus* from the Northern Territory
and an eastern clade which includes *L. fuscus* from New Guinea and the Queensland,
Australia region, the former which likely gave rise to *L. mackloti* (Rawlings et al., 2004).

*Liasis mackloti* is currently known to exist throughout the eastern outer Banda Arc islands (e.g. Sawu, Roti, Semau and Timor) as well as several inner Banda Arc islands proximal to Timor including Babar, Alor and Wetar (Rawlings et al., 2004).

The Indonesian Archipelago appeared approximately 15 million years ago (Hall, 1998) and has a volcanically active and unique geologic history. The southern part of the archipelago consists of an arc of major islands in an approximately east-west orientation, extending from Sumatera to Irian Jaya (see Appendix A). This sequence is geologically divided into two major components. The outer Banda Arc islands are volcanic and include Sumatera, Jawa (Java) and Timor may have originated in the Pliocene (Audley-Charles, 1987) and the inner Banda Arc of islands from Bali through Lombok, Sumbawa, and Flores to Banda, is Laurasian in origin and may be younger than the Miocene with Bali presumably being 3 million years old (Burrett et al., 1991). The outer Banda Arc islands which includes Sumba, Timor, Roti, Wetar, Sawu, Semau, Tanimbar, Kai, and Seram, have a Gondwanic basement overlayed with sediments of Permian to Pliocene age (Hall, 1998; Hamilton, 1979; Audley-Charles, 1981, 1987). The geologic history of the outer Banda Arc is somewhat contentious due to the complexity of this region, however it appears that the islands of Timor and neighboring islands began to emerge sometime in the Pliocene (Audley-Charles, 1987). Timor rose from 3-5 km below sea level (SL) since approximately 4.5 mya. Timor and Semau (and likely Roti, Robert Hall, pers. com.) were historically connected by dry terrestrial landscape (bridge) during the last glacial maxima 18,000 years ago (Heaney, 1991; Kitchener and Suyanto, 1996), which would have provided a mechanism of dispersal from Timor to Semau and
likely Roti. Sawu has risen from approximately 4 km (Ron Harris and Robert Hall, pers. com.) below SL since about 2 mya, however has only been emerged since approximately 200 ka. Harris (pers. com) recently extracted foraminifera from sediments deposited on Sawu during its ascent from water depths of approximately 4 km to the surface. This indicates that Sawu did not reach the surface until the Quaternary. Likewise, Quaternary coral terraces that overlie these sediments yield ages of approximately 200 ka. Seismic reflection profiles between Sawu and the other islands show thick sequences of synorogenic sediment without any evidence of unconformities and other irregularities associated with the existence of a land bridge. There is no evidence that any of the Sawu Sea region except for Sawu and Sumba have ever been within 1 km of the surface. Since sea level only varies by 200 m or so, it is highly unlikely that any land bridges existed that would have connected Sawu to any of the other islands under study (Roti, Semau, Timor, Wetar). Based on current evidence Wetar and Timor are not known to have been connected (Ron Harris and Robert Hall, pers. com.), even though this possibility was suggested to explain the existence of pygmy stegodonts (Robert Hall, pers. com.; unpubl. data) on both islands and therefore alternate hypotheses must be used to explain their historical distribution on these two islands (i.e., dispersion through rafting). Wetar is a volcanic island in the arc that has been historically thought to have collided with Timor about 4.5 Ma, although this has been recently scrutinized and does not appear to be the case (Ron Harris and Robert Hall, pers. com.). Land bridges also do not appear to have interconnected Wetar to Timor (Ron Harris and Robert Hall, pers. com.). The deep ocean basins between the islands outright preclude such connections within the most recent past (1 Ma). There is a record of deep marine sediment exposed on these islands.
that has been queried by analyzing forams (Harris, unpubl. data). The sequence of uplift is from east to west, and is associated with the local occurrence of plate boundary segments, such as the Sawu Thrust, north of Sawu. Most of these islands are emerging and their area above SL is increasing. Except for Wetar, all seem to have emerged very recently. Wetar and Timor appear to have emerged first, followed (in order) by Semau and Roti and lastly Sawu. Islands throughout the outer Banda Arc decrease in size from west to east with a marked decrease east of the island of Wetar. This may in part be due to the amount of oceanic crust that has subducted, or perhaps is an indication that the present volcanic arc east of Wetar is younger or that the original volcanic arc east of Wetar has been overridden by the Australian continental margin (Hall, 1998). The Australian continental crust appears to have subsequently collided with the islands of Alor and Wetar to create Timor in the early Pliocene after subduction removed the the oceanic lithosphere. This collision zone likely produced the geological evolution of other islands in the outer Banda Arc.

*Rafting and Oceanic Sea Currents*

The five islands are currently separated by a vast deep sea that further enhances reproductive isolation, however, there is no direct information currently available to elucidate the biogeographic mechanisms that gave rise to the current insular distribution of this species. Two models (vicariance and dispersal) could be used to explain the current insular biogeographic distribution. Vicariance models would infer that land bridges were historically present that interconnected the islands and allowed for movement of this species from one “island” to another, followed by a rising sea level that would have provided an effective reproductive barrier to preclude additional transient
movements between islands. This would explain the lack of sequence divergence between the three insular populations of *L. m. mackloti* on the islands of Timor, Semau and Roti. However, the geologic data presented herein precludes the possibility of land bridges that connect Sawu and Wetar to the Greater Timor island complex. Using the dispersal model to explain their current distribution, rafting could provide a possible explanation as to how founder populations could have seeded each of the islands where they are currently found that are not known to have been connected by way of land bridges (e.g. Sawu to Roti, and Wetar to Timor). Rafting includes both floating vegetation mats and movement of crustal elements through plate tectonics so that organisms could raft on terranes of Gondwana origin. Many species have been shown to have possibly migrated into Asia using the Lhasa and West Burma terrane in the Cretaceous and on the Indian plate in the Tertiary (Hall, 1998). Global oceanic currents have also been used to explain a number of ambiguous distributions that vicariance and terrane movement fails to explain. Large vegetation rafts following a climatic event (e.g. tsunami) have been shown to be effective dispersal agents to colonize new populations on islands (Hall, 1998). Circulation patterns of surface and near-surface waters in the Pacific ocean have been inferred by Creswell et al. (1993) at three stages during the Neogene as the Indonesian sea-way closed.

Based on historical circulatory patterns, the recent emergence of the five islands where *L. mackloti* inhabit, and the current topology inferred from molecular (Carmichael et al., 2003; Rawlings et al., 2004), morphological and behavioral (Carmichael et al., 2007; Carmichael unpubl. data) data, it would appear that rafting would be the most realistic scenario that could have allowed for the establishment of the populations of *L.*
mackloti throughout the insular range of this species, followed by dispersion throughout 'Greater Timor'. The major ocean current feature in this region is the Indonesian Throughflow current that originates from the Pacific Ocean and flows toward the Indian Ocean in a northeast to southwest direction across these islands (Fine et al. 1994). This current is carried through all of the passages along the Indonesian arc of islands from Sumatra to Timor to the Australian shelf. These surface currents have remained fairly stable and would appear to provide a likely route of dispersal throughout the current range of L. mackloti. CSIRO Marine Research has collected data from satellites that measure the sea surface topography (George Creswell, pers. corresp.). When comparing the sea surface topographic maps for 2004-2005, many eddies and cyclonic eddies in the sea surface of the Sawu Sea appear and drive sea currents anticlockwise around them and provide a possible dispersal route from Sawu to other islands north and east of Timor (e.g. Islands of Alor, Babar, Wetar). These eddies are periodic and have lifetimes of at least 18 months and a common diameter of 200 km. These eddies also frequently follow the northern coast of Australia and flow back to the north in the Timor Sea (George Creswell, pers. corresp.). Historical and current sea currents generally flow through this area in a westerly-southwesterly direction across the northern coasts of Wetar and Timor with eddies that flow along the northern coast of Australia in the Timor Sea in an easterly direction.

Our preliminary study demonstrates that the subspecies names currently used for L. mackloti appear to delineate the species boundaries as well, in that we have three well formed clades. We have not established a species-specific molecular clock and it would be necessary to calibrate the rate of molecular evolution for a gene suited to detecting
more recent divergences such as seen within the *L. mackloti* complex. Calibration of the rate of molecular evolution is also dependent on the availability of well dated fossils related to the taxa under study. Based on our use of two divergence rates (2% and 5.18% mya), we find that the latest divergence between the Australian (Northern Australia clade) water python (*L. fuscus*) and *L. m. savuensis* (Sawu Island) could have been 830,000 BP (before present) and the divergence between *L. m. savuensis* and *L. m. dunni* (Wetar) occurred 1.06 mya based on the higher divergence rate of 5.18% mya. This data is inconsistent with the emergence date of Sawu Island (~200 kya). If the emergence of Sawu Island occurred only recently, it would appear most likely that a geologically more ancient island from the inner Banda Arc islands provided founder animals that dispersed to Sawu only recently and therefore would be genetically similar to the island of origin. To resolve this dilemma, additional animals will be collected from islands proximal to (or in light of potential sea current pathways) Sawu and compared with both *L. m. savuensis* as well as *L. fuscus* from the Northern Territory of Australia in regards to molecular, morphological and behavioral data sets.

Preliminary behavioral data (based on courtship and pheromone trailing studies) shows congruent topologies when comparing phylogenetic trees derived from molecular, behavioral and morphological data (Carmichael unpubl. data). The *sawu* and *dunni/mackloti* (Sawu and Wetar/Greater Timor clades respectively) represent sister clades, therefore further resolution through the use of more genetic markers and increased sample size may be required to sufficiently delineate the pattern of dispersal between the various insular populations of *L. mackloti*. Carmichael et al. (2003) and Rawlings et al. (2004) both show that the Sawu population is phylogenetically basal to
the rest of the *L. mackloti* group and more similar to *L. fuscus* based on sequence divergence. Based on topologies derived from molecular phylogenies (Carmichael et al., 2003; Rawlings et al., 2004), *L. fuscus* from the Northern Territory of Australia likely gave rise to the Sawu population. Rawlings et al (2004) showed convincingly that the *L. mackloti* group likely originated from *L. fuscus* from the Northern Territory of Australia, and not New Guinea. Therefore it would appear that based on the proximity of Sawu to the northern coast of Australia, and it’s apparent phylogenetic closeness to *L. fuscus* from this area, that this insular population from Sawu later gave rise to the islands that make up the remaining part of its range or perhaps the seed island that gave rise to the current population we see today on Sawu. Northeasterly deflected eddies (George Cresswell, pers. com.) off the northern coast of Australia and along the south coast of Sawu could have provided a mechanism of dispersal to other islands throughout the known range of *L. mackloti*. An understanding of the geological evolution and historical sea currents in this region add support to this pattern of adaptive radiation we see with the *L. mackloti* complex.

Rafting-dispersal models appear to offer a plausible mechanism of radiation from Sawu to the inner Banda Arc islands including Wetar and subsequently Wetar to Timor. Although Auliya et al. (2002) have eluded that strong surface oceanic currents in this area likely represent geographic barriers aiding allopatric speciation, they do suggest that some large pythons, such as *Python reticulatus*, may be an excellent colonizer and could have dispersed across open oceans to inhabit new islands although it appears that surface currents would influence the direction of dispersal. Rawlings et al. (2004) also found evidence of several haplotypes in *L. mackloti* from Semau indicating recent dispersal.
around this region either through natural means or in correlation with human movement patterns. Nevertheless, historical sea currents and eddies likely played a role in the colonization of islands throughout this area.

Shine and Slip (1990) suggest that Australasian pythons represent a recent monophyletic radiation that originated from Asia during continental collision that occurred in the mid-Miocene and provided entrance into Australia. We concur with Rawlings et al. (2004) that *L. fuscus* radiated from Northern Australia through a possible land bridge between Lake Carpentaria (this area likely provided wet marshes preferred by *L. fuscus*) and New Guinea. Our behavioral data (Carmichael et al., 2007) appears to be highly congruent with the three clades presented in Rawlings et al. (2004) indicating RIMs that have formed in response to discrete evolutionary histories that have formed in response to isolation. In light of the mtDNA and morphological data presented here, it is possible that the three distinct clades also represent three unique evolutionary lineages and thus constitute distinct species.

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APPENDIX A

Distribution of *Liasis mackloti*, also showing the Indonesian Throughflow Sea Current. Eddies seasonally form throughout most of this region, giving rise to occasional sea current flows that are opposite the Indonesian Throughflow (see dashed arrow).
APPENDIX B

Plates of Liasis mackloti from the Islands of Sawu, Semau, Roti, Timor and Wetar

Plate 1. View of adult *L. m. savuensis* with eggs on the island of Sawu.

Plate 2. View of subadult *L. m. savuensis* from the island of Sawu approaching ontogenetic color change from a reddish color to a dark dorsal coloration.
Plate 3. View of neonate *L. m. savuensis* from the island of Sawu.

Plate 4. View of adult *L. m. dunni* from the island of Wetar.
Plate 5. View of adult *L. m. mackloti* from the island of Timor.

Plate 6. View of adult *L. m. mackloti* from the island of Semau.
Plate 7. View of *L. m. mackloti* from the island of Roti.

Plate 8. View of *L. m. mackloti* from the island of Roti.
CHAPTER II
GEOGRAPHIC VARIATION OF PHEROMONE TRAILING BEHAVIORS IN THE
INDONESIAN WATER PYTHON, *LIASIS MACKLOTI* SSP. (SQUAMATA: BOIDAE:
PYTHONINAE), OF INDONESIA'S LESSER SUNDAS ARCHIPELAGO

ABSTRACT

We conducted pheromone trailing experiments to investigate the level of
geographic variation present in this behavior in the macklot's python, *Liasis mackloti*
(Serpentes: Boidae). Three subspecies (*L. m. mackloti*, *L. m. savuensis*, and *L. m.
dunni*) are currently recognized and are found on several of the Lesser Sundas islands
of Indonesia including Sawu, Wetar, Timor, Semau and Roti. Based on prior studies,
three clades have been delineated (Wetar, Sawu, and a Timor-Semau-Roti clade). The
three subspecies display remarkable interpopulational (morphological, genetic and
behavioral) variation but only slight intrapopulational polymorphisms. A modified
Y-maze was used to test homotypic and heterotypic and male and female preferences
both within and between insular populations. The results of this study of the *L.
mackloti* complex indicate that during the breeding season male pythons were able to
discriminate between homotypic and heterotypic odors within each of the clades
(P<0.05). However, male pythons from Timor, Semau and Roti were unable to
differentiate pheromone trails produced by females from these three islands (P>0.05).
Male *L. m. dunni* from the island of Wetar are generally longer than females and also
exhibited specificity toward homotypic male trails. This homotypic male trailing
behavior, in addition to several male-male combat interactions observed during the
study suggests that males from Wetar may attempt to displace males for access to females. Pheromone trailing discrimination is an important pre-zygotic reproductive isolating mechanism that may have played an important role during speciation. We also present the role of dispersal and vicariance in shaping current patterns of geographic variation.

