To the Graduate Council:

I am submitting herewith a thesis written by Lisa Bailey Comeaux entitled "A Conservation Genetic Study and Phylogeny of Three Flying Fox (Chiroptera: Pteropodidae) Species." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Zoology.

Dr. Gary F. McCracken, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the council:

Associate Vice Chancellor
and Dean of the Graduate School

A Conservation Genetic Study and Phylogeny of Three Flying Fox (Chiroptera: Pteropodidae)Species

A Thesis

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Master of Science

Degree

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Abstract

Genetic diversity of three species of flying foxes (*Pteropus hypomelanus, P. pumilus, and P. rodricensis:* family Pteropodidae) was determined using Random Amplified Polymorphic DNA (RAPDs). Two of these species, *P. pumilus* and *P. rodricensis*, are in danger of extinction. The third species, *P. hypomelanus,* is not considered to be threatened. These bats are part of a captive breeding program in which genetic testing is of value in preventing the deleterious effects associated with loss of genetic diversity and inbreeding. Genetic similarity indices were calculated for individuals within each population. *Pteropus pumilus* displayed the least within-population similarity (0.906), *P. hypomelanus* displayed an intermediate value (0.916), and *P. rodricensis* had the greatest within population similarity (0.990). Mean genetic distances calculated from the similarity indices indicate that *P. pumilus* and *P. rodricensis* are more closely related to each other (0.205) than either is to *P. hypomelanus* (*P. hypomelanus* - *P. pumilus* (0.395); *P. hypomelanus* -*P. rodricensis* (0.484)) . The greatest heterozygosity was displayed by *P. pumilus* (0.207). *Pteropus hypomelanus* had a slightly smaller value (0.171), and *P. rodricensis* had the least heterozygosity (0.014). The genetic distances calculated from gene frequencies were only slightly less than those calculated from similarity indices.

Paternity exclusion was attempted for pups of *P. pumilus* and *P. rodricensis* that were born in captivity. For two *P. rodricensis* pups, all potential sires except one were excluded. For the seven *P. pumilus* pups, paternity exclusion results varied with different primers and are in conflict with data generated by other, more reliable methods.

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Chapter 1

Introduction

Flying foxes are members of the family Pteropodidae (Chiroptera) which is composed of 42 genera and 173 species. The geographical range of this family stretches from 37° north latitude to 38° south latitude and includes much of Africa, most of India, southern Asia, the Australian region, and associated Indian Ocean and western Pacific Islands (Fig 1; Mickleburgh *et al.*, 1992). Flying foxes feed almost exclusively upon fruit and floral products, and, thereby, play fundamental roles as pollinators and seed dispersers. According to Fujita & Tuttle (1991), over 280 plant species rely on flying foxes for propagation, many of which are essential to local economies. These plants serve many purposes, providing timber, foods, drinks, medicines, dyes, fibers, and fuels. Their value to local economies amount to millions of dollars annually.

Flying foxes also play important ecological roles. In several Pacific Island tropical forests, flying foxes are the sole vertebrate pollinators and seed dispersers. In fact, they may be responsible for the maintenance of entire Pacific island floras (Cox *et al.*, 1991). Despite their keystone status, flying foxes are poorly understood and suffer from poor reputations. They are often hunted for sport with losses counted in the thousands annually (Fujita and Tuttle, 1991). In many areas they are sold for food at high prices as a local delicacy, and in China, they are used medicinally.

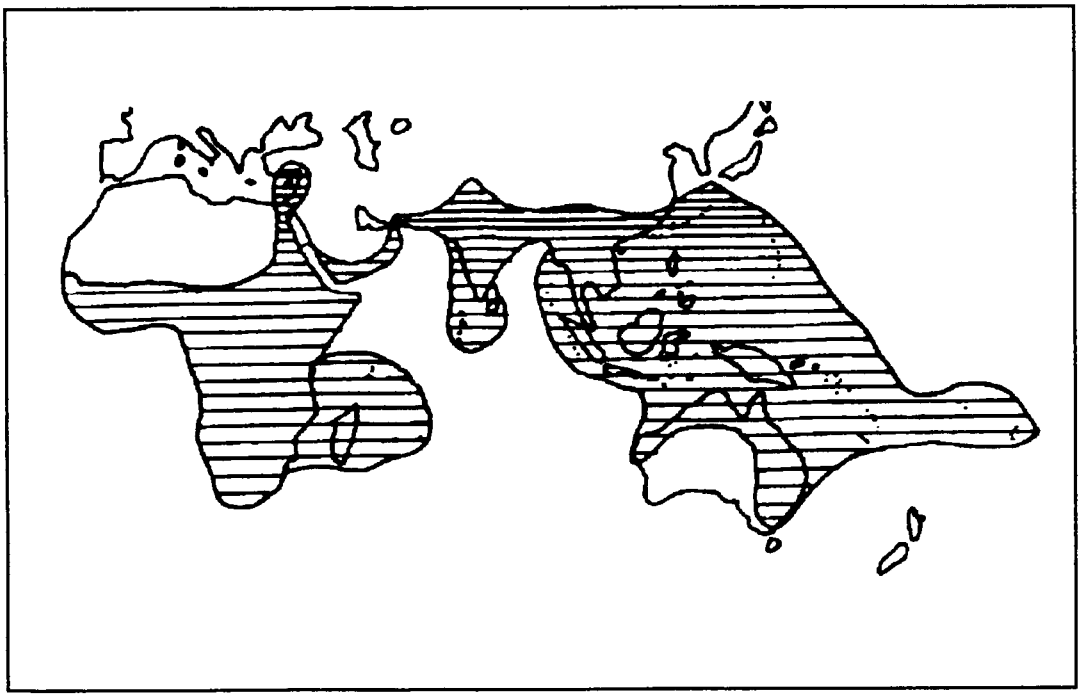


Fig 1. Global distribution of flying foxes of the genus *Pteropus* (Chiroptera: Pteropodidae) (Mickleburgh *et al.*, 1992).

The greatest threat to flying foxes comes from plantation owners who cultivate crops derived from their natural food. These plants often attract flying foxes, especially in areas where forests have been cleared for agricultural purposes. The bats often consume or cause damage to these crops resulting in the desire for large scale eradication of bats. In several instances, entire diurnal roosts have been wiped out in a single day (Mickleburgh *et al.*, 1992). If the bats survive these threats, their reduced numbers leave them increasingly vulnerable to eradication by introduced diseases and predators, by natural phenomena such as cyclones, and to the deleterious effects of genetic drift and inbreeding. Inbreeding in a small population can result in losses of heterozygosity, polymorphism and allelic diversity, which may result in the expression of deleterious alleles, lower individual fitness and decreased chances for survival (Allendorf and Leary, 1986).

The largest and most economically important genus, *Pteropus*, is primarily an island taxon. This taxon consists of 173 species, with over 96% of the species being entirely or partially distributed on islands (Brautigam and Elmqvist, 1990). Levels of endemism are high among these island flying foxes, with 61.4% of these species confined to single islands or small island groups (Mickleburgh *et al.*, 1992). Because of their low reproductive rates (usually 1 pup per year) and their limited distributions, many *Pteropus* species are in serious danger of extinction (Pierson and Rainey, 1992). In many cases, preservation may be dependant upon captive propagation.

Captive breeding programs should be designed to minimize further losses of genetic diversity due to inbreeding within a small population. In order to prevent losses of

diversity, the levels of remaining diversity must be assessed, and parentage, especially paternity, must be ascertained. This study was designed to determine these parameters for flying foxes held at the Lubee Foundation captive breeding program in Gainesville, Florida.

Three species of *Pteropus* (*P. hypomelanus*, *P. pumilus*, and *P. rodricensis*) were studied. These species constitute a wide array of population and biogeographical parameters, lending themselves to a study of genetic diversity which can be used to contribute to existing conservation strategies or develop new approaches.