INTRODUCTION

Indonesia's flora and fauna has evolved many unique elements during its long isolation from other landmasses. Indonesia represents a regional hotspot of biodiversity with exceptional endemism that is not only biologically rich, but zoogeographically complex. Wallace (1865) conducted numerous biological inventories of the eastern Malay Archipelago and was the first to detect an apparent and abrupt disjunction between Asian and Australian floral and faunal forms. The abrupt cline between these two landmasses has led to intense biogeographic research to identify "lines" that reflect faunal and floral boundaries between the islands that compose this region (Brown and Toft, 1999; de Boer and Duffels, 1996; Simpson, 1977). Since Darwin's (1859) time, insular populations have played an important role in our understanding of the nature of variation and the determinants of its patterns. Empirical studies of natural populations are benefited by a simplified population structure generally observed on islands with reduced or no migration between adjacent islands, spatiotemporal sequential relationships, and small population size. These natural coincidences have allowed critical insights into our understanding of the evolutionary processes (Carson and Kaneshiro, 1976; Gorman et al., 1975; Schmitt, 1978) that influence patterns of geographic variation.
Indonesia supports a rich diversity of pythons with many species occurring on numerous islands. There are numerous examples where several islands harbor conspecifics that have acquired unique and isolated evolutionary histories (Auliya et al., 2002; Carmichael et al., 2003; Carmichael et al., 2007; Harvey et al., 2000; Keogh et al., 2001; Rawlings and Donnellan, 2003; Shine et al., 1998; and Shine et al., 1999). These isolated island populations may represent diverse multi-species complexes depending on the degree of endemism (How and Kitchener, 1997; Kitchener and Suyanto, 1996).

How and Kitchener (1997) and Kitchener and Suyanto (1996) detected a major boundary between the Sulawesi and the Lesser Sunda islands along the western half of Indonesia and the southern and northern Maluku islands toward the eastern end of Indonesia. This boundary delineates and corresponds to Weber’s Line, a natural biogeographic boundary separating these two major geographic areas (How and Kitchener, 1997). Additionally, How and Kitchener (1997) confirmed that separation (or formation) of the islands of the Lesser Sunda archipelago in the Pleistocene had a major influence in determining current faunal distributions and likely has played a significant role in the patterns of speciation and high level of endemism that we see today.

Phylogeography and Python Diversity

Phylogeography, the science that is concerned with the evolutionary processes that affect geographic distributions of genealogical lineages, is a tool that can be used effectively to determine the amount of biodiversity currently present (Avise, 2000) and provide critical insights into the biogeographical mechanisms that have led to the current distributions we see today. Several recent studies have detected unique geographic patterns of genetic and morphological variation in Indonesian pythons including the
scrub pythons, *Morelia amethystina* group (Harvey et al., 2000), short-tailed pythons, *Python curtus* group (Keogh et al., 2001), green tree pythons, *Morelia viridis* (Rawlings and Donnellan, 2003), Australo-Papuan *Liasis* pythons (Rawlings et al., 2004), and the reticulated pythons, *Python reticulatus* (Shine et al., 1999). However, there seems to be a lack of experimental investigation of patterns of behavioral variation in the pythons throughout this region.

**Taxonomic Overview of *Liasis mackloti* ssp**

The *L. mackloti* complex is an insularized group that are robust moderately thick bodied snakes attaining lengths of up to 3-4 meters and demonstrate geographical variation with regard to morphological (size, coloration, markings, scalation), behavioral (courtship and premating distance reduction behaviors), and molecular (mtDNA) characters (Carmichael et al., 2003; Rawlings et al., 2004). Two subspecies of *L. mackloti* are found on the outer Banda Arc of islands, *L. m. mackloti* (Dumeril and Bibron, 1844), from the islands of Timor, Semau and Roti (referred to as ‘Greater Timor’), and *L. m. savuensis* (Brongersma, 1956), from the island of Sawu. One subspecies, *L. m. dunni* (Stull, 1932) is found on one of the inner Banda Arc of islands, Wetar. Additionally, How and Kitchener (1997) discovered *L. mackloti* to exist on the island of Alor located west of Wetar, also part of the inner Banda Arc. How and Kitchener (1997) found that the snake fauna on Alor and Wetar were more similar when compared to the outer Banda Arc islands as compared to the adjacent inner Banda Arc islands to the west, suggesting that a major barrier exists between Lembata and Alor. They also detected another barrier between Sumba and Sawu on the outer Banda Arc that delineates assemblages to the east and west. The Sawu snake fauna appear to be
more closely allied to the ‘Greater Timor’, Alor and Wetar islands at the level of species based on principal component analysis when comparing species/genera assemblages on the different islands (Rawlings et al., 2004).

*Liasis mackloti* are sexually dimorphic in size and color/pattern. In the Wetar population, males are typically larger in overall size than females, while females on the islands of Timor, Roti, Semau and Sawu are typically larger than males. This intersexual morphometric difference has interesting behavioral consequences. Typically male-male combat and territoriality (a behavior that has been difficult to identify in snakes) occurs more frequently in populations of animals where the males achieve greater size, and female choice for males in populations where females are larger than males (Gillingham, 1987). We have found that male *L. m. dunni* from the island of Wetar do engage in male-male combat, which has not been observed in *L. m. mackloti* and *L. m. savuensis* (Carmichael et al., 2003; Carmichael et al., 2007).

*Pre-Courtship Behavior (Pheromone Trailing)*

A snake’s survival and reproductive success is strongly dependent on its ability to locate necessary resources and recognize members of its own species. This conspecific recognition may play an important role in preventing hybridization between related species that are not spatially separated. Also this may act to prevent hybridization among individuals from isolated populations in the case of dispersal. Such prezygotic reproductive isolation prevents the waste of gametes and saves the energy normally spent during courtship (Ford, 1982). Many vertebrates, including snakes, use chemical signals to orient towards potential resources, which may include mates (Ford, 1986; LeMaster et al., 2001), food (Savidge, 1988), breeding sites, hibernacula, and refuge (Mason, 1992;
Mason et al., 1998; LeMaster et al., 2001). Chemical signals often used by vertebrates for orientation include pheromones. Pheromones are semiochemicals released by individuals that affect the physiology and behavior of members of the same species (Ford, 1986). Once deposited in the environment, a pheromone trail can guide a receiver directly to the location of the conspecific producer.

Trailing behavior has been recorded in field studies (Carpenter, 1977; Finneran, 1949; Fitzsimons, 1930; Ford, 1986) and in laboratory experiments (Devine, 1977; Ford, 1986; Gehlbach et al., 1971; Greene et al., 2001). Most cases involve males following trails laid by females; an activity that was considered to be associated with reproduction. However, trails also may function in forming non-reproductive aggregations (Costanzo, 1989). Pheromone trailing behavior between conspecifics is widespread in snakes, with a number of documented instances of species within five families demonstrating the behavior (Ford, 1986). In snakes, pheromone trailing is mediated by pheromones sequestered on the skin surface, which are laid down along with skin lipids as a snake passes across the substrate (Gehlbach et al., 1971; LeMaster et al., 2001). Subsequent snakes encountering the trail are able to transport the pheromonal cues via tongue flicking to the vomeronasal organ in the roof of the mouth where the cues are detected (Gehlbach et al., 1971).

Most reports document the occurrence of conspecific trailing behaviors during the breeding season and describe male snakes seeking out sexually receptive females (Ford, 1982, 1986). However, less is known about the presence of patterns of geographical variation in pheromone trailing behaviors within a species or between allopatric populations of a single-species complex. Pheromone trailing as a pre-zygotic isolating
mechanism, may play an important role in maintaining the genetic distinctiveness of unique populations and decrease the chance of hybrid matings. Furthermore, pheromone trailing specificity would also save the male the energy of trailing a heterospecific pheromone trail, trailing the wrong sex, or trailing a nonreceptive conspecific female and reduce the male’s exposure to predators (Costanzo, 1989).

*Liasis m. mackloti, L. m. savuensis, and L. m. dunni* represent distinct allopatric populations and no reported cases of hybridization between these subspecies are known. Gene flow appears to be minimal and does not appear to be occurring on a widespread basis or at least is limited by one or more RIMs (the ocean, for example) that exists between the five known insular populations of *L. m. ssp* (Carmichael et al., 2003; Carmichael et al., 2007; see Rawlings et al., 2004 for possible exceptions). RIMs play an important role in the initial formation of new species and having the ability to detect and locate a conspecific during the reproductive season would obviously have important consequences for the evolution of that species.

**Objectives of Study**

Very few studies employing multiple data sets (e.g. morphological and molecular) have incorporated behavioral data to accurately assess phylogeographic relationships between conspecific insular populations of squamate (lizards and snakes) reptiles. De Queiroz and Wimberger (1993) have argued convincingly that incorporating behavioral studies into multiple data sets provide the best means of obtaining robust estimates of phylogenetic relationships. A major goal of this project was to augment the existing genetic data (Carmichael et al., 2003; Rawlings et al., 2004) with behavioral data to determine whether or not the observed level of variation represents a potential multi-
species complex. In this chapter, we are reporting on unique patterns of geographic variation of pheromone trailing behaviors.

Pre-courtship behaviors (e.g. pheromone trailing) will be reviewed within this chapter to evaluate the extent of behavioral variation within and between the five insular populations that occur on the islands of Sawu, Wetar, Timor, Semau and Roti. Because of the implications regarding the evolution of reproductive isolating mechanisms (RIMs) within and between island populations, I was primarily interested in the geographic variation of courtship displays and pre-courtship distance reduction mechanisms (i.e. pheromone trailing) and the correlated differences in communication.

In this chapter, I will report on the results from the pheromone trailing experiments (other data currently being compiled for publication) as we provide additional insights of the biogeography of this group to support the existing literature (e.g., Rawlings et al., 2004).

MATERIALS AND METHODS

Sampling and Specimens

Pythons used for this study were collected and exported by Duncan MacRae of CV. Herpafauna Indonesia, Bali, Indonesia between 1995 and 2000. Only adult wild-caught animals were used for this project. Animals were used in the behavioral experimentation within 90 days after arriving in the United States. All snakes were quarantined following arrival and the animals were treated for potential ectoparasites and endoparasites. Five island populations of L. mackloti were included in this study; however, there is a high likelihood that additional populations of this species may inhabit...
nearby islands, such as Altauro and Alor (MacRae, pers. comm.), and Babar (Rawlings et al., 2004).

To examine pheromone trailing differences between the different island populations of *L. mackloti*, between 8-25 adult pythons were field collected from each island population (N=96) (*L. m. savuensis* from Sawu, N=20/8:12 male to female ratio; *L. m. mackloti* from Semau, N=8/4.4; Roti, N=25/10.15; *L. m. mackloti* from Timor, N=25/10.15; and *L. m. dunni* from Wetar, N=18/8.10). Pythons were maintained individually in appropriate sized Vision® reptile cages (ranging between 91 to 122 cm in length). A thermal gradient was maintained within each enclosure using 10.16 cm wide heat tape that was thermostatically controlled to provide a range of temperatures from 27°C to 35°C, and cages were maintained in a heated laboratory that was thermostatically controlled to give an ambient air temperature of approximately 27-29°C. Enclosures were maintained on a 14L:10D photoperiod. Water was offered ad libitum and snakes were fed every 12-16 days on appropriately sized thawed out frozen rodents, chicks, and quail. To induce pheromone trailing behaviors, the snakes were exposed to a three-month cool down period between October through January where the range of temperatures dropped to 25°C to 27°C, with a hotspot of 32°C available for 3 hours per day.

**Pheromone Trailing Experiments**

Snakes were tested using a modified Y-maze (three side arms instead of two) to determine whether *L. mackloti* ssp. could discriminate and make directional responses to homotypic and heterotypic gender biased pheromone trails. The modified Y-maze consisted of a base arm of the Y-maze that was 1.82 m long and 30 cm in diameter. At
the end of the base arm, three side arms (two experimental arms were positioned at a 45° angle from the base arm, and the third experimental arm was medially located between the two angled arms and projected straight out from the base arm) extended from the base arm. The base and experimental arms were constructed of wood which was lined with white butcher paper that was changed between runs. The paper was carefully applied so that the bottom and sides of the arms were completely covered. A waxy surface on the bottom of the paper eliminated the possibility of cross-contamination to the Y-maze structure. The configuration was arranged with coupling joints so that the arms could be easily removed between trials and re-papered before each run. Covering the Y-maze with new butcher paper between experimental tests was required since pheromones are known to be stable for a period of approximately 30-180 min (LeMaster et al., 2001). The following behavioral trials (treatments) were run to test the response of: 1) males to homotypic and heterotypic female trails (within and outside the breeding season), 2) males to homotypic and heterotypic male trails (within and outside the breeding season), 3) females to homotypic and heterotypic female trails, and 4) females to homotypic and heterotypic male trails. Additionally, a controlled experiment was established by subjecting a test snake to the modified Y-maze that had no snake odor and anthropogenic odors (perfumes, human odor, etc.).

A test snake was placed into an appropriate sized, opaque plastic container with a single opening facing the base arm. The Y-maze test arena was laid out facing in a northerly direction. This direction was selected following initial trials to assure that snakes are not orienting based on celestial or geomagnetic cues. For consistency between tests, all test arenas were positioned so that the escape hole on the holding box always
faced a northerly compass direction. For each trial, we randomly laid down a snake trail through one of three experimental arms to reduce the potential influence of "learning" or preference for a certain direction that could bias the trailing direction of the test snake. Trails were laid down by randomly selecting a stimulus snake (male or female, and homotypic or heterotypic, depending on the treatment) and allowing this snake to move through a specified experimental arm by capping off the other two arms. In addition to the pheromone trail secreted by the stimulus snake within one of the experimental arms, another had a heterotypic trail, and the third experimental arm was the control and did not contain any pheromone cues (or a different heterotypic trail). The pheromone trail of the stimulus snake was assumed to extend down the entire length of the experimental arm with each arm terminating at another plastic opaque container (i.e. "endpoint container") as the snake was assumed to have, and would have had to move the entire length of the arm to reach the endpoint container.

Once the stimulus trail was established, a randomly chosen test snake was placed into the opaque box and allowed the snake to acclimate for five minutes. The opening into the base arm was subsequently uncovered and the test snake was allowed to exit of its own accord. Trials were terminated when the test snake did not exit the opaque box after 30 min or trials in which the test snake stopped within the base arm and did not continue through the experimental arms. The endpoint containers were located at the end of each experimental arm so that the test snake could choose between one of the three experimental arms and eventually enter one of the endpoint containers. Using videography, once a test snake entered one of the three experimental endpoint containers, the trial was considered to be successful and the data recorded. We also noted whether
the test snake displayed a trail contact response (TCR), an overt response characterized
by careful investigation of the substrate by the snake with short, rapid tongue-flicks
followed by subsequent investigative movement along the pre-existing trail (Brown and
MacLean, 1983). Trail contact responses are commonly observed in snakes assumed to
be actively trailing (Ford, 1986; Mason, 1992; Mason et al., 1998). For each test snake,
the choice of experimental arm, entry of one of the three endpoint containers, and
number of tongue-flicks were used to evaluate pheromone trailing discrimination.

We performed 8-15 trials for each of the insular populations of *L. m. ssp.* within
each of the four treatments (e.g. male to female trails, male to male trails, female to male
trails, and female to female trails). Care was taken not to conduct more than one test per
snake within each of the experimental treatments. The effect of pheromone trails on arm
selection (i.e. experimental arms) was tested with chi-square tests of independence
(P<0.05) utilizing contingency tables (stimulus trail selection between the three
experimental arms of the modified Y-maze modified using Zar, 1984). Independence of
trials was assumed due to the completely random design. The pheromone trailing results
were qualitatively compared between insular populations. Various trials were used to
include both intra (homotypic) and interpopulational (heterotypic) introductions.
Statistical significance in male trailing behavior was analyzed utilizing one-way binomial
tests. Level of significance for each test was set at P < 0.05.

**RESULTS**

All of the male *L. m. dunni, L. m. savuensis,* and *L. m. mackloti* passed through
the experimental arms and selected an endpoint container. Fifty-seven percent of the
females of each subspecies completed the Y-maze and selected an endpoint container,

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albeit without significant preference (P>0.05). When males were exposed to male, and females exposed to both female and male homotypic and heterotypic trails, there was a less deliberate movement into the experimental arms and in many of the trials, the snakes seemed to move randomly throughout the experimental arms and base arm without any type of discriminate orientation toward a pheromone trail. Of the 50 males completing the experimental (trials present) trails, 90% demonstrated at least some inclination to follow homotypic female pheromone trails.

Control.—Several trials were conducted to test for experimental arm bias by presenting test snakes with three experimental arms lacking pheromone odor. Choice of experimental arms was not significantly different (P>0.05). Additionally, there was no significant difference (P>0.05) for experimental arms containing anthropogenic odors (P>0.05).

Male test snakes with homotypic and heterotypic female trails.—Male test snakes of *L. m. dunni*, *L. m. savuensis*, and *L. m. mackloti* entered the experimental arm containing homotypic female pheromone trails at significantly greater than chance frequency (P<0.001). There was no significant difference (P>0.05) when the male test snake was presented with pheromone trails of a heterotypic female. Male snakes from Timor, Semau and Roti did not significantly choose experimental arms with island specific homotypic trails over heterotypic trails from the other islands comprising the Timor-Semau-Roti island complex (P>0.05) but did choose females from one of these three islands over Sawu and Wetar (P<0.05). All but five of the males tested displayed TCRs immediately upon first exiting the box (Table 1) that leads into the base arm when a homotypic female pheromone trail was present in one of the three experimental arms.
TCRs were also observed at the junction into the three experimental test arms of the Y-maze.

Table 1. Discrimination of male snakes from each of the five island populations when presented with female pheromone trails during the breeding season (L. m. mackloti - T = Timor; L. m. mackloti - S = Semau; L. m. mackloti - R = Roti; LMS = L. m. savuensis; LMD = L. m. dunni; LMMT = L. m. mackloti - Timor; LMMS = L. m. mackloti - Semau; and LMMR = L. m. mackloti - Roti). The experimental arm (= Arm Contained) indicates a female from one of the five islands. The sequence of testing and which experimental arm received an island specific pheromone trail was randomly determined. Significance was determined using a binomial test with equal probability (N* = number of males tested; N** = number of individuals selecting that trail).

<table>
<thead>
<tr>
<th>N*</th>
<th>Male Subspecies Tested</th>
<th>Arm Contained</th>
<th>N** Arm Contained</th>
<th>N** Arm Contained</th>
<th>N** Arm Contained</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>L. m. savuensis</td>
<td>LMS</td>
<td>11 LMD</td>
<td>3 LMMT</td>
<td>1 P&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>L. m. dunni</td>
<td>LMS</td>
<td>3 LMD</td>
<td>10 LMMT</td>
<td>2 P&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>L. m. mackloti - T</td>
<td>LMS</td>
<td>1 LMD</td>
<td>2 LMMT</td>
<td>12 P&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>L. m. mackloti - S</td>
<td>LMS</td>
<td>1 LMD</td>
<td>1 LMMS</td>
<td>6 P&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>L. m. mackloti - R</td>
<td>LMS</td>
<td>2 LMD</td>
<td>2 LMMR</td>
<td>11 P&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>L. m. mackloti - T</td>
<td>LMMT</td>
<td>4 LMMS</td>
<td>5 LMMR</td>
<td>6 P&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>L. m. mackloti - S</td>
<td>LMMT</td>
<td>3 LMMS</td>
<td>6 LMMR</td>
<td>6 P&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>L. m. mackloti - R</td>
<td>LMMT</td>
<td>5 LMMS</td>
<td>4 LMMR</td>
<td>6 P&gt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

Male test snakes with homotypic versus heterotypic male trails.—When male test snakes of each of the three subspecies were presented with experimental arms containing pheromone trails of homotypic and heterotypic males, there was no significant difference (P>0.05) in their choice of experimental arms and there was no evidence of the exhibition of TCRs when presented with same sex trails, suggesting the movement through the experimental arms was random. The only exception was that males from Wetar show a significant choice (P<0.05) to enter experimental arms containing homotypic trails. Males from Wetar deviated from the normal reaction of males exposed to homotypic and heterotypic male trails. When males from Wetar were exposed to homotypic and
heterotypic male trails, the Wetar males significantly (P<0.05) chose the experimental arm containing the homotypic male trail (Table 2). Males from Wetar have also demonstrated aggression behavior toward conspecific males in ex-situ experimental tests (Carmichael et al., 2003).

Table 2. Discrimination of Male snakes from each of the five island populations when presented with male pheromone trails during the breeding season (L. m. mackloti – T = Timor; L. m. mackloti – S = Semau; L. m. mackloti – R = Roti; LMS = L. m. savuensis; LMD = L. m. dunni; LMMT = L. m. mackloti – Timor; LMMS = L. m. mackloti - Semau; and LMMR = L. m. mackloti - Roti). The experimental arm (= Arm Contained) indicates a male from one of the five islands. The sequence of testing and which experimental arm received an island specific pheromone trail was randomly determined. Significance was determined using a binomial test with equal probability (N* = number of males tested; N** = number of individuals selecting that trail).

<table>
<thead>
<tr>
<th>N*</th>
<th>Male Subspecies Tested</th>
<th>Arm Contained</th>
<th>N** Arm Contained</th>
<th>N** Arm Contained</th>
<th>N** Arm Contained</th>
<th>N** Probability</th>
</tr>
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<tbody>
<tr>
<td>12</td>
<td>L. m. savuensis</td>
<td>LMS</td>
<td>4</td>
<td>LMD</td>
<td>3</td>
<td>LMMT 5</td>
</tr>
<tr>
<td>14</td>
<td>L. m. dunni</td>
<td>LMS</td>
<td>0</td>
<td>LMD</td>
<td>12</td>
<td>LMMT 2</td>
</tr>
<tr>
<td>15</td>
<td>L. m. mackloti – T</td>
<td>LMS</td>
<td>3</td>
<td>LMD</td>
<td>6</td>
<td>LMMT 6</td>
</tr>
<tr>
<td>8</td>
<td>L. m. mackloti – S</td>
<td>LMS</td>
<td>3</td>
<td>LMD</td>
<td>2</td>
<td>LMMS 3</td>
</tr>
<tr>
<td>13</td>
<td>L. m. mackloti – R</td>
<td>LMS</td>
<td>3</td>
<td>LMD</td>
<td>4</td>
<td>LMMR 6</td>
</tr>
</tbody>
</table>

Male test snakes with male and female trails.—When males were exposed to both male and female pheromone trails, males significantly chose homotypic female trails (P>0.05) (Table 3). Although male L. m. dunni will significantly choose homotypic male trails over heterotypic male trails, when given the option between a homotypic male versus homotypic female trails, males will generally select the female trail (P<0.05).
Table 3. Discrimination of Male snakes from each of the five island populations when presented with conspecific male and female pheromone trails during the breeding season (L. m. mackloti – T = Timor; L. m. mackloti – S = Semau; L. m. mackloti – R = Roti; LMS = L. m. savuensis; LMD = L. m. dunni; LMMT = L. m. mackloti – Timor; LMMS = L. m. mackloti – Semau; and LMMR = L. m. mackloti – Roti). The experimental arm (= Arm Contained) indicates a male or female from each of the five islands. The sequence of testing and which experimental arm received an island specific pheromone trail was randomly determined. Significance was determined using a binomial test with equal probability (N* = number of males tested; N** = number of individuals selecting that trail).

<table>
<thead>
<tr>
<th>N*</th>
<th>Male Subspecies Tested</th>
<th>Arm Contained</th>
<th>N**</th>
<th>Arm Contained</th>
<th>N**</th>
<th>Arm Contained</th>
<th>N**</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>L. m. savuensis</td>
<td>LMS♂</td>
<td>11</td>
<td>LMS♀</td>
<td>3</td>
<td>No Trail</td>
<td>1</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>12</td>
<td>L. m. dunni</td>
<td>LMD♂</td>
<td>3</td>
<td>LMD♀</td>
<td>10</td>
<td>No Trail</td>
<td>2</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>15</td>
<td>L. m. mackloti – T</td>
<td>LMMT♂</td>
<td>1</td>
<td>LMMT♀</td>
<td>12</td>
<td>No Trail</td>
<td>2</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>8</td>
<td>L. m. mackloti – S</td>
<td>LMMS♂</td>
<td>1</td>
<td>LMMS♀</td>
<td>6</td>
<td>No Trail</td>
<td>1</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>15</td>
<td>L. m. mackloti – R</td>
<td>LMMR♂</td>
<td>2</td>
<td>LMMR♀</td>
<td>11</td>
<td>No Trail</td>
<td>2</td>
<td>P&lt;0.05</td>
</tr>
</tbody>
</table>

Test for temporal changes in male trailing response.—Male snakes tested outside of the breeding season (as determined by the presence of courtship behaviors) did not demonstrate a significant preference for males or females (P>0.05), thus indicating a potential temporal change in the semiochemical composition of the pheromone that is recognized by males during the breeding season, but not during the non-breeding season (Table 4).

Female test snakes with homotypic and heterotypic male trails.—Female test snakes for each subspecies randomly exited the base arm and into the experimental arms when tested with homotypic and heterotypic male pheromone trails (P>0.05) and did not display TCRs at any point during the trials (Table 4). Female test snakes for each subspecies also randomly exited the base arm and into the experimental arms when tested with a homotypic and heterotypic female pheromone trail (P>0.05). Females also did not display TCRs to homotypic and heterotypic pheromone trails.
Table 4. Discrimination of Male snakes from each of the five island populations when presented with male pheromone trails outside the breeding season (L. m. mackloti - T = Timor; L. m. mackloti - S = Semau; L. m. mackloti - R = Roti; LMS = L. m. savuensis; LMD = L. m. dunni; LMMT = L. m. mackloti - Timor; LMMS = L. m. mackloti - Semau; and LMMR = L. m. mackloti - Roti). The experimental arm (= Arm Contained) indicates a male from one of the five islands. The sequence of testing and which experimental arm received an island specific pheromone trail was randomly determined. Significance was determined using a binomial test with equal probability (N* = number of males tested; N** = number of individuals selecting that trail).

<table>
<thead>
<tr>
<th>N*</th>
<th>Male Subspecies Tested</th>
<th>Arm Contained</th>
<th>N**</th>
<th>Arm Contained</th>
<th>N**</th>
<th>Arm Contained</th>
<th>N**</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>L. m. savuensis</td>
<td>LMS 3 LMD 5 LMMT 2</td>
<td>P&gt;0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>L. m. dunni</td>
<td>LMS 4 LMD 3 LMMT 3</td>
<td>P&gt;0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>L. m. mackloti - T</td>
<td>LMS 4 LMD 3 LMMT 3</td>
<td>P&gt;0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>L. m. mackloti - S</td>
<td>LMS 2 LMD 1 LMMS 3</td>
<td>P&gt;0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>L. m. mackloti - R</td>
<td>LMS 3 LMD 4 LMMR 3</td>
<td>P&gt;0.05</td>
<td></td>
<td></td>
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</table>

_Tongue-flick response during tests._ Tongue flicks during the TCR, rather than being aerial and full length (as when actually following the trail in the base arm), were short and rapid and the tips touched the substrate. The TCR during male-female trials lasted ca. 5 s and involved ca. 2-15 tongue flicks. In trials where a TCR was not observed between homotypic and heterotypic male-male, female-male, and female-female trials, there was no significant difference (P>0.05) in their choice of experimental arms.

**DISCUSSION**

Our study demonstrates that pheromone trailing behaviors appears to exist based on the ability of reproductively active males to follow homotypic female pheromone trails indicating an ability of these snakes to locate potential mates during the mating season. Interestingly, male _L. m. dunni_ from the island of Wetar preferentially trail...
conspecific male trails over male trails produced by individuals from other insular populations, however, male \textit{L. m. mackloti} from Timor, Semau and Roti, and \textit{L. m. savuensis} from Sawu, did not preferentially trail homotypic male trails. Male-male combat was observed in \textit{L. m. dunni} but not in \textit{L. m. mackloti} and \textit{L. m. savuensis}, which is congruent with the pheromone trailing behavioral results that show that male \textit{L. m. dunni} are the only insular population that will trail homotypic male trails (Table 2).

Within the \textit{L. mackloti} complex, male-male ex-situ combat was only observed in \textit{L. m. dunni} during our artificially produced breeding season when females have been recently present in the test arena. When male \textit{L. m. dunni} are presented with both conspecific male and female pheromone trails, the test male will significantly (P<0.05) choose the female pheromone trail over the male, which in nature, would be the option that would put the male in close proximity to the female for conducting courtship behaviors. This trailing behavior appears to be seasonally (breeding) induced as pheromone trailing experiments conducted outside of the breeding season did not produce any significant preferences for conspecific male or female trails (P>0.05). This would certainly reduce the expenditure of energy required during male-male combat and trailing non-receptive females outside of the breeding season. Our results provide substantial support that male \textit{L. m. dunni} may attempt to exclude other males from a known area or at a minimum to displace a male for access to a female. This behavior needs to be further tested both in the field and in the laboratory to fully understand its complexity.

Males from the other islands seem to follow the boundary of each clade, in that males from the island of Sawu will significantly trail female trails from Sawu as
compared to the other island populations. This applies to Wetar as well, but Timor, Semau and Roti males do not seem to show a significant preference for their specific island of origin \((P > 0.05)\) when tested with females from Timor, Semau and Roti. Males from Timor, Semau, Roti and Sawu did not show any significant preference for males originating from their respective island and do not appear to show combat behaviors although this needs further testing.

We have observed different behaviors of courtship and pheromone trailing between insular populations that seems to demonstrate that there are currently three clades with unique evolutionary histories; Sawu Island and Wetar Island populations are separate clades, and the populations from the islands of Timor, Semau and Roti represent a single clade. These clade designations are also supported by molecular data (Carmichael et al., 2003; and Rawlings et al., 2003). Our results are congruent with the results of Rawlings et al. (2004) that suggest this subspecies complex may in fact represent distinct or at least incipient species and provides strong support for the monophyly of the three identified clades (Sawu, Wetar, and the Timor-Semau-Roti (TSR) complex).

Based on the level of behavioral differences (pheromone trailing) and unique evolutionary histories of each island population, we believe at minimum that each clade (Sawu clade, TSR clade, and the Wetar clade) represents an evolutionarily significant unit (ESU). These three well-supported clades (Carmichael et al., 2003 and Rawlings et al., 2004) demonstrate that the currently accepted subspecies do indeed represent three distinct evolutionary lineages.
Various species concepts have been thoroughly discussed in Frost and Hillis (1990) and Frost and Kluge (1994), however, because there have been numerous cases of ex-situ intergeneric hybridization, such as between *L. mackloti* and *Morelia spilota* (Dave Barker, pers. com.), reproductive isolation alone is not viewed as a necessary qualifier to determine the species status of each endemic population of *L. mackloti*. Instead, Kluge (1993) and Harvey et al. (2000) state that species complexes are best studied using phylogenetic analyses “in which demes or individuals are chosen as terminal taxa” (Kluge, 1993; Harvey et al., 2000). Preliminary behavioral data (based on pheromone trailing studies and unpublished data investigating geographic variation in courtship displays) shows congruent topologies when comparing phylogenetic trees derived from molecular, behavioral and morphological data (Carmichael et al., 2003; Rawlings et al., 2004).

Our pheromone trailing experiments indicate the presence of prezygotic RIMs that reduce the likelihood of hybridization and demonstrate conspecific (i.e., island) recognition. Since pheromone trailing has the main function of reducing distance between males and females during the breeding season so courtship can progress, these experiments validate our opinion that island-specific recognition for conspecifics demonstrates that speciation is in progress.

Information concerning the specificity of the pheromone trails produced by *L. mackloti ssp.* provide useful information for evaluating the mechanisms that are present to reduce hybridization between uniquely adapted populations. The data extracted from these procedures were not directly scored for phylogenetic evaluation, but rather was used to confirm the presence of prezygotic (premating) reproductive isolating
mechanisms that may play an important evolutionary mechanism leading to this potential species complex. Species-specific pheromone trails are a useful prezygotic reproductive isolating mechanism as they would decrease the chance of hybrid matings.