The first of these species, *P. rodricensis*, which has been called the rarest bat in the world, is limited in its distribution to Rodrigues Island, a small island located approximately 1500 km east of Madagascar. This species has suffered a severe decline due to hunting, habitat loss, and cyclones. Its population was estimated at less than 80 individuals in 1974 (Cheke and Dahl, 1981). In 1976, a captive breeding program was established by the Jersey Wildlife Preservation Trust in cooperation with the Mauritian government using 18 bats captured from the wild. Two colonies were established, one at the Jersey Zoo founded by three males and seven females, and one in Mauritius founded by three males and five females (Carroll and Mace, 1988). By 1991, this population had increased to over 250 individuals housed at nine different facilities (Mickleburgh *et al.*, 1992). The Lubee Foundation's *P. rodricensis* colony was founded using three males and five females which were descended from the original captive population. At the time of this study, this population had increased to 20 bats. On Rodrigues Island, the only remaining wild habitat for *P. rodricensis*, the population has increased to an estimated

1500 individuals (Mickleburgh *et al.*, 1992). The survival of this population is precarious because severe habitat loss has left it particularly vulnerable to cyclones. Not only do cyclones directly cause the deaths of large numbers of bats which are unable to find adequate shelter, but they also destroy vegetation and the bats' few food resources (Cheke and Dahl, 1981).

Pteropus pumilus, a native of the Philippine Islands, is listed as vulnerable by the International Union for Conservation of Nature and Natural Resources (IUCN) (Mickleburgh *et al.*, 1992). This species is fairly intolerant to human disturbance, and the major threat to its survival is deforestation. Because *P. pumilus* flies and forages high in the canopy, it is seldom observed, and population numbers may be greater than sightings and capture rates imply. For the same reason, relatively little is known of the life history and status of this species (Mickleburgh *et al.*, 1992). The Lubee *P. pumilus* colony was begun with seven males and six females which were wild-caught. Seven offspring were subsequently produced.

Pteropus hypomelanus, which has a wide geographical range including Peninsular Malaysia, Indonesia, Thailand, Burma, the Philippines, and several Indian and Pacific Ocean islands, is listed by the IUCN as not threatened. This species seems to be tolerant of human disturbance and often persists in agricultural areas (Mickleburgh *et al.*, 1992). The Lubee colony was founded with 12 males and 26 females which were wild caught. These bats have proliferated in captivity and had produced 23 offspring when this study was begun.

RAPD (Random Amplified Polymorphic DNA) analysis is a PCR based technique

which uses primers of arbitrary sequence to produce unique banding patterns similar to those produced by RFLPs (Restriction Fragment Length Polymorphisms) (Williams et al., 1990; Welsh and McClelland, 1990) (Fig. 2). This technique has been used to estimate genetic diversity in numerous taxa including species of mammals (Kappe *et al.*, 1995), fish (Bardakci and Skibinski, 1994), insects (Kambhampati *et al.*, 1992), fungi (Huang *et al.*, 1994), and plants (Chalmers *et al.*, 1993). RAPD data have also been used in the construction of phylogenetic trees (Kambhampati *et al.*, 1992, Bardakci and Skibinski, 1994). Recently, a controversy has been generated over the use of RAPDs in paternity testing. Riedy *et al.* (1991) found a large number of non-parental bands in offspring of known pedigree and, therefore, found paternity identification impossible. Scott *et al.* (1992) presented several guidelines for analysis which may make paternity determination using RAPDs feasible in some cases.

The goals of this study were to use RAPDs to: 1) estimate the levels of genetic variation in the Lubee Foundation colonies of *P. rodricensis*, *P. pumilus*, and *P. hypomelanus*; 2) determine the genetic distances between these three species; and 3) attempt to determine the paternity of pups born into the *P. pumilus* and *P. rodricensis* colonies.

It was hypothesized that the levels of genetic variation would be lowest for *P. rodricensis* due to its history of population bottlenecks and inbreeding. It was expected that *P. pumilus* should show intermediate levels of variation due to its limited distribution and relatively small population size, whereas it was expected that *P. hypomelanus* would have the greatest levels of genetic variation. Based on morphology, it was hypothesized

that the genetic distance should be the least between *P. pumilus* and *P. rodricensis*, and the greatest between *P. hypomelanus* and *P. rodricensis*. Finally, it was hypothesized that paternity exclusion should be possible for *P. pumilus*, but may be limited by a lack of variation in *P. rodricensis.*

Chapter 2

Materials and Methods

Sample Collection

Approximately 2 ml of blood was obtained from each of the 20 *P. rodricensis* and the 20 *P. pumilus* individuals housed at the Lubee Foundation (Tables 1 and 2). Two ml of blood was also taken from twenty wild caught *P. hypomelanus* adults that were arbitrarily selected from the Lubee colony (Table 3). The blood was mixed with 0.3 ml of sterile 75% EDTA and stored at -80°C.

DNA Extraction

DNA from blood cells was extracted with phenol / chloroform and concentrated using Microcon 30 microconcentrators (Amicon). Detailed description of DNA extraction procedures is outlined in Appendix A. DNA concentration was estimated with a TKO 100 fluorometer and stock solutions were diluted to 10 ng/μl with 0.1X TE. All samples were stored at -20°C.

RAPD PCR

Primer kits A, B, and C were obtained from Operon Technologies. Each kit contained 20 random sequence decamers of >50% GC concentration. These 60 primers were screened to identify 25 that produced clear bands that were easily interpretable for all

Table 1. *P. rodricensis* individuals used in this study. An asterisk indicates a pup born in captivity at the Lubee Foundation. An ISIS number is assigned to each captive animal, and because it is unique and constant, it is useful for identification.

Number	Sex	ISIS #
r1	M	904564
r2	M	904574
r3	M	904576
r4	F	904561
r5	F	904562
r6	F	904563
r7	F	904560
r8	F	904559
*r9	M	914524
*r10	M	914528
*r11	F	914529
*r12	F	914527
*r13	F	914525
*r14	M	929422
*r15	M	929424
*r16	M	929423
*r17	M	929425
*r18	M	929485
*r19	F	929505
*r20	F	929524

Table 2. *Pteropus pumilus* individuals used in this study. An asterisk indicates a pup born in captivity at the Lubee Foundation. An ISIS number is assigned to each captive animal, and because it is unique and constant, it is useful for identification.

number	Sex	ISIS #
p1	M	914523
p2	M	929216
p3	M	929215
p4	M	929214
p5	M	929213
p6	M	929212
p7	M	929211
p8	F	929210
p9	F	929209
p10	F	929208
p11	F	929207
p12	F	929206
p13	F	929205
*p14	F	929474
*p15	M	929472
*p16	F	929490
*p17	F	929514
*p18	F	929522
*p19	M	929539
*p20	M	929543

Table 3. *Pteropus hypomelanus* individuals used in this study. An asterisk indicates a pup born in captivity at the Lubee Foundation. An ISIS number is assigned to each captive animal, and because it is unique and constant, it is useful for identification.

Band #	Sex	ISIS #
620	F	904533
622	F	904536
623	F	904537
626	F	904515
627	M	904525
633	F	904517
635	M	Deceased
638	F	904534
639	F	904553
645	F	904541
647	F	904516
648	F	Deceased
649	F	904520
652	F	904539
656	F	904546
661	F	904549
666	F	904552
667	M	Deceased
684	F	904557
688	F	904558

All *P. hypomelanus* were captured from the wild.

three species. Analysis of all samples was conducted using these primers (Table 4). Each 10μl reaction mixture consisted of 100 μM/μl nucleotide triphosphates (Idaho Technologies), 0.4 pM/μl primer, 20 ng DNA template, 0.06 u/μl Taq DNA Polymerase (Promega), and 1X medium $MgCl_2$ buffer (Idaho Technologies) in sterile, distilled water. PCR was performed using an Air Thermo Cycler 1605 (Idaho Technologies). The amplification procedure (adapted from Williams *et al.*, 1990) consisted of 2 cycles with a denaturing temperature of 92°C for 60 seconds, an annealing temperature of 42°C for 7 seconds, and a period of elongation at 72°C for 70 seconds, followed by 38 cycles of 92°C for 1 second, 42°C for 7 seconds, and 72°C for 70 seconds. The amplification products were separated by horizontal electrophoresis using 1.4% agarose gels stained with ethidium bromide. Because there is controversy over the repeatability of RAPD bands, each individual was tested twice with each primer.