Reproductive isolation between insular populations of *L. m. savuensis* on Sawu and *L. m. dunni* on Wetar, and *L. m. mackloti* within the TSR ('Greater Timor') complex, based on the results of our behavioral tests and published molecular data (Rawlings et al., 2004), would advocate recognizing these three clades as potentially separate species.

Future studies will include a multi-character based phylogeny of the *L. mackloti* species complex which will be generated using additional behavioral data (e.g. courtship), morphological data and molecular markers to provide a robust phylogeny to more thoroughly resolve the systematics of this insular group of pythons and to make stronger inferences relative to the biogeography of this potential species complex. Additionally, potential behavioral variation within a specific island (e.g. Semau) where multiple genetic haplotypes have been observed (Rawlings et al., 2004) will be investigated to test for intrapopulational behavioral variation. A molecular clock will also need to be developed as the sequence divergence appears to be very rapid based on how long some of these islands have been above sea level (e.g. Sawu has only been above sea level for about 200ka). Furthermore, phylogeographic comparisons between our study and published data of other vertebrate species will be made to investigate whether or not historical geologic events have uniformly shaped the distribution of life forms throughout the Indonesian Archipelago. A comparison of our study with other phylogeographic investigations of the Indonesian floral and faunal communities could
provide invaluable information to conservation managers faced with the growing challenge of conserving habitat versus isolated species.

ACKNOWLEDGMENTS

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LITERATURE CITED


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CHAPTER III

GEOGRAPHIC VARIATION IN MALE COURTSHIP BEHAVIORS OF THE
INDONESIAN WATER PYTHON, *Liasis mackloti* ssp. (Squamata: Boidae: Pythoninae)

ABSTRACT

Courtship behaviors were compared between three subspecies of the Indonesian water python, *L. m. mackloti, L. m. savuensis, and L. m. dunni,* to elucidate patterns of geographic variation in male courtship behavior in standard laboratory conditions. The three subspecies are found on a series of islands that are part of the Outer Banda Arc and Inner Banda Arc of the Lesser Sundas Archipelago. The insular populations of *L. mackloti* ssp. within this archipelago indicate that separation between islands during the Pleistocene played a role in determining current assemblages and variation within species. The islands of eastern Indonesia form biogeographic subregions that have relatively high levels of endemism and evidence of incipient speciation as a consequence of changes in sea-levels and climate during the Pleistocene. Two predominant models to explain the biogeography of *L. mackloti* ssp. include vicariance and dispersion, both of which likely played an important role in shaping the distribution pattern of these pythons that we currently observe and are discussed in this chapter. *L. mackloti* generally adheres to the triphasic schema including tactile-chase, tactile-alignment, and intromission and coitus. However, the use of the pelvic spurs during tactile-chase and tactile-alignment is a unique boid-typical motor pattern and likely plays an important premating reproductive isolating mechanism that prevents interbreeding between conspecific individuals. Behavioral sequence chains were derived from videotaped experimental tests and subjected to transition analysis. We observed variation in both the
frequency of occurrence and the sequence of the principal courtship behaviors and, when compared statistically, most of these behaviors differed between populations. We observed geographically unique island patterns in the sequence in which male courtship behaviors are displayed. Our data yielded the following information: 1) the courtship sequence in all three subspecies is not random; 2) the sequence of *L. m. savuensis* is much less complex than that of *L. m. mackloti* and *L. m. dunni*; and 3) the tactile pelvic spurring target sites used by males on the females' dorsum is different between the three subspecies. We also determined whether sexual isolation among selected populations existed. In the first study, we performed male-female reciprocal crosses of pythons between two different insular populations and measured mating success and whether eggs were produced. The results of this test reveal that sexual isolation occurs between the three defined subspecies. The behavioral differences in regards to courtship sufficiently delineate the subspecies and no significant difference was detected when comparing the three different island populations of *L. m. mackloti* (Timor, Semau and Roti), however, they were significantly different than *L. m. savuensis* and *L. m. dunni*. The historical isolation of Sawu and Wetar Islands, and connection of Timor, Semau and Roti Islands reveals interesting biogeographic patterns and the snake fauna of islands within the Lesser Sunda group indicate that separation between islands during the Pleistocene played a role in determining current assemblages and variation within species detected in this study.

INTRODUCTION

Patterns of geographic variation often exists between conspecific populations in traits associated with fitness, metabolic rates, acclamatory abilities, thermal and osmotic tolerances and life-history parameters (Travis, 1994a). There also exists wide variation in
certain types of behavior patterns, such as those involved with migration (Dingle, 1994),
nest construction (Lynch, 1994), and reproductive behavior (Gillingham, 1977). Such
extensive variation between populations raises interesting questions relating to the
causality of sexual and/or behavioral reproductive isolation and evolutionary
differentiation of populations leading to speciation. Four important questions emerge
from the literature (Kaneshiro, 1976; Verrell and Arnold, 1989; How and Kitchener,
1997): (1) How much of this variation is under genetic control? (2) To the extent that
there is genetic control, have the observed patterns of variation between populations been
produced by natural selection? If so, then such patterns are strong evidence for the power
of selection to match local phenotypes to local ecological conditions. (3) What
evolutionary processes give rise to sexual isolation and what genetic mechanisms
underlie the process and what behavioral mechanisms maintain isolation. (4) Do these
patterns of intraspecific variation mirror interspecific variation? If so, then studies of this
type of intraspecific (or interpopulational) variation can be used as model systems for
large-scale evolutionary processes and to elucidate process of incipient speciation. Much
is known about the existence of such trait associations, and the reproductive behaviors of
snakes have been well studies, however, little is known about the differences between
conspecific boid populations and their evolutionary significance. Genetic divergence
arising from selection for adaptations to local environments among allopatric populations
and genetic drift may lead to sexual isolation when such divergence is correlated with
behavior (Mayr, 1963; Koepfer, 1987). These behavioral differences when present in
newly formed sympatric populations lead to genetic divergence that might strengthen
isolation. This divergence may occur in sympatric subpopulations that have adapted to
different microhabitats. The mechanism of speciation, and whether or not this is a result of allopatric or sympatric speciation, can be readily obtained from comparisons of the temporal and sequential components of courtship behavior exhibited between and within populations of geographically isolated groups (Markow & Hanson, 1981), and therefore might provide important insights of specific behavioral patterns exhibited during the process of speciation. Additionally, in snakes, there are some evolutionarily interesting questions that can be explored regarding the role of sexual selection in the mating systems present between conspecific populations, and the correlatory selective pressures that enhance behavioral differences in regards to courtship.

Sexual and/or behavioral reproductive isolation has been proposed as an important step in the evolutionary differentiation of populations leading to speciation. Groups in which this has been observed include *Drosophila* (e.g. Kaneshiro, 1976; Watanabe and Kawanishi, 1979; Wasserman and Koepfer, 1980), lacewings (Futuyma and Mayer, 1980; Tauber and Tauber, 1997), salamanders (Houck et al., 1988; Verrell and Arnold, 1989); and spiders (Stratton and Uetz, 1981, 1983; Schmidt, 1990). The questions of principal interest with regard to sexual selection are: (1) what evolutionary processes give rise to sexual isolation, (2) what genetic mechanisms underlie the process and (3) what behavioral mechanisms maintain isolation.

Understanding the nature of geographical variation in behavior, particularly courtship behavior, is essential to answering these questions. Genetic divergence arising from selection for adaptations to local environments among allopatric populations and genetic drift may lead to sexual isolation when such divergence is correlated with behavior (Mayr, 1963; Koepfer, 1987). Behavioral differences in newly sympatric
populations that have undergone genetic divergence might undergo reinforcement
(Dobzhansky, 1970) thereby strengthening isolation. This has been demonstrated in
laboratory selection studies with *Drosophila* (Wasserman and Koepfer, 1977; Ringo et
al., 1985). Isolation may also occur in sympatric subpopulations that have adapted to
different microhabitats or in races of organisms adapted to different hosts (Bush, 1992;
Feder et al., 1994). Multilocus genetic simulation models suggest that sympatric
reproductive isolation may occur under a wide variety of conditions (Johnson et al.,
1996). Clues to the mechanism of speciation and whether speciation is occurring
sympatrically or allopatrically, can be obtained from comparisons of the temporal and
sequential components of courtship behavior exhibited between and within populations
that are geographically isolated. Such studies have the potential to reveal not only the
geographical pattern of species differentiation, and thus the importance of underlying
geological processes, but also the importance of specific behavioral patterns in the
process of speciation.

The *L. mackloti* complex is an insularized group that are robust moderately thick
bodied pythons attaining lengths of up to 3-4 meters and demonstrate geographic
variation in regards to morphological (size, coloration, markings, scalation), behavioral
(courtship and premating distance reduction behaviors) (Carmichael et al., 2007), and
molecular (mtDNA) attributes (Carmichael et al., 2003; Rawlings et al. 2004). Two
subspecies of *L. mackloti* are found on the outer Banda Arc of islands, *L. m. mackloti*
(Dumeril and Bibron, 1844), from the islands of Timor, Semau and Roti (referred to as
‘Greater Timor’), and *L. m. savuensis* (Brongersma, 1956), from the island of Sawu (see
Fig. 1). One subspecies, *L. m. dunni* (Stull, 1932) is found on one of the inner Banda Arc
of islands, Wetar (see Plates 1-7 in Figure 1 for picture and description of each subspecies; and Fig. 2). Additionally, How and Kitchener (1997) discovered *L. mackloti* to exist on the island of Alor located west of Wetar, also part of the inner Banda Arc. How and Kitchener (1997) found that the snake fauna on Alor and Wetar were more similar when compared to the outer Banda Arc islands as compared to the adjacent inner Banda Arc islands to the west, suggesting that a major biogeographical barrier exists between Lembata and Alor. They also detected another barrier between Sumba and Sawu on the outer Banda Arc that delineates assemblages to the east and west. The Savu snake fauna appear to be more closely allied to the ‘Greater Timor’, Alor and Wetar islands at the level of species based on principal component analysis when comparing species/genera assemblages on the different islands (Rawlings et al., 2004).

*Liias mackloti* are sexually dimorphic (in size and color/pattern). In the Wetar population, males are typically larger in overall size than females, while females on the islands of Timor, Roti, Semau and Sawu are typically larger than males. This intersexual morphometric difference has interesting behavioral consequences; typically male-male combat and territoriality (a behavior that has been difficult to identify in snakes) occurs more frequently in populations of animals where the males achieve greater size, and female choice for males in populations where females are larger than males. We have found that male *L. m. dunni* from the island of Wetar do engage in male-male combat (unpubl. data), which has not been observed in *L. m. mackloti* and *L. m. savuensis* (Carmichael et al., 2003; Carmichael et al., 2007).

Because of the implications regarding the evolution of reproductive isolating mechanisms within and between island populations of *L. mackloti*, we were primarily
interested in the geographic variation of courtship displays. Carmichael et al. (2007) investigated patterns of geographic variation of pre-courtship distance reduction mechanisms such as pheromone trailing within the *L. mackloti* complex and found significant differences in male-female recognition between several groups of islands. These differences generally followed three natural clades: Sawu clade (Sawu Island; *L. m. savuensis*); Wetar Clade (Wetar Island; *L. m. dunni*) and the Great Timor clade (Timor, Semau and Roti Islands; *L. m. mackloti*).

The characteristic actions of the courting male python while lying over the female is the use of the pelvic spurs or hooks (vestigial hind appendages) to stroke, scratch, or vibrate against the female in her cloacal region to induce her to twist her cloaca lateral and gape it so the male can effect intromission. The colubrid courtship appears to be more complex, but this may reflect the greater amount of information available on these forms (Gillingham, 1977). Characteristics of courtship (primarily by the male) for colubrids and boids which appear to varying degrees in the species observed are: following or chasing, dorsal mount with parallel alignment, lateral trunk loops which move along dorsally over the female (cephalocaudal waves, although these are not as near as intense as observed in colubrids), movement of the adpressed chin (chin rubbing) along the dorsum of the female, lateral or ventrolateral jerks or rippling movement of the trunk wall which generally move anterior (caudocephalic waves), tail looping (tail search) movements which move anterior, and biting (in some a bite hold).

Communication during courtship in snakes appears to be primarily tactile, once species and sex identification have been made (Gillingham, 1977). Chin rubbing and dorsal crawling with cephalocaudal waves by the male help to achieve parallel alignment.
while at the same time serving to passify the female and maintain this alignment. The body jerks or rippling muscle actions in the form of caudocephalic waves by the male in contact with the female posteriorly may contribute to keeping the female submissive and also stimulate her to be receptive to the tail actions. The searching or pushing movements by the tail of the male, usually starting posterior and moving anteriorly in contact with the tail and cloacal region of the female appear to function to induce the female to raise her tail and gape her cloacal opening, and at the same time bring the erect hemipenis into position for intromission. The synchronization of the cloacal gaping and intromission is paramount to successful coupling and thus must depend upon precisely timed tactile communication signals.

All of the above actions appear to be a form of tactile communication, taking place in the different regions along the length of the female snake. In analyzing the sequences of actions during courtship and copulation, three phases emerge (Gillingham, 1977) – tactile-chase, tactile-alignment and intromission and coitus. Each of these phases involves tactile signals. Although it is possible that behaviors that occur less frequently may be important in female recognition, our main purpose in this study was to compare the overall male pattern between insular populations as an indication of geographical variation in courtship behavior. We were interested in elucidating the differences in combined motor pattern transition frequencies as well as the differences in the location, on the females dorsolateral surface, where males actively pelvic spur using intense spur movements (which are very deliberate spurring movements directed at the soft interscalar tissue of the female). We investigated courtship and mating in the five insular populations of L. mackloti with three objectives in mind: (1) to elucidate the behavioral repertoire of
courtship between the insular populations; (2) to make qualitative and quantitative comparisons of the insular populations as to spatial (interpopulational) differences in courtship behavior; and (3) to determine if the basic triphasic behavioral schema of Gillingham et al. (1977) is applicable to *L. mackloti*. Male pythons typically "spur" the dorsum of females during courtship using a pair of pelvic spurs located lateral to the cloaca. Spurring represents a unique motor pattern and are boid-typical (Murphy et al., 1978). The behavioral (courtship, presence/absence of male-male combat, and pheromone trailing specificity) studies were conducted to determine the extent of geographic variation of courtship behaviors within a moderate to large python species, *Liiasis mackloti*, that inhabits several islands of Indonesia's Lesser Sundas Archipelago of the Outer and Inner Banda Arc and to provide additional insights of the biogeography of this group to support the existing literature (e.g. Rawlings et al., 2004).

**MATERIALS AND METHODS**

*Sampling and Specimens*

Sexually mature pythons used for this study were collected from each of the five islands studied (Wetar, Sawu, Roti, Semau, and Timor) and exported to the author by Duncan MacRae of CV. Herpafauna Indonesia, Bali, Indonesia between 1995 and 2003. Snakes were quarantined for a period of 90 days prior to use in this study. Dave Barker of Vida Preciosa International, Inc. (V.P.I.) assisted in verification of the origin of these animals and has traveled to and spent exhaustive field hours studying endemic python populations throughout Indonesia (Harvey et al., 2000; Keogh, 2001; Barker and Barker, in prep). Only adult animals were legally exported from Indonesia and used for this
project. Animals were used in the behavioral experimentation within 90-180 days after arriving in the United States.

Sixteen to 25 adult pythons were field collected (N=96) from each island population (Sawu, N=50; Semau, N=20; Roti, N=49; Timor, N=50; Wetar, N=38) (Table 1). Pythons were directly transported from Indonesia to the author and maintained individually in appropriate sized Vision® reptile cages (ranging between 91 to 122 cm in length). A thermal gradient was maintained within each enclosure using 10.16 cm wide heat tape that was placed underneath the cage and thermostatically controlled to provide a range of temperatures from 27°C to 35°C and cages were maintained in a heated laboratory that was thermostatically controlled to give an ambient air temperature of approximately 27°C to 28°C. Enclosures were maintained on a 14L:10D photoperiod. Water was offered ad libitum and snakes were fed every 12-16 days on appropriately sized thawed out frozen rodents, chicks, and quail. To induce reproductive behaviors, the snakes were exposed to a three-month cool down period between October through January where the range of temperatures dropped to 26°C to 27°C, with a hotspot of 35°C available for 3 hours per day. Newspaper was used as a maintenance substrate in the enclosures for all of the snakes.

Table 1. Summary of collection sites for video studies of male courtship (study of sexual isolation performed as separate tests) behavior of the Indonesian water python, *L. mackloti ssp.* Locations/islands are arranged east to west.

<table>
<thead>
<tr>
<th>Island location of each python studied</th>
<th>Number of males observed</th>
<th>Number of courtships recorded</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wetar (<em>L. m. dunnii</em>)</td>
<td>28</td>
<td>13</td>
</tr>
<tr>
<td>Timor (<em>L. m. mackloti</em>)</td>
<td>35</td>
<td>18</td>
</tr>
<tr>
<td>Semau (<em>L. m. mackloti</em>)</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>Roti (<em>L. m. mackloti</em>)</td>
<td>34</td>
<td>16</td>
</tr>
<tr>
<td>Sawu (<em>L. m. savuensis</em>)</td>
<td>30</td>
<td>19</td>
</tr>
<tr>
<td>Total</td>
<td>143</td>
<td>72</td>
</tr>
</tbody>
</table>

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Collection and video observation

The basic male courtship pattern for each subspecies (and insular population) was determined using the following protocol. All mating trials were conducted in an enclosure measuring 6 meters in length and 3 meters in width and 2 meters in height in order to maintain the pairs in close proximity to each other during behavioral observations and trials were conducted during the scotophase period (2100 to 2300 h) as this represented a period of time of peak activity. Behavioral observations generally lasted approximately 60 minutes. Cypress mulch was used as a substratum in the test arena to allow for better traction and mimic natural conditions used by pythons in-situ. Heat lamps fitted with 75-100w ceramic heat bulbs were used to eliminate light but to raise the ambient air temperature to match conditions of the animals’ maintenance enclosures. Behavior patterns were selected from an ethogram (see Table 2) based on analyses of videotaped encounters conducted prior to this study and following the triphasic behavioral schema of Gillingham et al. (1977). No new behavior patterns were identified during the course of the present study and all could be assigned to predetermined ethogram categories. We simultaneously videotaped (using a Sony Digital 8 Handycam Camcorder, Model DCR-TRV720NTSC) and audio recorded the first six trials for a period of 60-minutes to determine whether or not the frequency and sequence of courtship behaviors was consistent throughout this test period. Comparisons of these recordings confirmed that we could identify all behavior patterns at the times and in the orders in which they occurred. Before a robust interpopulational comparison of the frequency distributions and the sequence of male courtship displays could be made between the five insular populations of *L. mackloti*, we used the G test for homogeneity.
among repeated observations and compared the repeated sequences of each male (Table 3). For those males for which there was no significant difference in the frequency of occurrence of the courtship behavior between repeated 60-min segments of the same bout, we used the average of the 60-min bouts observed to represent that male in further analyses. In all cases, the frequency of occurrence of the principal displays was invariant over the length of the courtship bout. We deduce from these results that a random choice of a 60-min scoring segment is acceptable. Males that did not show temporal homogeneity in frequency of courtship display were excluded from all further analysis.

We also wanted to know whether or not males within each population were homogeneous in the frequency of occurrence of their courtship displays. This information was required because at the outset of the study we did not know the extent at which species differentiation might occur.

Table 2. Description of male courtship motor patterns of *L. mackloti* (from Gillingham, 1979) and was used as an ethogram for behavioral observation

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Description of behavior</th>
</tr>
</thead>
<tbody>
<tr>
<td>Touch (TO)</td>
<td>Male approaches female and makes contact with her trunk using his snout.</td>
</tr>
<tr>
<td>Mount (MT)</td>
<td>Male places his head on dorsum of female and keeps his chin in close contact with her.</td>
</tr>
<tr>
<td>Chase (CH)</td>
<td>Rapid pursuit by male of fleeing female. Male not in contact with female.</td>
</tr>
<tr>
<td>Chase-mount (CM)</td>
<td>Male follows or moves with fleeing female while maintaining chin and/or trunk contact with her dorsum.</td>
</tr>
<tr>
<td>Dorsal-advance movement (DM)</td>
<td>Anteriorly directed movement of mounted male with chin adpressed to medial or lateral dorsum of female.</td>
</tr>
<tr>
<td>Writhe (WR)</td>
<td>Rhythmic sliding, twisting and turning movement performed while completely mounted on female.</td>
</tr>
<tr>
<td>Tail-search copulatory attempt (TSCA)</td>
<td>Complex tail movements as male attempts cloacal juxtaposition and intromission.</td>
</tr>
<tr>
<td>Intromission (IN)</td>
<td>Insertion of one hemipenis in cloaca of female.</td>
</tr>
<tr>
<td>Biting (BY)</td>
<td>Male grasps female in neck or trunk region with subsequent release.</td>
</tr>
<tr>
<td>Discontinuation (DS)</td>
<td>Male dismounts female without copulation and resumes investigative behavior or assumes a coiled position.</td>
</tr>
<tr>
<td>Fast-spurring (FS)</td>
<td>Use of the pelvic spurs however they barely make contact with the females dorsum and generally are targeted throughout the sagittal and/or parasagittal regions. Average stroke rate is approximately 3.57 strokes/sec (stroke rate &gt; 2.5/sec is fast spurring)</td>
</tr>
</tbody>
</table>
Slow-spurring (SS) Use of the pelvic spurs and occurs during writhing movement of male as tail is slowly dragged back and forth over the females dorsum in a zig-zag fashion. Spurs make lateral contact with the female and oscillated at an average rate of 1.9 strokes/sec. Pelvic spurring directed within 30 scale rows anterior to the vent, and is initiated prior to the first tail-search copulatory attempt and carried out throughout the tactile-alignment phase of courtship. Typically spurs are directed at the soft interscalar tissue and has an average spur rate of 0.94 strokes/min.

Intense spur movements (ISP) Pelvic spurring directed within 30 scale rows anterior to the vent, and is initiated prior to the first tail-search copulatory attempt and carried out throughout the tactile-alignment phase of courtship. Typically spurs are directed at the soft interscalar tissue and has an average spur rate of 0.94 strokes/min.

<table>
<thead>
<tr>
<th>Population</th>
<th>Male</th>
<th>Number of repeated observations</th>
<th>G</th>
<th>df</th>
<th>P&lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wetar</td>
<td>LMD004</td>
<td>2</td>
<td>1.741</td>
<td>3</td>
<td>0.628 NS</td>
</tr>
<tr>
<td></td>
<td>LMD028</td>
<td>2</td>
<td>2.103</td>
<td>3</td>
<td>0.551 NS</td>
</tr>
<tr>
<td>Sawu</td>
<td>LMS017</td>
<td>2</td>
<td>2.528</td>
<td>3</td>
<td>0.470 NS</td>
</tr>
<tr>
<td></td>
<td>LMS006</td>
<td>2</td>
<td>2.976</td>
<td>3</td>
<td>0.322 NS</td>
</tr>
<tr>
<td>Timor</td>
<td>LMMT012</td>
<td>2</td>
<td>1.569</td>
<td>3</td>
<td>0.666 NS</td>
</tr>
<tr>
<td></td>
<td>LMMT032</td>
<td>2</td>
<td>2.507</td>
<td>3</td>
<td>0.868 NS</td>
</tr>
<tr>
<td>Semau</td>
<td>LMMS009</td>
<td>2</td>
<td>5.372</td>
<td>3</td>
<td>0.146 NS</td>
</tr>
<tr>
<td></td>
<td>LMMS021</td>
<td>2</td>
<td>2.239</td>
<td>3</td>
<td>0.635 NS</td>
</tr>
<tr>
<td>Roti</td>
<td>LMMR001</td>
<td>2</td>
<td>1.091</td>
<td>3</td>
<td>0.770 NS</td>
</tr>
</tbody>
</table>

Table 3. Homogeneity of repeated observations of selected males of *L. mackloti* (Wetar = *L. m. dunni*; Sawu = *L. m. savuensis*; Timor, Semau and Roti = *L. m. mackloti*)

G test for homogeneity among repeated observations

Recordings were transcribed into text format using JWatcher software, a program that converts a computer keyboard into an event-recorder (Ottoni, 1995). All behavior patterns were considered as instantaneous events (having no duration) in order to avoid confounding factors (e.g. the length of time required for a male to approach the female depended on the initial distance between them, a variable that we could not control). However, we did record the duration of spurring episodes, because males exhibited this behavior pattern only when in direct contact with females. If a male displayed an instantaneous behavior pattern twice, or more, consecutively, this was considered as a single episode. We calculated the duration of these episodes as the time (in seconds) between the first instance of the specified behavior pattern and the first instance of a
different one. In order to include in calculations the time that a male spent performing
single instantaneous behavior patterns (those not contained in episodes), we assigned the
mean time taken to exhibit one complete action. Furthermore, male behavior patterns
were classified as either "courtship/precopulatory" or "non-courtship"; the latter included
behavior patterns that are not elements of courtship but may be associated with
recognition of a conspecific. Because courtship always occurred in close proximity to the
female, we considered the start of courtship to be the first instance of a
"courtship/precopulatory" behavior pattern shown within 10 cm of the female. Male
courtship was continuous (i.e. males did not show multiple courtship bouts in a single
trial). Courtship ceased when: 1) attempted mating occurred; 2) a trial ended after 60
minutes; or 3) a male lost interest and ceased to display "courtship/precopulatory"
behavior patterns. Only such behavior patterns are included in the analyses reported
here.

Over a seven-year period (between 2000 and 2007) 72 of 143 tests resulted in
courtship and/or mating. The behavioral observations were made at various hours during
the day; however after the first eight hours of videotaping in 2000 it was clear that most
courtship behaviors occurred during the scotophasic period between 2100 and 2300
hours. Due to a decreased availability and increased cost of purchasing additional
females, each female may have participated in more than one trial. Although this may
lead to problems of non-independence, a pilot study revealed little inter-individual
variation in female behavior patterns (Carmichael, unpubl. data). In addition, subsequent
analyses detected no significant effects of female identity on male mating/courtship
frequencies and courtship content.
Males (Table 1) were each tested with approximately 8 females from each insular population in randomized order for a total of 143 observations. Each male experienced at least 1 wk of rest between consecutive trials and was never paired with the same female more than once. So that multiple courtship trials could be completed, females were prevented from mating during the trial period by placing a small piece of surgical tape over the female’s cloaca immediately prior to a trial (Gillingham, 1977). A preliminary study showed that this technique effectively prevented intromission without significantly altering either male or female behavior patterns (Gillingham, 1977). The tape was immediately removed after the trial using luke warm water with minimal disturbance to the snake. To initiate the mating trials, a single male and female were individually contained in cloth snake bags, which were loosely tied, and placed at opposite ends of the glass-enclosed test arena. Substrate was replaced between individual trials and the test arena was thoroughly sanitized and cleaned prior to the subsequent trial run. Fresh substrate (cypress mulch) was added for each trial event. All courtship trials were recorded on videotape and a verbal account of the test was audiorecorded. If courtship did not commence within 60 minutes after introducing a male and female into the test arena, the snakes were removed and tested at a later time. In order to see the entire test arena clearly, string was attached to one of the corners of each bag containing a snake which was looped over the top of the enclosure and made accessible outside the test arena. After 10 minutes of acclimation, the string was pulled from outside the enclosure in order to gently remove the snake bag to aid in videography. One minute after both snakes emerged out of the bags, the focal sampling event commenced in order to visually assess courtship interactions. Focal sampling was used in preliminary trials to
describe and record the frequency of several different categories of courtship behaviors. Courtship and mating behavior of *L. mackloti* generally follows the triphasic pattern observed in colubrids (Gillingham et al., 1977; Gillingham, 1980; Gillingham and Chambers, 1982). The triphasic pattern includes tactile-chase (Phase I), tactile-alignment (Phase II), and intromission and coitus (Phase III) (Gillingham et al., 1977; Gillingham, 1980). However, there are two key differences observed between colubrid and pythonid courtship behaviors: 1) there are fewer primary behaviors elicited during male-female encounters, and 2) the pelvic spur is actively used during courtship and represents a novel and unique motor pattern. The use of the pelvic spur is common throughout most all boid species (Murphy et al., 1978). An ethogram listing all of the salient stereotypic behaviors used during courtship (Gillingham and Chambers, 1982), was used during each trial and three phases of motor patterns were defined as basic components of the courtship sequence (see Table 2). These motor patterns can be generally organized into three “phases” following Gillingham (1987): 

*Phase 1: Tactile-Chase* - The first phase of snake courtship includes initial contact and preliminary courtship acts. This phase is accompanied by much tongue flicking, and although visual cues are at least partly responsible for bringing the individuals together, tongue flicking is necessary for species recognition and sexual recognition. Additionally, in many courtship will not occur without the transfer of the appropriate pheremone(s) to the male’s vomeronasal organ. The male typically makes a posterio-anterior advancement along the females dorsum using his chin, after which he begins rapidly spurring the females dorsum in the interscale membrane regions. This phase typically progresses from approach and contact, to either chase or chase-mount, to
mount. Once the male python has mounted the female, he will use an array of tactile movements including chin rubbing in a posterio-anterior direction up the females dorsum and spurring using the pelvic spur typically within 4-30 scale rows anterior to the cloaca. These pelvic spurs are used actively by males during courtship and during male-male combat and likely play an important role in facilitating cloacal gaping just before hemipenial intromission (Murphy et al., 1978; Gillingham and Chambers, 1982). During chin rubbing, the male closely adpresses his chin to the dorsum of the female while moving the head forward, backward, or laterally, but generally achieving an overall anterior direction during dorsal advance. No courtship biting, undulation, dorsal body looping, caudocephalic waves, and cephalocaudal waves were observed during test trials using pythons, however these are commonly observed behaviors with colubrids (Gillingham, 1987). For the purpose of analyzing differences in the sequence of key behaviors exhibited by males for the purpose of detecting patterns of geographic variation in courtship, several closely related patterns were combined within the tactile-chase phase to give the following five main classes of behaviors, each symbolized by four letters:

1) Touch-mount (TOMT): Male approaches female, makes contact with tongue and/or snout and mounts female.
2) Chase-chase mount (CHCM): Pursuit of fleeing female by male, with or without actual contact.
3) Dorsal-advance-writhe (DMWR): Male proceeds anteriorially along the medial and/or lateral dorsum of the female.
4) Fast-Spurring-writhe (FSWR): Male moves spurs at a frequency greater than 2.5 times per second accompanied by a writhing-like movement where the male’s tail is slowly dragged back and forth over the female’s dorsum in a zig-zag fashion with spurs making contact on females dorsum.
5) Slow-Spurring-writhe (SSWR): Male moves spurs at a frequency lower than 2.5 times per second where the male’s tail is slowly dragged back and forth over the female’s dorsum in a zig-zag fashion.
Phase 2: Tactile-Alignment – The second phase of snake courtship begins with the first copulatory attempt, often referred to as a tail-search copulatory attempt or TSCA (Gillingham, 1977). The TSCA is a ubiquitous behavior seen in most snakes. The result of such a complex movement is the juxtaposition of the cloacal apertures so that, following female cloacal gaping, the male can introduce a single hemipenis and effect copulation. This maneuvering is not unlike that exhibited by male tuatara as cloacal apposition is attempted (Gillingham et al., 1995). Attendant to the TSCAs are the same tactile acts that occur during Phase 1. In some species, the male snake’s head and anterior body employ these acts while the tail maintains an ostensibly independent action on that portion of the female. More commonly, TSCAs are separated by periods of intense tactile stimulation. In mating aggregations of eastern garter snakes (Thamnophis sirtalis), it is not uncommon for a male’s head to be in contact with a female other than the one with which his tail makes contact (Gillingham and Dickinson, 1980). Even if a male “mistakenly” maintains head contact with other individuals, it is ultimately the activity of his tail that determines mating success. One of the unique behaviors seen in pythons in Phase 2 is the intense spur movement (ISP), a behavior initiated by the male prior to the first tail-search copulatory attempt and throughout the tactile-alignment phase of courtship. This behavior is directed at the soft interscalar tissue of the female and often lifts the scale appreciably and in many cases brings about local forward-jerking movements by the female. The male only exhibited this motor pattern when his spurs were in lateral contact with the female and within 30 scale rows of the female’s vent. The scale-lifting occurred at an average 0.89 strokes/min (Table 1) and varies between insular populations of L. mackloti.
Phase 3: Intromission and Coitus – The final phase begins when hemipenial penetration of the female is effected. Although some authors have speculated that copulatory adjustment is brought about by the male through the use of hemipenial spines to lift the female’s cloacal scales, with little or no female participation, it is now well established that female cloacal gaping is necessary for intromission (Gillingham, 1980; Schuett and Gillingham, 1989). Because of the proximity of the ventral surface of the juxtaposed tails just before intromission, the actual process is rarely seen. On the other hand, it is not uncommon for a receptive female to gape her cloaca before TSCA initiation or even simply in the presence of a potential mate (Gillingham, 1977).