Analysis

Bands were scored as 1 if present and 0 if absent. Only those bands which were visible in both runs were scored. The total number of loci produced and the proportion of those loci which produced polymorphic products were calculated. Because there is no general consensus as to the most acceptable method of RAPD data analysis, these data were analyzed using two different methods which yield slightly different but comparable measures of within- and between population genetic diversity and genetic distances. The first method involved a calculation of an index of similarity or Band Sharing Index (BSI) using the formula:

Table 4. Sequences and codes for primers used in this study.

Primer Code	Primer Sequence (5' to 3')
OPA-2	TGCCGAGCTG
OPA-5	AGGGGTCTTG
OPA-7	GAAACGGGTG
OPA-8	GTGACGTAGG
OPA-9	GGGTAACGCC
OPA-11	CAATCGCCGT
OPA-14	TCTGTGCTGG
OPB-1	GTTTCGCTCC
OPB-3	CATCCCCCTG
OPB-6	TGCTCTGCCC
OPB-7	GGTGACGCAG
OPB-8	GTCCACACGG
OPB-9	TGGGGGACTC
OPB-10	CTGCTGGGAC
OPB-13	TTCCCCCGCT
OPB-14	TCCGCTCTGG
OPB-15	GGAGGGTGTT
OPC-1	TTCGAGCCAG
OPC-2	ATGGGTCCAG
OPC-5	TACGGCTGAG
OPC-6	GAACGGACTC
OPC-8	GTGAACTCCG
0PC-10	TGTCTGGGTG
OPC-11	AAAGCTGCGG
OPC-18	TGAGTGGGTG

$$S_{ij} = 2S_{ij}/(N_i + N_j)$$

where S_{ij} is the number of bands shared by individuals i and j and N_i and N_j are the number of bands scored for each individual (Lynch, 1990). The BSI is a measure of similarity between two individuals and is based upon the assumption that shared bands are homologous and are indicative of common inheritance. Thus, two individuals that share many bands are more closely related than two individuals that share fewer bands. When the BSI is equal to one, the banding patterns of the two individuals are exactly the same, and when the BSI value is zero, no bands are shared by the two individuals (Bardakci and Skibinski, 1994). Within population similarity was calculated as the average of S_{ij} across all pairwise comparisons between individuals. This value indicates the relative number of bands shared by members of a population or sample (Lynch, 1990). When this value approaches one, the individuals of the population are very similar to each other and, therefore, the genetic diversity is low. As this value approaches zero, genetic diversity increases. Between population similarity was calculated using the formula:

$$S_{ij} = 1 + S'_{ij} - 0.5(S_i + S_j)$$

where S'_{ij} is the average similarity between all paired individuals from populations i and j, and S_i and S_j are the values of S for populations I and j (Lynch, 1990). This value indicates the average numbers of bands shared between populations and is interpreted in the same manner as within population similarity. It is possible for this value to exceed one. Genetic distance between populations i and j can be calculated using the formula for Roger's genetic distance using band frequencies instead of allele frequencies:

$$D_{ij} = (1/n) \Sigma^{n}_{k=1} (X_{ij} - X_{jk})$$

where n is the number of bands, X_{ij} is the frequency of the j^{th} band in the i^{th} population, and X_{jk} is the frequency of the k^{th} band in the i^{th} population (Nei, 1987). Nei (1978) noted that using small numbers of individuals can lead to negative genetic distance values and suggested the following bias correction to counteract the problem:

$$S_i = (2n_xS_x - 1)/(2n_x - 1) \text{ and } S_j = (2n_yS_y - 1)/(2n_Y - 1)$$

where n_x and n_y are the numbers of individuals sampled from population i and j, respectively, and S_x and S_y are the squared averages of the shared band frequencies in populations i and j. This bias correction was implemented after preliminary tests yielded several negative distance values.

The second method (Lynch and Milligan, 1994) involved the calculation of more conventional measures of population structure such as gene frequencies and heterozygosity. This analysis relies on the assumption that each locus can be treated as a two-allele system, where only one (dominant) allele is amplifiable by PCR. In order to avoid a downward bias in the estimation of null allele frequency, a locus was used only if $Nq^2 > 3$ where Nq is the number of individuals of a given population displaying the null allele. Gene frequency of the null allele (q) was estimated using the formula:

$$q = x^{1/2}(1 - Var(x) / 8x^2)^{-1}$$

where x is the fraction of individuals in the population without the marker, $Var(x) = x(1 - x)/N$ is the sampling variance of the frequency of null homozygotes, and N is the number of individuals sampled. This formula was used instead of $q = x^{1/2}$ which yields a downwardly biased estimate of q (Lynch and Milligan, 1994).

When the population is locally inbred, like *P. rodricensis*, $x = q^2(1 - F_{is}) + qF_{is}$. In

this situation, there is no way to determine if F_{is}=0 with RAPD analysis of one generation. In this case, it was suggested that random mating be allowed in the population so that Hardy-Weinberg equilibrium could be attained in the offspring population, and disequilibrium could be revealed by a change in x across generations. Furthermore, it is recommended that if the value of F_{is} for the parental generation was equal to or very near zero, the estimates of q could be pooled for both generations. If, however, the value of F_{is} was not equal to zero, then the q value should be included only for the offspring generation. In the case of *P. rodricensis*, two generations were available for this analysis, but other factors warranted consideration. First, although the population is inbred, there is no reason to believe that the parental generation was not produced from random mating. Secondly, because of the extreme inbreeding experienced by *P. rodricensis*, F_{is} is almost certainly not equal to zero for either generation. Finally, if the parental generation were excluded, only 12 individuals would be available for analysis. For these reasons, the estimates of q were pooled from both generations. Expected heterozygosity was calculated by the formula:

$$H_j(I) = 2q_j(I)[1 - q_j(I)] + 2Var[q_j(I)]$$

which is the probability that two genes, randomly drawn from population j, differ at the ith locus. Averaging this value over all observed loci (L) gives the mean observed gene diversity of the jth population

$$H_j = 1/L\sum^{L}_{j=1} He(I).$$

The probability that a gene randomly drawn from population j differs from one randomly drawn from population k (termed heterozygosity between populations j and k at the ith

locus) was estimated by the formula:

$$H_{jk}(I) = q_j(I) + q_k(I) - 2q_j(I)q_k(I)$$

An estimate of Nei's genetic distance between populations was calculated using the formula:

$$D_{jk} = -\ln(j_{jk}/(j_j j_k)^{1/2}) - \mathrm{Var}(H'_{jk})/2j^2_{jk} + \mathrm{Var}(H_j)/4j^2_j + \mathrm{Var}(H_k)/4j^2_k .$$

This formula provided an estimate of the mean number of mutations separating the genes from two populations.

Paternity Analysis

Mother-pup relationships were obtained from Lubee for individuals of *P. pumilus* and *P. rodricensis*. These individuals and potential sires were examined using the previously mentioned RAPD protocols and suggestions made by Scott *et al.* (1992) to help avoid misinterpretation due to non-parental bands. These suggestions included making sure that bands from all amplifications were reproducible, and when possible, using only bands unique to the putative sires, making the examination of other loci unnecessary. Each locus was examined for the situation in which the pup had the dominant allele and the dam had the null allele. This configuration is the only one which can yield paternity exclusion data using RAPDs. In this case, all putative sires which did not possess the dominant allele were excluded. More loci were examined in the same manner until all potential sires except the true father were eliminated (see Appendix D for an example).

Chapter 3

Results

All primers except primers OPB-13 and OPB-14 yielded variable products for at least one species. Twelve primers yielded variable products for all three species. The number of bands produced per individual by each primer ranged from 2 to 15 with an average of 8, resulting in a total of 218 presumptive loci in the three species. *Pteropus pumilus* was the most polymorphic of the species, followed by *P. hypomelanus,* with *P. rodricensis* the least polymorphic (Fig. 2).

Repeatability was high for most primers. Primer OPB-8 produced two bands in six individuals in the first run that were not present in the second run. Primer OPB-15 produced two bands in one individual and three bands in two individuals which were not reproduced in the second run. Primers OPC-2, OPC-5, OPC-6, OPB-1, OPB-3, OPA-5, and OPA-7 all produced single bands in one or two individuals which were not present in both runs. The aberrant bands varied among individuals and species. In only one instance was a non-reproducible band produced in the same individual for two different primers. All other primers yielded identical results in both runs. In all cases, bands which were not produced in both runs were omitted from the analysis.

Similarity Index Analysis

The values of the within population similarities for each species varied across

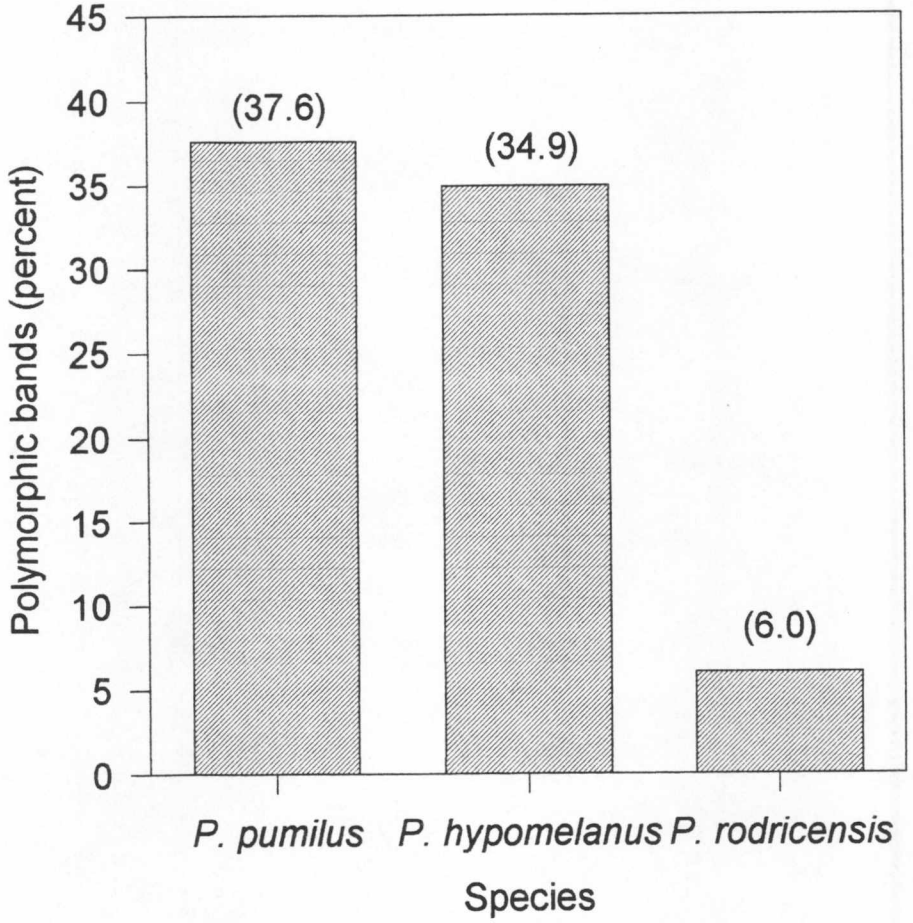


Fig. 2. Percentage of the 218 RAPD loci which were polymorphic in three species of bats of the genus *Pteropus*.

primers (Table 5). The ranges and averages for each species were as follows:

	range	average
P. hypomelanus	0.797 - 1.000	0.916
P. pumilus	0.516 - 1.000	0.906
P. rodricensis	0.896 - 1.000	0.990.

The between population similarity values indicate that on average, individuals of *P. pumilus* and *P. rodricensis* share more bands in common than either does with *P. hypomelanus* (Table 6). These between population similarity values were used to calculate genetic distances (Table 7). The genetic distance is greatest between *P. hypomelanus* and *P. rodricensis,* and the least between *P. pumilus* and *P. rodricensis.* The ranges and averages of genetic distances between species are as follows:

	range	average
P. hypomelanus - P. pumilus	0.033 - 1.100	0.395
P. hypomelanus - P. rodricensis	0.081 - 1.125	0.485
P. pumilus - P. rodricensis	0.000 - 0.970	0.206.

Gene Frequency and Heterozygosity Calculation

For this part of the analysis, it was presumed that each band of a specific size is a separate locus. Gene frequencies and heterozygosities varied across loci both among and within primers. For *P. hypomelanus* the square of number of individuals displaying the null allele (Nq^2) exceeded 3 for 113 of the 218 loci. The numbers of loci where $Nq^2 > 3$ for *P. pumilus* and *P. rodricensis* were 110 and 78, respectively. These loci were used to

Table 5. Within population similarities for three species of bats of the genus *Pteropus* calculated for each primer.

Primer	Species		
	P. hypomelanus	*P. pumilus*	*P. rodricensis*
OPA-2	0.870	0.566	0.985
OPA-5	0.904	0.944	0.981
OPA-7	0.860	0.964	1.000
OPA-8	0.821	0.853	1.000
OPA-9	0.961	0.914	1.000
OPA-11	0.855	0.887	0.986
OPA-14	0.912	0.925	0.973
OPB-1	0.948	0.901	0.980
OPB-3	0.954	1.000	1.000
OPB-6	0.980	0.973	0.986
OPB-7	0.859	0.816	0.896
OPB-8	0.852	0.932	0.978
OPB-9	0.931	0.906	1.000
OPB-10	0.932	0.931	1.000
OPB-13	1.000	1.000	1.000
OPB-14	1.000	1.000	1.000
OPB-15	0.960	0.925	0.986
OPC-1	1.000	1.000	1.000
OPC-2	0.797	0.890	0.979
OPC-5	0.855	0.691	0.985
OPC-6	0.954	0.908	0.995
OPC-8	0.922	0.946	1.000
OPC-10	1.000	0.973	1.000
OPC-11	0.855	0.924	1.000
OPC-18	0.914	0.891	1.000
Average	0.916	0.906	0.990

Table 6. Between population similarities for three species of bats of the genus *Pteropus* calculated for each primer.

Primer	Species Compared		
	hyp-pum	hyp-rod	pum-rod
OPA-2	0.569	0.540	0.775
OPA-5	0.707	0.660	0.839
OPA-7	0.547	0.449	0.825
OPA-8	0.837	0.672	0.847
OPA-9	0.677	0.544	0.849
OPA-11	0.843	0.798	0.993
OPA-14	0.582	0.532	0.996
OPB-1	0.879	0.683	0.785
OPB-3	0.968	0.569	0.615
OPB-6	0.854	0.856	1.000
OPB-7	0.774	0.479	0.604
OPB-8	0.707	0.656	0.899
OPB-9	0.725	0.924	0.778
OPB-10	0.759	0.696	0.995
OPB-13	0.500	0.667	0.800
OPB-14	0.667	0.667	1.000
OPB-15	0.689	0.636	0.829
OPC-1	0.333	0.286	0.889
OPC-2	0.631	0.647	0.630
OPC-5	0.797	0.556	0.901
OPC-6	0.921	0.805	0.901
OPC-8	0.603	0.876	0.396
OPC-10	0.852	0.857	0.999
OPC-11	0.939	0.881	0.991
OPC-18	0.684	0.481	0.903
Average	0.747	0.657	0.842

hyp = *P. hypomelanus*
pum = *P. pumilus*
rod = *P. rodricensis*

Table 7. Genetic distances calculated for each primer for three species of bats of the genus *Pteropus* calculated from a similarity index