We examined the interaction between male and female “conspecific” *L. mackloti* using pairwise combination mating trials for each known population, however we were mainly interested in determining the sequence of male behaviors as they are generally more complex and often are a better predictor of ultimate female response and as a result made for a more interesting and robust interpopulational comparison that could be placed into an evolutionary context. The female’s behavioral repertoire can and does alter the transition of one phase of male behavior to another within the triphasic scheme, however male behaviors were more predictable and allowed for a more robust phylogenetic comparison between the five insular populations of *L. mackloti*. The continuous recording (or “all-occurrences” recording) method was used to provide an exact and faithful record of the behavior, measuring true frequencies and durations and the times at which behavior patterns stopped and started. All behavioral sequences were video recorded in the absence of light using infrared detection technology on a Sony Digi-8 video camera to avoid under-estimating the duration of various behavioral events and to
maximize scotophasic conditions. Narration was recorded throughout the behavioral trials. Voice-recorded transcriptions and videotapes were analyzed for overall behavioral patterns.

*Data analysis: frequency distributions of male courtship display*

A replicated goodness-of-fit analysis (Sokal & Rohlf, 1995) was used to examine differences in the frequency distributions of male courtship behaviors between the populations where the populations were viewed as replicates. The analysis involved the following steps. First, for each population, we tested (log-likelihood method, G test) the hypotheses of a uniform distribution of courtship behaviors. The uniform distribution was employed because it is the most parsimonious and no other plausible distribution has been suggested. This analysis is conducted to determine if populations differed from the expected distribution. To determine the extent of departure from expectation for the group of populations as a whole, we calculated the total G statistic, which is the sum of the individual population G statistics. A significantly high total G statistic suggests that there is heterogeneity among populations in the frequency of occurrence of behaviors when compared to the expected frequency. We then calculated the pooled G statistic from the pooled frequency data from all populations to determine whether the trend in the departure from the expected was the same in all populations. If the departures from the expected frequency distribution between the populations compensated for one another in some way (e.g. one group of populations had high frequencies of fast-spurring and low frequency of intense spurring movements, and another group of populations had low frequencies of fast-spurring and high frequencies of intense spurring movements; see Table 5), we would expect a small pooled G. Finally, to determine whether the
magnitude of the departure from expected was similar among populations, we calculated the heterogeneity G statistic. If populations that differed from expected did so with the same magnitude, we would expect a low value for this statistic.

Analysis of the Sequence of Male Display

To delineate patterns in the sequence of male courtship behaviors within and between populations, we constructed a transition matrix of preceding and following behaviors for each population using the methods of Dingle (1969), Chatfield and Lemon (1970), and Clark (1994). We performed an analysis of each transition matrix by first calculating a $X^2$ value for the entire matrix to determine whether 'preceding' behaviors were independent of 'following' behaviors. For all matrices for which the independence of preceding and following behaviors was rejected, we calculated the row $X^2$. The presence of row $X^2$ values greater than 11.34 (P<0.01; here we follow the conservative approach advocated by Clark, 1994) indicates the possibility of significantly different behavioral dyads (i.e. a pair of behaviors that occur more or less frequently together than expected if pairs of behaviors were chosen at random). To identify significant behavioral dyads, we calculated the cell $X^2$ values using the equation of Clark (1994), which adjusts the cell degrees of freedom to 1. We assumed that behaviors that occurred significantly more frequently than expected for a particular following behavior would facilitate following behavior, whereas behaviors that occurred significantly less frequently than expected would inhibit following behavior. This type of transitional analysis is useful for assessing whether sequential events are independent of one another (in the sense of probability theory). However, past events can entirely determine the next event to happen, meaning that the last thing the animal did predicts well (but not completely) what
its next act will be. Transition matrices can then be compared for a suite of behavioral characters (tongue flicks, frequency and location of spurring activities) between insular populations. The male’s behavior was recorded as a sequence of discrete actions and treated as a series of first-order transitions: first act to second act, second act to third act, etc. Included in these transition matrices are transitions between the behavioral phases, and also transitions from display act to orientation acts and to spurring. These behavioral characters can then be statistically compared to examine differences in this behavior and to evaluate this data in a phylogenetic context.

Duncan’s multiple range test was performed on those data showing significant F values (Steel and Torrie, 1960). Motor patterns were arranged into sequences for further analysis by the methods described by Chatfield and Lemon (1970). Matrices of observed and expected motor pattern transition frequencies were set up and tested for random deviations using chi square. Sequence diagrams for each species were constructed both on the basis of transition frequencies and relative deviations (observed – expected/expected) as a measure of transition probability between behavioral events (Altmann, 1968). A subjective analysis of all data was used in creating the qualitative descriptions, and rare events eliminated from transition matrices for statistical reasons were included in these descriptions.

Indices of mating success, dismount frequencies and total copulatory attempts were used as measures of female receptivity. Interpopulational and intrapopulational (i.e. between and within populations) trials were made. Volleys of tongue-flicking by males were common throughout the courtship trials, but were excluded from sequence analysis because of temporal overlap with the motor patterns.
Various phases of courtship (e.g. dorsal-anterior advance, tactile-chase, tactile-alignment, tail-search copulatory advance, intromission, coitus) were defined so that a comparison could be made between the known insular populations. All matrices discussed in the results use combined motor pattern transition frequencies and the corresponding sequence diagrams are based on frequency of occurrence of each combined motor pattern and the relative deviation (probability) of each behavior being expressed.

The behavioral data was then compared to the molecular phylogenies generated by Carmichael et al. (2003) and Rawlings et al. (2004) to investigate congruencies between these two sets of data (behavioral and molecular). These studies have shown that there are three unique clades: (1) *L. m. savuensis* is only found on the island of Sawu and is referred to as the Sawu clade, (2) *L. m. dunni* is only found on the island of Wetar and referred to as the Wetar clade. (3) *Liasis m. mackloti* inhabits the islands of Timor, Semau and Roti and is referred to as the Timor-Semau-Roti clade, within the *L. mackloti* ssp. complex based on mitochondrial cytochrome b sequences.

**Spurring movement site specificity**

Various aspects of courtship behavior were noted during the observation period. Specifically, spurring movement frequency (e.g. fast-spurring, slow-spurring, scale-lifting) was carefully evaluated (following Gillingham and Chambers, 1982) and the location of spurring activity was identified. The use of pelvic spurs during courtship is a unique boid-typical motor pattern that is not found in other snakes. The pelvic spurs are used actively during courtship and appear to be species-specific in the frequency of how often spurs are moved, and there appears to be a specific target site on the females.
dorsum that a male would attempt to spur (ISP) prior to a tail-search copulatory attempt (TSCA). Approximately 24 hours before the courtship trials commenced, a randomly determined female from one of the five insular populations was selected and guided into an appropriately sized restraining tube so that the posterior half of the body could be freely handled and a grid system marked. The grid system was marked on the dorsolateral aspect of the test female using non-toxic acrylic paint dots that were applied to the center and apical surface of each scale in the approximate range of scales that would typically be targeted by the male during pelvic spurring (within 30 scale rows anterior of the cloaca). Spurring typically occurs throughout the last half of the females dorsolateral surface, however, prior to coitus, intense spur movements (ISPs) primarily occur within a small concentrated area of scales along the dorso-lateral surface of the female and within approximately 30 scale rows anterior to the cloaca. The frequency and duration of male spurring within specific patches of scales was noted during courtship. Additionally, after each trial the female was placed into a restraining tube and the individual dots inspected using a dissection microscope to investigate which of the dots had been scratched by the pelvic spurs. Characteristic pelvic spur scratch marks on the painted dots were very obvious and the pattern of scratched dots strongly correlated with the videos made during the trials to determine which scales the males spent most of their time pelvic spurring. We attempted to determine whether or not there were significant differences between different patches of scales targeted by the males during ISP that elicited the highest frequency of copulatory attempts and compared these results between the five insular populations of *L. mackloti*. We used a single factor ANOVA and Tukey statistic to compare interpopulational differences in the target sites used by males on the females.
dorsum using ISPs. Each scale that the male spurred using ISP was identified according to the scale row and distance from the cloaca, and then this distance was converted into a ratio (log spurred scale distance from cloaca/log length of 30 scale rows anterior of the cloaca in cm) to allow for interindividual comparisons. Preliminary observations determined that there was an increased chance of successful coitus if the male progressed to ISP (as this was the most common spurring behavior prior to coitus) and if he concentrated his spurring efforts on specific groups of scales on the dorsum of the females.

*Collection and video observation methods for sexual isolation experiments*

Snakes used in the first sexual isolation experiments were produced through captive breeding programs from wild-caught adults collected between 1995 and 1999 from the islands of Sawu, Wetar, Timor, Semau, and Roti. All snakes used for this experiment were derived from captive born hatchling snakes that were raised to sexual maturity while in isolation from other individuals (one snake per enclosure), and therefore were considered to be virgins during the experiment.

The videotaping procedures for the sexual isolation experiments were similar to those used for the study of geographical variation in male courtship except that (1) both the male and female snakes were included in each video recording, and (2) because we were interested principally in whether mating would occur, the length of the taping bout was determined by the activities of the snakes, with a single bout lasting until the beginning of the copulation or until 12 hours after taping commenced, whichever came first.
Experimental design for sexual isolation studies

To determine whether sexual isolation exists among the five insular populations studied, and to discover whether there is asymmetry of sexual isolation if isolation exists, we employed a crossing experiment in which mature male and female snakes from the same (homotypic) and different (heterotypic) populations were paired and their behaviors were videotaped. For each population pair (Sawu-Wetar; Sawu-Roti, Sawu-Semau; Sawu-Timor; Wetar-Roti; Wetar-Semau; Wetar-Timor; Semau-Roti; Semau-Timor, Roti-Timor), we observed 20 heterotypic male-female pairings. In each case, 10 of the heterotypic pairings involved a female from the population and 10 involved a male. For example, in the case of the Sawu-Wetar cross, 10 of the pairings involved Sawu females and Wetar males and 10 of the pairings involved Sawu males and Wetar females. We conducted 10 homotypic pairings for each population.

Mating Success

We noted whether copulation occurred (successful mating) and whether eggs were produced. We estimated the binomial parameter separately for the homotypic encounters combined and the heterotypic encounters combined by setting 95% confidence intervals on $P$, the ratio of the number of successful matings to the total number of encounters attempted (an estimator of the binomial parameter). We counted the number of pairings resulting in the production of eggs hatched for each cross.

The author maintains an approved license through the Florida Fish and Wildlife Conservation Commission (License No. EPB35458) and during the duration of the study maintained an approved plan through the The University of Southern Mississippi’s Institutional Animal Care and Use Committee (IACUC Protocol Approval No. 190-001).
RESULTS

Frequency Distributions of Display and Sequence of Display

Our examination of intrapopulational variation in the frequency of courtship display revealed a high degree of homogeneity within each of the five insular populations studied and therefore no significant difference in the frequency distributions of male behaviors was observed between males within any of the studied insular populations (see Tables 4, 5, 6 and 7). The courtship and mating behaviors of *L. mackloti (savuensis, dunni* and *mackloti*) can be divided into three phases according to Gillingham et al. (1977): Tactile-chase (I), tactile-alignment (II) and intromission and coitus (III).

Table 4. G statistic analysis of intrapopulation homogeneity of male courtship behavior in *L. mackloti*

<table>
<thead>
<tr>
<th>Population</th>
<th>G</th>
<th>df</th>
<th>P</th>
<th>Group size (no. of males)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wetar (<em>L. m. dunni</em>)</td>
<td>7.67</td>
<td>6</td>
<td>NS</td>
<td>3</td>
</tr>
<tr>
<td>Timor (<em>L. m. mackloti</em>)</td>
<td>6.85</td>
<td>6</td>
<td>NS</td>
<td>3</td>
</tr>
<tr>
<td>Semau (<em>L. m. mackloti</em>)</td>
<td>6.04</td>
<td>6</td>
<td>NS</td>
<td>3</td>
</tr>
<tr>
<td>Roti (<em>L. m. mackloti</em>)</td>
<td>3.40</td>
<td>6</td>
<td>NS</td>
<td>3</td>
</tr>
<tr>
<td>Sawu (<em>L. m. savuensis</em>)</td>
<td>4.93</td>
<td>6</td>
<td>NS</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 5. Average frequency of occurrence of courtship behaviors, and the test of the hypothesis of a uniform frequency distribution of the behaviors in five populations of *L. mackloti* on the islands of Sawu, Wetar, Semau, Roti, and Timor.

<table>
<thead>
<tr>
<th>Population</th>
<th>No Active Spurring</th>
<th>Fast-Spurring</th>
<th>Slow-Spurring</th>
<th>Intense Spur Movements</th>
<th>G</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wetar (<em>L. m. dunni</em>)</td>
<td>1</td>
<td>32</td>
<td>8</td>
<td>34.5</td>
<td>55.75</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Timor (<em>L. m. mackloti</em>)</td>
<td>3.3</td>
<td>10</td>
<td>23</td>
<td>14.2</td>
<td>8.46</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Semau (<em>L. m. mackloti</em>)</td>
<td>12</td>
<td>7</td>
<td>20</td>
<td>21</td>
<td>9.64</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Roti (<em>L. m. mackloti</em>)</td>
<td>9</td>
<td>12</td>
<td>22</td>
<td>26</td>
<td>19.46</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sawu (<em>L. m. savuensis</em>)</td>
<td>15</td>
<td>45</td>
<td>20</td>
<td>48.5</td>
<td>21.49</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Table 6. Comparison of homotypic and heterotypic matings of *L. mackloti*. Means (±SD). Each of the populations in the table correspond to the island name (subspecies in parentheses): Sawu (*L. m. savuensis*), Wetar (*L. m. dunni*), Timor (*L. m. mackloti*), Semau (*L. m. mackloti*) and Roti (*L. m. mackloti*) represent the island populations.