Primer	Species		
	hyp-pum	hyp-rod	pum-rod
OPA-2	0.894	0.683	0.306
OPA-5	0.381	0.447	0.183
OPA-7	0.686	0.895	0.197
OPA-8	0.216	0.442	0.178
OPA-9	0.423	0.625	0.171
OPA-11	0.199	0.246	0.006
OPA-14	0.607	0.686	0.004
OPB-1	0.140	0.399	0.259
OPB-3	0.033	0.581	0.486
OPB-6	0.162	0.158	0.000
OPB-7	0.314	0.900	0.621
OPB-8	0.397	0.469	0.112
OPB-9	0.355	0.081	0.264
OPB-10	0.299	0.378	0.005
OPB-13	0.693	0.406	0.223
OPB-14	0.406	0.406	0.000
OPB-15	0.400	0.469	0.197
OPC-1	1.100	1.253	0.118
OPC-2	0.574	0.502	0.503
OPC-5	0.300	0.657	0.111
OPC-6	0.088	0.224	0.109
OPC-8	0.555	0.138	0.970
OPC-10	0.163	0.154	0.001
OPC-11	0.070	0.134	0.009
OPC-18	0.431	0.780	0.106
Average	0.395	0.485	0.206

hyp = *P. hypomelanus*
pum = *P. pumilus*
rod = *P. rodricensis*

calculate estimated gene frequencies and heterozygosities (Tables 8-10). The range of heterozygosity values for *P. hypomelanus* is 0.000 - 0.519 with an average of 0.171. The range for *P. pumilus* is 0.000 - 0.519 with an average of 0.207, and the values for *P. rodricensis* range from 0.000 to 0.517 with an average of 0.014.

The genetic distance values calculated from gene frequencies are as follows:

P. hypomelanus - *P. pumilus*	0.373
*P. hypome*lanus - *P. rodricensis*	0.500
P. pumilus - *P. rodricensis*	0.263.

Paternity Exclusion

For paternity exclusion analysis in *P. rodricensis*, only one locus (primer OPB-1; Band size ~ 700 bp) was informative. All dams were null homozygotes, and two pups, r9 and r15 displayed the dominant allele. Of the three putative sires (r1, r2, and r3), only r3 possessed the dominant allele. Thus r1 and r2 were excluded, and r3 was identified as the sire of r9 and r15.

Sufficient variation was present to permit paternity exclusion for all *P. pumilus* pups using the criteria of Scott *et al.* (1992), however paternity exclusion using different sets of loci were in conflict with one another. As examples, dominant alleles at different loci were unique to only one of the putative sires (Tables 11 and 12). However, in all cases, the use of different loci led to the identification of different putative sires for each pup.

Table 8. Estimated gene frequencies and heterozygosities for *Pteropus hypomelanus.*

primer	band	frequency	heterozygosity
OPA-2	506	0.975	0.050
OPA-2	650	1.000	0.000
OPA-2	750	0.745	0.391
OPA-2	1000	1.000	0.000
OPA-2	1060	1.000	0.000
OPA-2	1350	1.000	0.000
OPA-2	1500	0.868	0.236
OPA-5	506	1.000	0.000
OPA-5	600	0.556	0.511
OPA-5	700	0.402	0.502
OPA-5	750	1.000	0.000
OPA-5	930	1.000	0.000
OPA-5	1000	1.000	0.000
OPA-5	1060	0.459	0.517
OPA-5	1100	1.000	0.000
OPA-5	1636	0.402	0.502
OPA-7	600	1.000	0.000
OPA-7	800	0.809	0.318
OPA-7	1050	1.000	0.000
OPA-7	1060	1.000	0.000
OPA-7	2050	0.402	0.502
OPA-7	3000	0.510	0.519
OPA-8	506	1.000	0.000
OPA-8	650	0.402	0.502
OPA-8	700	0.949	0.099
OPA-8	1000	0.556	0.511
OPA-9	450	0.923	0.146
OPA-9	1060	1.000	0.000
OPA-9	1800	1.000	0.000
OPA-9	2000	1.000	0.000
OPA-11	550	0.510	0.510
OPA-11	1000	1.000	0.000
OPA-11	2000	0.896	0.192
OPA-11	2050	0.459	0.517
OPA-14	900	0.949	0.099
OPA-14	1080	1.000	0.000
OPA-14	1300	0.402	0.502
OPB-1	600	1.000	0.000
OPB-1	810	1.000	0.000
OPB-1	900	0.949	0.099
OPB-1	1400	0.868	0.236
OPB-1	2500	0.839	0.278
OPB-3	900	0.778	0.356
OPB-3	1020	1.000	0.000
OPB-3	1150	1.000	0.000
OPB-3	1600	1.000	0.000
OPB-6	750	1.000	0.000
OPB-7	700	0.896	0.192
OPB-7	1000	0.778	0.356
OPB-7	1160	1.000	0.000
OPB-7	1200	0.599	0.497
OPB-7	1250	1.000	0.000
OPB-8	310	1.000	0.000
OPB-8	410	0.638	0.477
OPB-8	730	1.000	0.000
OPB-8	750	0.745	0.391
OPB-8	800	1.000	0.000
OPB-8	1100	0.638	0.477
OPB-8	1500	1.000	0.000
OPB-8	2000	0.638	0.477
OPB-9	600	0.510	0.519
OPB-9	645	1.000	0.000
OPB-10	990	1.000	0.000
OPB-10	2000	0.868	0.236
OPB-13	850	1.000	0.000
OPB-13	1050	1.000	0.000
OPB-15	600	1.000	0.000
OPB-15	620	1.000	0.000
OPB-15	1100	1.000	0.000
OPB-15	1300	0.459	0.517
OPB-15	1700	1.000	0.000
OPC-1	506	1.000	0.000
OPC-1	1100	1.000	0.000
OPC-1	1600	1.000	0.000
OPC-1	1800	1.000	0.000
OPC-2	298	0.975	0.050
OPC-2	344	1.000	0.000
OPC-2	400	1.000	0.000
OPC-2	506	0.556	0.511
OPC-2	600	0.459	0.517

Table 8. (continued)

primer	band	frequency	heterozygosity
OPC-2	650	0.556	0.511
OPC-2	700	0.459	0.517
OPC-2	1000	0.712	0.423
OPC-2	1025	0.556	0.511
OPC-2	1050	0.459	0.517
OPC-2	1200	0.599	0.497
OPC-5	396	0.974	0.053
OPC-5	520	1.000	0.000
OPC-5	600	0.974	0.053
OPC-5	750	0.693	0.439
OPC-5	1250	0.974	0.053
OPC-5	1400	0.860	0.247
OPC-6	475	0.778	0.356
OPC-8	600	1.000	0.000
OPC-6	850	1.000	0.000
OPC-6	1300	1.000	0.000
OPC-8	700	0.556	0.511
OPC-8	800	1.000	0.000
OPC-8	925	0.599	0.497
OPC-8	1000	1.000	0.000
OPC-8	1200	1.000	0.000
OPC-8	1300	1.000	0.000
OPC-8	1500	1.000	0.000
OPC-8	1636	1.000	0.000
OPC-10	950	1.000	0.000
OPC-11	506	1.000	0.000
OPC-11	600	0.402	0.502
OPC-11	950	0.556	0.511
OPC-18	500	0.402	0.502
OPC-18	750	1.000	0.000
OPC-18	1000	1.000	0.000
OPC-18	1800	0.839	0.278
OPC-18	2000	1.000	0.000