<table>
<thead>
<tr>
<th>Population</th>
<th>Mean time (days) to</th>
<th>Ovideposit</th>
<th>Hatching (@ 32.2°C Incubation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>Female</td>
<td>No. crosses attempted</td>
<td>No. copulations observed</td>
</tr>
<tr>
<td>Sawu</td>
<td>Sawu</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Sawu</td>
<td>Wetar</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Wetar</td>
<td>Sawu</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Sawu</td>
<td>Timor</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Timor</td>
<td>Sawu</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Sawu</td>
<td>Semau</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Semau</td>
<td>Sawu</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Sawu</td>
<td>Roti</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Roti</td>
<td>Sawu</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Wetar</td>
<td>Wetar</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Wetar</td>
<td>Timor</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Timor</td>
<td>Wetar</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Wetar</td>
<td>Semau</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Semau</td>
<td>Wetar</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Wetar</td>
<td>Roti</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Roti</td>
<td>Wetar</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Timor</td>
<td>Timor</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Timor</td>
<td>Semau</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Semau</td>
<td>Timor</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Timor</td>
<td>Roti</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Roti</td>
<td>Timor</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Semau</td>
<td>Semau</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Roti</td>
<td>Roti</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>
Table 7. Comparison of spurring locations targeted between three subspecies of male *L. mackloti* (**L. m. savuensis**, **L. m. dunni** and **L. m. mackloti**) on the homotypic female dorsum. The values represent distance (scale count anterior to cloaca) from the cloaca where the male spent the highest duration ISP (proportion of each observed ISP in parentheses). Each data point represents the scale that had the highest frequency of occurrence of spurring and where the largest duration of time was spent spurring by the males (single factor ANOVA and Tukey; *P*<0.05; HSD 0.0708).

<table>
<thead>
<tr>
<th>Subspecies (distance of highest duration ISP in scale counts from cloaca)</th>
<th><strong>L. m. dunni</strong> (Wetar, N = 30)</th>
<th><strong>L. m. savuensis</strong> (Sawu, N = 30)</th>
<th><strong>L. m. mackloti</strong> (Timor, N = 30)</th>
<th><strong>L. m. mackloti</strong> (Semau, N = 30)</th>
<th><strong>L. m. mackloti</strong> (Roti, N = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 (0.4)</td>
<td>17 (0.57)</td>
<td>8 (0.27)</td>
<td>9 (0.3)</td>
<td>7 (0.23)</td>
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</tr>
<tr>
<td>16 (0.53)</td>
<td>16 (0.53)</td>
<td>13 (0.43)</td>
<td>14 (0.47)</td>
<td>10 (0.33)</td>
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<td>12 (0.4)</td>
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<td>13 (0.43)</td>
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<td>7 (0.23)</td>
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<td>7 (0.23)</td>
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<td>7 (0.23)</td>
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<td>12 (0.4)</td>
<td>26 (0.87)</td>
<td>8 (0.27)</td>
<td>6 (0.2)</td>
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<tr>
<td>11 (0.37)</td>
<td>28 (0.93)</td>
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<td>12 (0.4)</td>
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<tr>
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<td>7 (0.23)</td>
<td>6 (0.2)</td>
<td>9 (0.3)</td>
<td></td>
</tr>
</tbody>
</table>

**Phase 1.** – The tactile-chase phase began when the male initiated courtship activities, and ended at the first copulatory attempt. Successful trials showed average tactile-chase durations to be highest for **L. m. savuensis** (12.5 min). **L. mackloti mackloti** with the lowest value (5.0 min), was significantly lower than both **L. m. savuensis** and **L. m. dunni** (8.0 min.) with the greatest difference between **L. m. mackloti** and **L. m. savuensis**.
A female, when contacted by a male, reacted in one of two ways, with responses differing among species. In negative (unsuccessful) trials, a female either showed a forward-jerking response or was passive (no response, NR); female *L. m. savuensis* exhibited a higher forward-jerking incidence on contact than the other two subspecies ($X^2 = 31.40, P<0.005, df = 2$).

In trials that led to successful mating, female *L. m. savuensis* responded to the male’s first contact with forward-jerking behavior 85.7% of the time, while female *L. m. dunni* had a response of 34% while *L. m. mackloti* from each of the islands of Timor, Roti and Semau, made this response 26.6%, 23.2% and 21.1% respectively ($X^2 = 20.36, P<0.005, df = 2$). The rate of forward-jerking ranged between 1.4 and 1.8 jerks per sec with no significant differences among species or between sexes.

Courtship began with the male pressing his chin to the female’s dorsum and moving toward her head (dorsal advance movements). This movement proceeded directly along the dorsal midline of the female (sagittally) or to the right or left of the midline (para-sagittally or laterally). As this action progressed, the female moved rapidly forward, often dislodging the courting male. If the male was dislodged, a chase resulted; if he managed to maintain his dorsal position as she moved, a chase-mount resulted. When the female’s flight ceased, the male resumed his courtship in the same sequence as before (i.e., contact, mount and dorsal advance movement).

The fast-spurring and slow-spurring were independent behaviors that although used sometimes interchangeably, were distinct in the frequency of use prior to certain following behaviors and therefore remained unpoled for matrix analysis. The spurring first appeared late in Phase I for *L. m. savuensis* and *L. m. dunni*, and averaged 1.7 and
1.4 spurs per second, significantly higher (P<0.01) than the 1.1 per sec spurring for *L. m. mackloti* from the three insular populations.

FSWR was used frequently by *L. m. savuensis* to transition directly from FSWR to ISP (P<0.05), while *L. m. mackloti* from the three insular populations used SSWR to transition to ISP (P<0.05). *L. m. dunni* used both FSWR and SSWR to transition into ISP in Phase II. The importance of ISP in all five insular populations is obvious as this behavioral modality significantly results in progression to TSCA and subsequent coitus (P<0.05).

*Phase II.* — The tactile-alignment phase began with the ISP immediately prior to the first tail-search copulatory attempt (TSCA) and ended at hemipenial intromission. The combination of interchangeable fast-spurring (FASP) and slow-spurring (SLSP) with ISP and TSCA are the most frequent motor patterns exhibited during this phase, comprising an average of 86.4, 79.9 and 75.9% of *L. m. mackloti, L. m. dunni* and *L. m. savuensis* Phase II motor pattern transitions, respectively.

The TSCA began as a slow twitching of the last few centimeters of the male’s tail as it lay parallel to that of the female. The tail-twitching became more rapid and within seconds involved the entire tail and about 30 scale rows anterior of the cloaca. At this time a loop of the male’s tail was thrown over the female’s tail and the region just posterior to this loop was pushed beneath the female’s tail. Her tail was then held between a dorsal-anterior loop and a ventral-posterior loop with the latter twisted in such a way that the ventral scutes and cloacal area made contact with the ventral region of the female. The configuration was usually formed posterior to the female’s vent and was then slid anteriorly beneath the cloaca of the female. If intromission did not take place,
the tail of the male straightened parallel to her tail, and the loops reformed and again moved anteriorly. Two or three such formations in rapid succession made up one TSCA, each of which was followed by a period of tactile movements from one to several minutes. The TSCA’s occurred irregularly at the beginning of Phase II, but became more evenly spaced just prior to intromission.

The number of TSCA’s needed by the male to reach intromission varied between subspecies: *L. m. savuensis* required an average of 2.8 per trial, significantly lower than the other two subspecies (P<0.01). In regards to transition from Phase I to ISP movements (as compared to an alternate behavior in Phase II, such as TSCA), there was no significant difference between the three insular populations of *L. m. mackloti* (P>0.05). However, *L. m. dunnii* and *L. m. savuensis* primarily used FSWR as a preceding behavior to transition to ISP, while *L. m. mackloti* from the three insular populations significantly used SSWR to transition to ISP. There was a significant difference (P<0.05) between the frequency of transition to ISP when comparing *L. m. savuensis* and *L. m. dunnii*, as both used FSWR to transition to ISP but *L. m. savuensis* displaying this transition more frequently. These ISP movements often lifted the scale appreciably and in many cases brought about local forward-jerking movements by the female. The male typically exhibited this motor pattern when his spurs were in lateral contact with the female and within 30 scale rows anterior of the cloaca. This scale-lifting occurred at an average of 0.82 strokes/min.

As courtship progressed toward intromission in Phase III the tongue-flick rate of the male increased markedly in all three subspecies, with the greatest increase for *L. m.*
*savuensis* going from an average of 1.5 per sec in Phase I to an average of 2.4 per sec in Phase II. The rate increases in all subspecies were significant (P<0.05).

During Phase II very little fast-spurring with writhing movement behavior was shown by *L. m. mackloti* or *L. m. dunni*, and none by *L. m. savuensis*. Phase II was shorter for *L. m. savuensis* (4.5 min, P<0.01) than *L. m. mackloti* (18.3 min) or *L. m. dunni* (14.9 min). Phase II was terminated at hemipenial intromission.

*Phase III.* – Intromission of a hemipenis marked the beginning of this phase and was accomplished when the female opened her cloacal aperture during the final TSCA by the male. During coitus (Phase III) the male *L. m. mackloti* from the three insular populations held their heads an average of 10.3 and 8.5 cm respectively, behind that of the female, while *L. m. savuensis* and *L. m. dunni* usually held their head an average of 4.3 and 7.6 cm, respectively, behind the head of the female. All three subspecies showed similar coital positions and an anterior advancement of their head position from courtship intitiation to coitus.

No tongue-flicking was observed during coitus and the only movements observed were caudal where both tails (male and female) slowly moved back and forth, dorso-ventrally and laterally. Occasionally a slight lateral pulsation of the male’s body could be observed just anterior to the vent. On a few occasions the female edged forward slightly and the male responded by moving with her.

Phase III lasted an average of 20.3 and 24.7 min in *L. m. dunni* and *L. m. savuensis*, respectively, significantly longer (P<0.05) than the three insular populations of *L. m. mackloti* (18.1 min). Actual separation was achieved either by the tails moving away from one another or the snakes themselves moving in opposite directions until the
hemipenis was pulled from the females cloaca. Hemipenial inversion (retraction) occurred after removal from the cloaca and was not apparently responsible for separation. All three subspecies showed little variation during this act and all demonstrated post-copulatory tail-raising behavior for several minutes following.

*Frequency distribution of displays*

The absolute frequencies for each population relative to the principal male behaviors (spurring) are shown in Table 5. The analysis of these distributions indicate that there was significant heterogeneity between the three major clades in the way in which they departed from the expected uniform distribution ($G_{\text{total}}=205.46$). The pooled data departed significantly from the expected frequency ($G_{\text{pooled}}=58.74$) indicating that, although populations were heterogeneous in their departure from expected, the pattern of the departure was similar across populations. While the pattern of departure among populations was the same, the magnitude of the departure differed ($G_{\text{heterogeneity}}=146.75$).

*Behavioral sequences.* — Reproductive behaviors in *L. mackloti* can be divided into a triphasic schema: tactile-chase, tactile-alignment, and intromission and coitus. Mutually exclusive motor patterns exhibited by males during courtship were arranged into intraindividual sequences and transition motor pattern pairs from each sequence were tallied into appropriate cells of a transition matrix. This resulted in a single matrix for each species containing motor pattern transition totals. The transition matrix of preceding and following behaviors, along with the population (entire population matrix) and row values are presented for each population in Figures 3-7. In all but three populations (Timor-Semau-Roti clade), significant row $X^2$ values were observed in at least one row of the population matrix, indicating the possible presence of significant
behavioral dyads. The analysis of the significant rows suggests the presence of a geographically consistent pattern in the temporal sequence of male courtship behavior.

Sequence diagrams were drawn for each subspecies showing a grouped motor pattern transition matrix using the number of occurrences as a measure of transition frequency and relative deviation (Altmann, 1968) as a measure of transition probability. *L. m. mackloti* from Timor, Semau and Roti displayed very similar matrices of combined motor pattern transition frequencies and sequence diagrams (P>0.05). *L. m. dunni* appears to be transitional between *savuensis* and *mackloti* although they were more closely aligned with the transition matrix displayed by *L. m. mackloti* more so than *L. m. savuensis* (Appendix; Fig. 1-5).

**Female receptivity.** – Several measures of female receptivity were made. Following entry into the test chamber the contact latency and courtship latency were measured in the three species and no significant differences were detected. However, three additional parameters showed significant differences (P<0.01). An index of mating success (IMS) was calculated following Gillingham (1977).

With the IMS averages for all trails for each subspecies, *L. m. savuensis* has an IMS of 5.1, significantly higher (P<0.01) than the other two species.

When the number of dismounts per trails was compared, *L. m. savuensis* females displaced the courting male significantly fewer (P<0.01) times than *L. m. mackloti* and *L. m. dunni* and the number of TSCA’s required for intromission was significantly lower.

**Pelvic Spurring.** – The location of targeted pelvic spurring activity by males on the females dorsolateral surface was significantly different (P<0.05) when comparing the five insular populations of *L. mackloti* based on the incidence of “scratch” marks made...
on the painted dots in the middle of each scale within the three predetermined quadrats and analysis of video tapes. Males from each of the three clades spend a significant amount of time using ISP, spurring very distinctly different groups of scales when looking at the three subspecies. There was no significant difference in the target site used by males during ISP when comparing the three insular populations of *L. m. mackloti* (P>0.05) (Table 6).

*Sexual isolation between insular populations*

Homotypic encounters resulted in matings over 80% of the time (binomial test: 95% confidence limits for all homotypic encounters combined: 0.49, 0.84, N=30, P=0.71; Table 7). Females of the Timor, Semau and Roti populations were unable to distinguish homotypic and heterotypic males from those populations; with a majority of the pairings resulting in copulation. There was also no evidence of asymmetry in sexual recognition. For example, Roti male-Timor female pairs were just as likely to copulate as Roti female-Timor male pairs (Table 6). The number of eggs produced in these two crosses also produced the same number of eggs irregardless of the island of origin of the female within the Timor-Semau-Roti clade (TSR clade). Heterotypic encounters did not result in successful matings.

**DISCUSSION**

The results of our study strongly indicate that there is nearly complete sexual isolation between what we identify to be three clades, the Sawu, Wetar and Greater Timor clades, which are also delineated by the subspecies designation currently used and supported by morphological and molecular data sets (Rawlings et al., 2004; Carmichael, unpubl. data). Based on these results, we believe that each clade represents a distinct
species although it is possible that our laboratory protocol did not include the appropriate set of environmental conditions to stimulate interbreeding. This seems unlikely, however, due to the fixity of the behaviors elucidated during our study and the successful copulatory events we observed between homotypic crosses.

The analysis of successive segments of male courtship in lengthy male courtship bouts and the test of intrapopulation variation in male display indicated the presence of considerable homogeneity in male courtship within the populations we examined. This suggests that, generally, local variation in male courtship behavior is not pronounced.

We analyzed two types of male courtship information: the frequency of display of each behavior relative to other important behaviors and the sequence in which displays are presented to the female. Our results revealed considerable variation between the three subspecies in these two aspects of their courtship behavior. With respect to the sequence of male courtship, we observed the most significant differences in the pattern of transition from FSWR to ISP in the Sawu and Wetar populations when compared with the Greater Timor clade which typically transitioned from SSWR to ISP, although L. m. savuensis had the highest frequency of occurrence when analyzing the transition sequence for this transition matrix. These results suggest that different selection, environmental pressures, genetic drift or independent sexual selection events may have influenced the evolution of these two types of courtship patterns however the functional significance of the sequence of behaviors (especially in regards to the transition from SSWR to ISP in L. m. mackloti as compared to FSWR to ISP in L. m. savuensis and L. m. dunni representing an intermediate usage of FSWR to ISP and SSWR to ISP) is not well understood.
The male courtship behavior of the *L. mackloti* ssp. is complex, and it is likely that our analysis, which was intended to elucidate broad geographical patterns, lacked the resolution to diagnose subtle differences in the courtship of males from different populations, both intrapopulational differences on each of the five insular populations we studied, as well as between the three insular populations of *L. m. mackloti*. For example, some of the behaviors are distinctive but are often given with variable amounts of speed and vigor, which may alter the information content of these displays. Furthermore, we have preliminary evidence of differences in the response of males to the pheromones of females from their own and different populations (Carmichael et al., 2007). The scale of variation in male courtship behavior may depend on habitat heterogeneity, the intensity of microhabitat preferences, local and geographical dispersal patterns and genetic difference among populations. If distinctive microhabitats exist within the normal population range of this subspecies complex, divergent courtship types could arise if there is disruptive selection on traits related to microhabitat preference. This idea also merits further investigation in this group.