Table 9. Estimated gene frequencies and heterozygosities for *Pteropus pumilus.*

primer	band	frequency	heterozygosity
OPA-2	506	0.839	0.278
OPA-2	525	1.000	0.000
OPA-2	650	0.638	0.477
OPA-2	750	0.949	0.099
OPA-2	1000	0.949	0.099
OPA-2	1060	0.402	0.502
OPA-2	1100	1.000	0.000
OPA-2	1250	0.676	0.452
OPA-2	1350	0.676	0.452
OPA-2	1500	0.599	0.497
OPA-5	600	0.402	0.502
OPA-5	700	1.000	0.000
OPA-5	750	1.000	0.000
OPA-5	950	0.402	0.502
OPA-5	1060	1.000	0.000
OPA-5	1100	0.895	0.192
OPA-5	1636	1.000	0.000
OPA-7	750	1.000	0.000
OPA-7	800	1.000	0.000
OPA-7	850	1.000	0.000
OPA-7	3000	1.000	0.000
OPA-8	506	0.556	0.511
OPA-8	700	1.000	0.000
OPA-8	1000	1.000	0.000
OPA-9	750	0.402	0.502
OPA-9	1800	0.510	0.519
OPA-9	2600	0.402	0.502
OPA-11	550	1.000	0.000
OPA-11	1000	0.459	0.517
OPA-11	2000	1.000	0.000
OPA-11	2050	0.510	0.519
OPA-14	750	1.000	0.000
OPA-14	900	0.459	0.517
OPA-14	1100	0.975	0.050
OPB-1	700	0.896	0.192
OPB-1	810	0.975	0.050
OPB-1	900	0.949	0.099
OPB-1	1100	1.000	0.000
OPB-1	1400	0.745	0.391
OPB-1	1500	0.402	0.502

primer	band	frequency	heterozygosity
OPB-1	2500	1.000	0.000
OPB-3	1020	1.000	0.000
OPB-3	1150	1.000	0.000
OPB-3	1600	1.000	0.000
OPB-7	700	1.000	0.000
OPB-7	1150	0.510	0.519
OPB-7	1160	0.556	0.511
OPB-7	1200	1.000	0.000
OPB-7	1250	0.459	0.517
OPB-7	1350	1.000	0.000
OPB-7	2050	0.693	0.439
OPB-8	310	0.402	0.502
OPB-8	300	1.000	0.000
OPB-8	700	1.000	0.000
OPB-8	750	1.000	0.000
OPB-8	800	1.000	0.000
OPB-8	950	0.638	0.477
OPB-8	1500	1.000	0.000
OPB-8	2050	1.000	0.000
OPB-9	1000	0.459	0.517
OPB-9	2000	1.000	0.000
OPB-9	2050	1.000	0.000
OPB-9	3000	1.000	0.000
OPB-10	750	0.949	0.099
OPB-10	2000	0.459	0.517
OPB-10	2050	1.000	0.000
OPB-10	2100	1.000	0.000
OPB-14	350	1.000	0.000
OPB-15	350	1.000	0.000
OPB-15	400	0.459	0.517
OPB-15	600	0.868	0.236
OPB-15	1300	1.000	0.000
OPB-15	1700	0.949	0.099
OPC-1	506	1.000	0.000
OPC-1	750	1.000	0.000
OPC-2	396	0.712	0.423
OPC-2	506	0.510	0.519
OPC-2	600	0.459	0.517
OPC-2	650	0.975	0.050
OPC-2	725	1.000	0.000

Table 9. (continued)

primer	band	frequency	heterozygosity
OPC-2	1025	1.000	0.000
OPC-2	1200	1.000	0.000
OPC-2	1636	1.000	0.000
OPC-5	396	0.839	0.278
OPC-5	506	1.000	0.000
OPC-5	520	0.638	0.477
OPC-5	600	0.949	0.099
OPC-5	700	1.000	0.000
OPC-5	750	0.402	0.502
OPC-5	1050	1.000	0.000
OPC-5	1250	0.638	0.477
OPC-5	1300	0.599	0.497
OPC-5	1400	0.638	0.477
OPC-6	450	0.510	0.519
OPC-6	475	0.923	0.146
OPC-6	850	0.839	0.278
OPC-6	1300	0.459	0.517
OPC-6	1250	1.000	0.000
OPC-8	600	0.510	0.519
OPC-8	900	1.000	0.000
OPC-8	925	1.000	0.000
OPC-8	1000	1.000	0.000
OPC-8	1300	0.949	0.099
OPC-8	1500	0.745	0.391
OPC-11	506	0.556	0.511
OPC-18	500	1.000	0.000
OPC-18	720	0.402	0.502
OPC-18	1000	0.510	0.519
OPC-18	1800	0.975	0.050
OPC-18	2000	0.402	0.502

Table 10. Estimated gene frequencies and heterozygosities for *Pteropus rodricensis.*

primer	band	frequency	heterozygosity
OPA-2	506	1.000	0.000
OPA-2	750	1.000	0.000
OPA-2	1250	1.000	0.000
OPA-5	600	1.000	0.000
OPA-5	700	1.000	0.000
OPA-5	930	1.000	0.000
OPA-5	1060	1.000	0.000
OPA-5	1100	1.000	0.000
OPA-5	1636	1.000	0.000
OPA-7	750	1.000	0.000
OPA-7	800	1.000	0.000
OPA-7	850	1.000	0.000
OPA-7	1060	1.000	0.000
OPA-7	3000	1.000	0.000
OPA-8	1000	1.000	0.000
OPA-9	300	1.000	0.000
OPA-9	2600	1.000	0.000
OPA-11	550	1.000	0.000
OPA-11	2000	1.000	0.000
OPA-14	750	1.000	0.000
OPA-14	1100	1.000	0.000
OPB-1	800	1.000	0.000
OPB-1	810	1.000	0.000
OPB-1	950	1.000	0.000
OPB-1	1100	1.000	0.000
OPB-1	1200	1.000	0.000
OPB-1	1400	1.000	0.000
OPB-1	2500	1.000	0.000
OPB-3	1200	1.000	0.000
OPB-7	500	1.000	0.000
OPB-7	700	1.000	0.000
OPB-7	1000	1.000	0.000
OPB-7	1160	1.000	0.000
OPB-7	1200	1.000	0.000
OPB-7	1350	1.000	0.000
OPB-7	2050	0.676	0.452
OPB-8	300	1.000	0.000
OPB-8	700	1.000	0.000
OPB-8	750	1.000	0.000
OPB-8	1100	0.459	0.517

primer	band	frequency	heterozygosity
OPB-9	645	1.000	0.000
OPB-9	2000	1.000	0.000
OPB-10	750	1.000	0.000
OPB-10	2050	1.000	0.000
OPB-10	2100	1.000	0.000
OPB-15	350	1.000	0.000
OPB-15	600	1.000	0.000
OPB-15	620	1.000	0.000
OPB-15	1700	1.000	0.000
OPC-1	750	1.000	0.000
OPC-2	400	1.000	0.000
OPC-2	650	1.000	0.000
OPC-2	1000	1.000	0.000
OPC-2	1050	0.975	0.050
OPC-2	1200	1.000	0.000
OPC-2	1636	1.000	0.000
OPC-5	506	1.000	0.000
OPC-5	600	1.000	0.000
OPC-5	750	1.000	0.000
OPC-5	1150	1.000	0.000
OPC-5	1300	1.000	0.000
OPC-6	475	0.975	0.050
OPC-6	850	1.000	0.000
OPC-6	1000	1.000	0.000
OPC-6	1250	1.000	0.000
OPC-6	1636	1.000	0.000
OPC-8	396	1.000	0.000
OPC-8	600	1.000	0.000
OPC-8	700	1.000	0.000
OPC-8	800	1.000	0.000
OPC-8	1000	1.000	0.000
OPC-8	1200	1.000	0.000
OPC-8	1300	1.000	0.000
OPC-8	1500	1.000	0.000
OPC-8	1636	1.000	0.000
OPC-18	350	1.000	0.000
OPC-18	500	1.000	0.000
OPC-18	1800	1.000	0.000

Table 11. Paternity exclusion data for *Pteropus pumilus.*

Pup	Primer	Locus*	Potential sires excluded
p14	OPC-5	1300	p2 and p6
	OPC-8	1500	p1, p4, p5, and p7
	OPC-5 and OPC-8		(All but p3)
p15	OPC-6	850	All but p5
p16	OPC-8	1300	All but p3
p17	OPC-6	850	All but p5
p18	OPB-1	700	All but p3
p19	OPC-6	475	All but p4
p20	OPC-6	475	All but p4

* Approximate band size (bp)

Table 12. Alternative paternity exclusion data for *Pteropus pumilus.*

Pup	Primer	Locus*	Potential sires excluded
p14	OPA-2	1350	p4, p5, p6 and p7
	OPA-8	700	p2 and p3
	OPA-2 and OPA-8		(All but p1)
p15	OPB-15	600	All but p6
p16	OPB-15	1700	All but p5
p17	OPC-5	600	All but p2
p18	OPB-8	1500	All but p2
p19	OPB-7	1160	All but p7
p20	OPA-2	1000	All but p5

* Approximate band size (bp)

For the remaining *P. pumilus* pup, p14, none of the potential sires displayed a unique dominant allele at any informative locus, making paternity exclusion more difficult. In this case, two different sets of loci can be used to exclude potential sires of the pup. In one case, at locus 1300 of primer OPC-5, the dam displayed the null allele, pup # 14 had the dominant allele, and all putative sires except p2 and p6 had the dominant allele. At locus 1500 of primer OPC-8, the dam displayed the null allele, the pup displayed the dominant allele and all potential sires except p1, p4, p5, and p7 had the dominant allele. Based on these data, all individuals except p3 were excluded using at least one locus (Table 11, Fig 3). However, when different primers were examined in the same manner, a different male also can be identified as the sire of pup # 14 (Table 12).

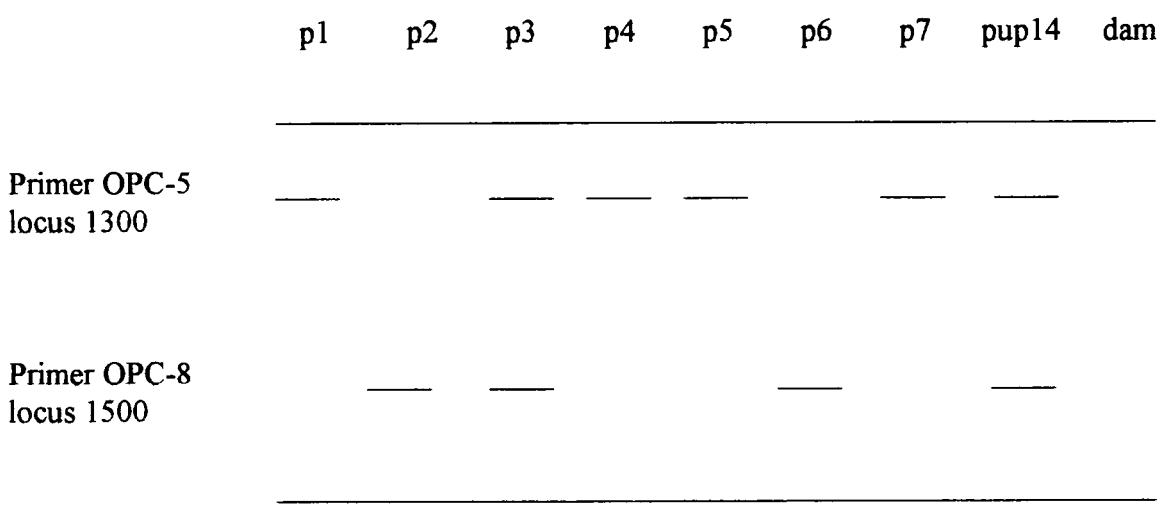


Fig. 3. Paternity exclusion for *P. pumilus* pup # 14. This illustration shows the banding patterns for pup # 14, the dam, and the seven putative sires at two loci used for paternity exclusion. The short horizontal lines following the locus designations represent bands produced at that locus. These loci are shown in juxtaposition for explanatory purposes, and were not present on the same gel.

Chapter 4

Discussion

This study demonstrates the utility of RAPD markers in determining relative amounts of genetic variation both within and among species. Because RAPD analysis can detect small changes in DNA sequence, it can be used to identify variation where other techniques like allozymes, which rely on functional differences, have failed. For example, using allozymes McCracken (unpublished data) found no variation in *P. rodricensis*, whereas RAPDs clearly demonstrate that variation is present in this species, albeit in levels that are substantially lower than in *P. hypomelanus* and *P. pumilus*.

Similarity Index

The within-population similarity values obtained from this study are comparable to values reported from other taxa, including fish (0.969) (Bardacki and Skibinski, 1994), nematodes (0.920) (Folkertsma *et al.*, 1994), and plants (0.977) (Chalmers *et al.*, 1993). As expected, *P. rodricensis* has the least genetic diversity of the three species. The finding that *P. pumilus* has slightly greater genetic diversity than *P. hypomelanus* is not as was expected. Levels of diversity obtained from preliminary microsatellite analysis (Comeaux, unpublished data) provide the same rank orders of genetic diversities as observed using RAPDs. The hypothesis that *P. pumilus* should be less diverse than *P. hypomelanus* was based upon the IUCN's classification of *P. pumilus* as vulnerable and the reports that its numbers are seriously declining. If, indeed, population numbers

declined rapidly, inbreeding and genetic drift were likely to occur. Because *P. hypomelanus* has not experienced serious population declines, and hence, very little or no genetic drift or inbreeding due to small population size were expected, it was expected that *P. hypomelanus* should have the greatest amount of genetic diversity.

The results showing that *P. pumilus* is more variable than *P. hypomelanus* may be due to an underestimate of the actual population numbers of *P. pumilus*. Another explanation may be that the genetic diversity in a population which has experienced population declines or bottlenecks can remain high for several generations due to historically large effective population sizes and large amounts of genetic diversity prior to the population decline (Dinerstein and McCracken, 1990). In this regard, the long generation time (four to five years) and low reproductive rates of flying foxes make this scenario plausible. If the population declines were relatively recent, enough time may not have passed to allow serious declines in genetic diversity. Finally, the lower diversity in *P. hypomelanus* versus *P. pumilus* may be a result of the manner in which the *P. hypomelanus* and *P. pumilus* individuals were captured. It is possible that the *P. hypomelanus* individuals were all captured from a single roost, whereas the *P. pumilus* were taken from diverse locations. If this was the case, the *P. hypomelanus* individuals may be more closely related and are therefore, less genetically diverse than individuals caught from more dispersed locations. Information on capture histories was not available at the time of this writing.

The genetic distance measurements that were calculated from between population similarities and gene frequencies indicate that *P. pumilus* and *P. rodricensis* are the

genetically most closely related species, while *P. hypomelanus* and *P. rodricensis* are the genetically most distantly related species. This is inconsistent with expectations from the geographical ranges of these species. The minimum distance between the ranges of *P. pumilus* and *P. rodricensis* is greater than 6000 km. The ranges of *P. hypomelanus* and *P. pumilus* overlap on the Philippine Islands, and the minimum distance between the ranges *P. hypomelanus* and *P. rodricensis* is approximately 4300 km. The geographical distances between the ranges of these species suggest that from biogeographical expectations *P. hypomelanus* and *P. pumilus* should be more closely related to each other than either should be to *P. rodricensis.* Based on morphology and geographical ranges, Koopman (1994) supports this latter grouping. He places *P. hypomelanus* and *P. pumilus* in the *Subniger* group of *Pteropus*, while *P. rodricensis* is placed in the *Molossinus* group. Corbet and Hill (1992) also support the grouping of *P. hypomelanus* with *P. pumilus,* considering both species as members of the *hypomelanus* group. Corbet and Hill (1992) did not consider *P. rodricensis*. The classification of Corbet and Hill follows that of Anderson (1912) and is based on the relative measurements of several characteristics including bone lengths, ear length, and dentition. Other studies (Krutzsch, 1962; Lanza, 1963; Ingle and Heaney, 1992) have used the analysis of other characters to supplement Anderson's work but, to date, there is no published study on the genetic relationships among these three species. The results of this study demonstrate the need for genetic studies of *Pteropus* systematics.

Gene Frequency and Heterozygosity

Only two other published studies, one on insects (Skinner and Comacho, 1995), and one on fish (Naish *et al.*, 1995) have measured gene frequencies and heterozygosity using the methods employed in this thesis. The heterozygosity values obtained for all three bat species were lower than but comparable to values reported for potato leafhoppers (0.299) (Skinner and Camacho, 1995) and tilapia (0.37) (Naish *et al.*, 1995). These results from RAPDs are as expected because mammals are known from both allozyme (Hartl and Clark, 1989) and mitochondrial DNA studies (Avise, 1994) to have lower levels of heterozygosity than most other groups of animals, including most insects and fish.