In our survey of the geographical pattern of male courtship behavior between the five insular populations we studied of *L. mackloti*, we found (1) large geographic differences between the three clades (Sawu, Wetar and the Greater Timor clades) in the frequency of occurrence, sequence, and spurring activity during male courtship displays, and (2) considerable intrapopulational homogeneity in the sequence with which male displays are given, and significant heterogeneity when considering the subspecies. While we did observe interpopulational differences in the frequency of occurrence of behavior in male courtship and in the sequence of male behaviors, we do not yet know the
significance of these behavioral patterns, if any, in mate selection and sexual isolation. Our study emphasizes the complexity of the courtship behavior of *L. mackloti*, and likely pythons in general. When males and females of different populations were paired experimentally, patterns of sexual isolation emerged. The three populations of the TSR clade in ex-situ experiments appear to interbreed freely. No intrapopulational differences in the frequency of male courtship behavior were observed. There is a high probability that *L. mackloti* occur on other islands such as Alor, Bambar (How and Kitchener, 1997; Rawlings et al., 2004) besides the five that we studied, and therefore the extent to which sexual isolation is present among other populations in the region has yet to be determined although due to the geographical isolation of these islands, it would appear that similar behavioral heterogeneity would be present between insular populations. This needs further examination within each insular population to measure levels of heterogeneity that might exist on each island.

All three subspecies of *L. mackloti* can be placed into the behavioral triphasic schema of Gillingham et al. (1977). The considerable amount of tongue-flicking observed, with its rate increased from Phase I to Phase II, implies an olfactory role in species and sex recognition. This activity ceases entirely once the snakes are united. In light of the probable olfactory role in species isolation, the importance of the interspecific behavioral variations observed is open to some question. The tactile movements of *L. m. mackloti* males while mounted on the female are of a different type than that exhibited by *L. m. savuensis* males in the same position. In most cases, *L. m. dunni* represents an intermediate set of behaviors shared by *L. m. mackloti* and *L. m. savuensis*, with a greater congruence of behaviors with *L. m. mackloti*. The slow and fast spurring not only show
a difference in intensity, but also in rate with *L. m. mackloti* primarily using SSWR to transition into Phase II, while *L. m. savuensis* use FSWR and *L. m. dunni* use both SSWR and FSWR. Close examination of the ventral tactile movements of the male while mounted on the female reveal decided differences between subspecies, especially in regards to the general area on the females’ dorsolateral region that is targeted by males during spurring activities. The importance of these patterns in species recognition, especially being that each of the three subspecies are isolated from each other, remains to be determined. Individual variation in pulse frequency of the spurring movements was small, but present.

Tactile movements are not restricted to spurring movements. Males press their chins tightly against the dorsal and dorsolateral surface of the female. Tongue-flicking undoubtedly has olfactory implications, but the contact of each flick with the female’s dorsum may also be a possible source of tactile stimulation during courtship. As courtship proceeds there is a similar trend in the males of all three subspecies toward anterior advancement, and at intromission the males’ head is just behind that of the female. If she moves forward and he loses position, he endeavors to advance again to the position described. It appears that his head orientation is important if for no other reason than to ensure that there is an anterior-anterior, posterior-posterior alignment between the trunks and tails.

The transition analyses on the observed behavioral sequences reject the null hypothesis that these motor patterns occur at random and that there are no differences between the transition matrix frequency between the three subspecies. The sequence diagrams created from the combined motor units demonstrate that *L. m. savuensis* has a
less complicated pattern with less active spurring in combination with writhe-bump motor patterns, while *L. m. dunni* represents a transitional taxon sharing behavioral sequences with both *L. m. savuensis* and *L. m. mackloti*. Here also, the frequency of chasing and chase-mounting is reduced. *L. m. mackloti* shows more complexity early in courtship, with the chase and chase-mount frequency increased. All of the early courtship motor patterns revolve around slow-spurring, fast-spurring, and zig-zag spurring where the spurs are lightly moved over the surface of the females dorsum longitudinally and imply its essential role to the courtship behavior as a whole.

Each phase in *L. m. mackloti* shows a Phase I cycle between TOMT and DMWR, while *L. m. dunni* and *L. m. savuensis* show a significant progression from TOMT to CHCM then to DMWR. There is also a significant difference in the use of slow versus fast spurring in order to progress to ISP in Phase II, a significant preceding behavior to coitus in Phase III. *L. m. savuensis* primarily use fast-spurring to transition into Phase II, while the three insular populations of *L. m. mackloti* primarily use slow-spurring. *L. m. dunni* uses both slow- and fast-spurring to transition to ISP, however there is a marked increase in the use of fast-spurring for this progression. Once Phase II is reached by all three subspecies, a low probability of return to Phase I motor patterns exist, while *L. m. dunni* and *L. m. savuensis* shows a greater tendency toward immediate progression from Phase I to Phase II (more so in *L. m. savuensis*).

*L. m. savuensis* courtship and mating behavior is distinguished by the high probability of fast-spurring with writhe movements. *L. m. mackloti* exhibits less fast-spurring with writhe movements but overall is similar to *L. m. savuensis* and *L. m. dunni* in that they show a simple direct sequence of mating behavior. Furthermore, the former
has a significantly lower number of dismounts per trial, requires fewer TSCA’s to reach intromission and has a higher index of mating success than the other two subspecies. The relative importance of these behavioral differences to species recognition, if any, is not known and its relation to behavioral isolation will not be resolved until the role of olfaction and visual modalities are firmly established. *Liasis mackloti* ssp. generally fit the triphasic schema however with reduced tactile-chase and tactile-alignment phases. There are generally no writhe-bumps (in isolation of spurring), forward-jerking movements or caudocephalic waves.

An interesting research question is how does sexual or ecological selection influence the duration of copulation. Although not specifically addressed in this project, we believe that there is a compromise between predator pressure and competition for mates, but the details of this compromise await further study. Following coitus, there is little if any detectable social behavior between the sexes however this also requires further analysis.

An examination of mating success and the extent of asymmetry in the sexual isolation among the populations that we compared provides some clues about the phylogenetic affinities of the populations of *L. mackloti* in this region. We conclude that each clade (Sawu, Wetar and Greater Timor), which follows the current subspecies designation, may each represent a distinct species according to the evolutionary species concept and is in agreement with other studies showing this clade boundary (Rawlings et al., 2004). The phylogeny generated from DNA sequence data (Rawlings et al., 2004 and Carmichael et al., 2003) further indicates that the molecular and morphological based topology (Carmichael unpubl. data; Rawlings et al., 2004) is congruent with a behavioral
phylogenetic topology, as in both cases *L. m. dunni* appear to be more closely related to 
*L. m. mackloti* than *L. m. mackloti* and *L. m. dunni* are to *L. m. savuensis*. Further 
interpopulation pairing studies are needed to determine the relationship between these 
and other unidentified populations throughout this region of Indonesia.

*Liasis mackloti* is composed of three clades using congruent data sets: *L. m.
savuensis* (Sawu Island), *L. m. dunni* (Wetar Island), and *L. m. mackloti* (Timor, Semau 
and Roti Islands or Greater Timor). Based on current geological interpretation (Heaney, 
1991), Wetar Island has remained isolated from all islands in the inner and outer Banda 
Arc and appears to be volcanic in origin. Sawu has also remained isolated from all the 
other islands in the Lesser Sunda region. Roti, Semau and Timor were all connected as 
‘Greater Timor’ but remained separated by sea from Sawu to the west and Wetar on the 
adjacent inner Banda Arc (Heaney, 1991). The junctions between these enlarged 
Pleistocene island groupings have an important relationship to the variation found in 
many vertebrate species and populations throughout the Lesser Sunda islands and parallel 
the pattern of geographic variation we detected in *L. mackloti ssp*. Interestingly, How 
and Kitchener (1997) examined the zoogeographic relationships of eastern Indonesian 
islands using information on snake assemblages available in the literature and from their 
survey, and found that the snake fauna on Wetar and Alor (to the west of Wetar) on the 
inner Banda Arc islands are more similar to the outer Banda Arc islands than to adjacent 
inner Banda Arc islands to their west. This suggests that a major barrier occurred 
between the islands of Lembata and Alor in the inner Banda Arc and Sumba and Sawu on 
the outer Banda Arc that differentiated assemblages to the east and west. The snake 
fauna of Sawu is allied to that of ‘Greater Timor’, Alor and Wetar at the level of species,
due primarily to the presence of *L. mackloti* on all these islands. The separation of Lombok from the other Lesser Sunda islands results from many Asian species terminating their distribution on Lombok, implying a previous closer connection to the Sunda Shelf islands to the west as has been suggested by Kitchener et al. (1990) for mammals. The clearest example of this is in the species *Dendrelaphis pictus*, which is now regarded on Lombok and islands to the west as specifically different from congeners on the Lesser Sunda islands to the east (How et al., 1996).

Several snake species in the Lesser Sunda region also show morphological variation between islands consistent with observations made by the author (Carmichael et al., unpubl. data; Carmichael et al., 2007), with the most marked variations apparent between regions that were separated by sea barriers during the Pleistocene glacial maxima. For example, populations of *Dendrelaphis inornatus* on Sumba are the same subspecies as those on Sawu, Sumbawa, Flores and Lembata, while those on Alor, Pantar and Wetar are taxonomically allied to those on Timor, Semau and Roti. Musters (1983) noted a similar island distribution for subspecific differentiation in the agamid *Draco volans* Linnaeus, with *D. v. boschmai* Hennig, occurring on the islands of Sumbawa, Sumba, Flores and Adonara and *D. v. timoriensis* Kuhl, occurring on Roti, Semau, Wetar and Alor. These two studies suggest that a major barrier occurred between the islands of Lembata and either Pantar or Alor that differentiated forms to the east and west. Seas separated both these islands throughout the Pleistocene.

Studies of other species suggest that morphological differentiation occurred at different junctures in the Pleistocene geography, but almost always between islands that had remained separated during the glacial maxima. On Sawu the population of *L. m.*
Savuensis has been shown to be subspecifically distinct from the nominate form that occurs on the other islands of the eastern Lesser Sunda region (Brongersma, 1956). Thus Sawu, which was geographically isolated throughout the Pleistocene, has a unique subspecies of boid but shares the same subspecies of colubrid with islands of the western Lesser Sunda region. How et al. (1996) examined morphological variation in four species of snake throughout the Lesser Sunda islands and showed that the greatest morphological variation within species occurred between populations isolated by sea throughout the Pleistocene rather than those isolated on present configurations.

This emphasis on the significance of Pleistocene sea barriers to morphological differentiation has been reinforced by studies on numerous mammalian taxa (Kitchener and Suyanto, 1996). The chiropteran, Hipposideros didema, showed subspecific variation between the islands of Lombok and Sumbawa (Kitchener et al., 1992), a case that parallels the recent specific separation of the snake Dendrelaphis from these two islands (How et al., 1996). However, in the other twenty-five recognized subspecies of ten mammalian taxa the separation occurred where island populations had been isolated throughout the Pleistocene (Kitchener and Suyanto, 1996).

The level of endemism among the snakes of eastern Indonesia is higher than that documented for the butterflies of the region (Vane-Wright, 1991) but similar to that being documented for the mammals (Kitchener and Suyanto, 1996). Endemism of above 20% in the Lesser Sunda islands suggests that these islands are an important region for speciation in snakes. Endemicity is one of the most significant features of biogeographic distributions (Rosen, 1988) and within the islands of eastern Indonesia there have been major barriers to dispersal, both past and present, that have enhanced the levels of
vertebrate endemicity. Kitchener and Suyanto (1996) concluded that the high endemicity among mammals in the Lesser Sunda and Maluku regions, as well as Sulawesi, was strongly supportive of Simpson’s (1977) view that Wallacea should not be regarded as a transitional zone between the Asian and Australo-Papuan region.

There is evidence within the snakes of Indonesia for considering the Lesser Sunda islands and the southern and northern Maluku islands as unique biogeographic subregions with differing regions of origin for their assemblages, a relatively high degree of endemism and areas for incipient speciation. The patterns of geographic variation we detected in regards to courtship behaviors in *L. mackloti* ssp. is congruent with the patterns detected in regards to molecular and morphological data sets (Carmichael et al., 2003; Rawlings et al., 2004). We have also identified that the three data sets (molecular, morphological and behavioral) yield equivalent patterns of phylogeny. Current studies are focusing on *L. mackloti* from the islands of Alor (which is presumably at the western edge of a major biogeographic barrier), Babar and other smaller islands in this vicinity to continue to gain a more thorough understanding of the range of phenotypic variation present, so that ultimately a robust phylogeny can be generated using a comparative method to elucidate the taxonomic status of this subspecies complex. This information will also give further insights into the biogeography of this insularized python species.

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LITERATURE CITED


### APPENDIX A

#### Table 1

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Figure 1. Matrices of combined motor pattern transition frequencies and corresponding sequence diagrams for *L. m. mackloti* from the island of Timor. Rows correspond to preceding acts and columns to following acts. Values above represent observed frequencies with their expected values below and numbers in parentheses represent standard deviations. Percent (%) occurrence is given next to totals. In sequence diagram below, thickness of arrows represents relative deviation (probability) and circle diameters represents frequency of occurrence. Roman numerals denote behavioral phases.
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Figure 2. Matrices of combined motor pattern transition frequencies and corresponding sequence diagrams for L. m. mackloti from the island of Roti. Rows correspond to preceding acts and columns to following acts. Values above represent observed frequencies with their expected values below and numbers in parentheses represent standard deviations. Percent (%) occurrence is given next to totals. In sequence diagram below, thickness of arrows represents relative deviation (probability) and circle diameters represents frequency of occurrence. Roman numerals denote behavioral phases.
APPENDIX A

Figure 3. Matrices of combined motor pattern transition frequencies and corresponding sequence diagrams for *L. m. mackloti* from the island of Semau. Rows correspond to preceding acts and columns to following acts. Values above represent observed frequencies with their expected values below and numbers in parentheses represent standard deviations. Percent (%) occurrence is given next to totals. In sequence diagram below, thickness of arrows represents relative deviation (probability) and circle diameters represents frequency of occurrence. Roman numerals represent behavioral phases.

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Figure 4. Matrices of combined motor pattern transition frequencies and corresponding sequence diagrams for *L. m. savuensis* from the island of Sawu. Rows correspond to preceding acts and columns to following acts. Values above represent observed frequencies with their expected values below and numbers in parentheses represent standard deviations. Percent (%) occurrence is given next to totals. In sequence diagram below, thickness of arrows represents relative deviation (probability) and circle diameters represents frequency of occurrence. Roman numerals represent behavioral phases.

![Sequence Diagram](image_url)
### Figure 5

Matrices of combined motor pattern transition frequencies and corresponding sequence diagrams for *L. m. dunni* from the island of Wetar. Rows correspond to preceding acts and columns to following acts. Values above represent observed frequencies with their expected values below and numbers in parentheses represent standard deviations. Percent (%) occurrence is given next to totals. In sequence diagram below, thickness of arrows represents relative deviation (probability) and circle diameters represents frequency of occurrence. Roman numerals represent behavioral phases.

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