The heterozygosity values obtained from this study are much greater than heterozygosities typically estimated from allozymes. The overall average heterozygosity calculated for mammals, in general, using allozymes is 0.037 (Hartl and Clark, 1989). The values provided in this analysis greatly exceed this value for *P. hypomelanus* and *P. pumilus*. As mentioned previously, allozyme analysis failed to detect any variation in *P. rodricensis*. It is expected that RAPD analysis should detect more variation than allozymes because RAPDs are sensitive to very small changes in DNA sequence which can facilitate or prevent the annealing of a primer, and thereby affect banding patterns. Allozyme analysis detects functional and conformational changes in DNA products, making it less sensitive to small sequence changes. A change in a single base pair often does not affect the conformation or charge of the protein product, so it is not detected by allozyme analysis. Furthermore, RAPDs can detect changes in all parts of the genome,

including intron regions and repetitive sequences which are not expressed, whereas allozymes can only detect changes in regions of the genome which encode specific enzymes (Williams *et al.*, 1990; Haymer, 1994). Because repetitive DNA is not acted upon by selection, as are exon and regulatory regions, mutations accumulate and are propagated during replication (Haymer, 1994). Because RAPDs can detect changes in these highly dynamic regions, it is expected that greater amounts of variation will be revealed by RAPDs than by allozymes. These two techniques rely on the detection of very different types genetic changes, making a direct comparison unjustified.

Microsatellite DNA analysis is another technique that reliably detects small differences in repetitive DNA. Preliminary microsatellite analysis of the same three *Pteropus* populations demonstrates very high heterozygosity values, and supports the results obtained in this study (Comeaux, unpublished data). Although the average heterozygosity values obtained from microsatellite analysis are higher in all cases (*P. pumilus* H_o= 0.61; *P. hypomelanus* H_o = 0.57; *P. rodricensis* H_o = 0.33) than those obtained using RAPDs, these separate estimates of genetic diversity display the same relative relationships.

Paternity Exclusion

Paternity exclusions obtained from RAPDs were unreliable. Depending upon which locus was examined, different putative sires were identified as the only individual which could donate the dominant allele to the pup in question (Tables 11 and 12). For example, for pup p20, if primer OPA-2 locus 1000 is examined first, p5 appears to be the

only individual which could donate the dominant allele at this locus, and p5 is identified as the sire of p20. However, if primer OPC-6 locus 474 is examined first, p4 is identified as the sire of p20. In all cases, it was possible to exclude all putative sires by combining the results of two contradictory loci. Tables 11 and 12 do not reflect all of the paternity exclusions that were possible.

As another problem, paternity exclusion using microsatellite analysis yields very different results than RAPDs. RAPDs (depending on which locus is examined) indicated that p5 or p6 sired pup p15, but subsequent microsatellite analysis excludes both p5 and p6. Although microsatellite analysis of this population is not complete, the paternity exclusion results are contradictory for all 7 pups. Because microsatellites produce codominant markers which are reliably interpreted due to single locus amplification, the results obtained from microsatellite analysis are much more reliable than those obtained from RAPD analysis.

Non-parental bands were obviously produced in the RAPD amplification leading to a misinterpretation of paternity exclusion despite the fact that the guidelines set forth by Scott *et al.* (1992) were strictly followed. These bands are most likely PCR artifacts due to polymerase slippage during replication or non-template directed addition of nucleotides by Taq polymerase (Riedy *et al.*, 1991). Errors may also be caused by comigration of DNA fragments produced from different parts of the genome (Rieseberg, 1996).

Reliability

Despite the fact that non-parental bands can confound paternity exclusion, RAPDs

appear to be valuable for detecting and estimating genetic variation. Mating and hybridization studies indicate that non-parental bands are most likely produced at the same rate in similar species (under identical amplification conditions) and should result in no species specific bias in variability estimates (Riedy *et al.*, 1991; Kazan *et al.*, 1992; Haymer, 1994). Also, bands which reflect authentic variation appear to greatly outnumber artifacts (Black, 1993). Thus, estimates of relative genetic diversity should have validity (Bardakci and Skibinski, 1993). Values generated by RAPDs are only relative and, care must be taken when making comparisons between data from different studies. Therefore, RAPDs can be seen as a method for determining whether or not genetic variation exists, and in what relative amounts. Because of the possibility of comigration of nonhomologous fragments, and the unknown genetic basis of RAPDs, phylogenies created using RAPDs should be carefully examined, and if possible substantiated with other types of data.

Chapter 5

Conclusions

Populations of many flying fox species have been in serious decline since the turn of the century (Mickleburgh *et al.*, 1992). Their status as keystone species makes them vital to the maintenance of Old World tropical ecosystems (Cox *et al.*, 1991), and their conservation is of utmost importance. Recently, it has been necessary to bring several species into captivity because their numbers had dropped so drastically that extinction seems an immediate possibility. To date, no reintroductions to the wild have been attempted for captive bred flying foxes, although, reintroductions are the ultimate goal and are likely in the future (Mickleburgh *et al.*, 1992).

Genetic analyses such as in this study can be of use to managers of captive breeding programs in establishing guidelines for the maintenance of genetic diversity. However, continued existence of flying foxes can not be totally reliant upon captive propagation. In many cases, demographic factors are more pressing than the maintenance of genetic diversity. Stronger attempts should be made to preserve natural habitat. Hunting and trade should be controlled or banned entirely in areas like American Samoa where flying fox populations are highly endangered. In some areas where legislation to control hunting and trade has been passed, it has not been enforced (Mickleburgh *et al.*, 1992). Efforts to educate plantation owners, hunters, and local consumers about the importance of flying foxes may aid in conservation efforts.

When conservation efforts fail, and captive propagation becomes a necessity, genetic testing becomes essential for wise management. The case of *P. rodricensis* provides one illustration of the value of genetic testing. From their demographic history and history of captive propagation, it might be expected that little or no genetic diversity is likely to remain in the captive populations of *P. rodricensis*. If so, management strategies would not need to emphasize provisions to prevent inbreeding. This study clearly shows that substantial genetic variation remains even in this highly inbred species. These results have now been confirmed by microsatellite analysis, as well. Thus we now believe that it is of utmost importance to design captive breeding programs with precautions to prevent or reduce inbreeding, and, thereby conserve the remaining amounts of genetic diversity for this and other endangered species. The maintenance of this genetic diversity may be crucial to their continued existence.

RAPDs are a highly variable class of molecular markers which can be assayed rapidly and require no prior sequence information from the species of interest. Methods of analysis developed by Lynch and Milligan (1994) allow the calculation of traditional measures of genetic diversity, like heterozygosity, making RAPD analysis more informative and more easily comparable to other molecular markers. These factors, along with the relatively low cost of RAPD analysis, make RAPDs an attractive tool for ecological genetic studies.

However, there are several drawbacks to RAPD analysis. First, slight variations in the amount of template, magnesium chloride, and Taq polymerase can result in differences in the intensity and number of bands, making the repeatability of RAPDs a

serious concern. Differences in band intensity and banding patterns can also result from small thermocycler malfunctions like fluctuations in temperature and ramp time, which are often undetected. It is necessary to repeat each reaction in order to minimize PCR artifacts. Lastly, and most importantly, even when conditions are carefully controlled, "stray" or non-parental bands evidently occur and can confound paternity analysis. For this reason, I believe that RAPDs should not be used for paternity determination. These factors should also be taken into consideration when calculating and comparing estimates of genetic diversity. Values generated from RAPD analysis should in no way be considered absolute, but rather as indicators of relative relationships. Finally, due to the possibility of comigration of non-homologous bands, RAPDs should not be used as the sole determinant of phylogenetic relationships.

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Appendices

Appendix A

DNA Extraction Protocol

(For Whole Blood Stored in EDTA or High Salt Solutions)

This protocol typically takes 2 days to complete.

1) Add 1 ml (can use 250-500ul) whole blood to autoclaved 10ml centrifuge tube.

2) Add 5 ml sterile dH2O --- vortex.

3) Add immediately 300ul 20x SSC --- vortex.

4) Centrifuge @8000 RPM for 5 min.

5) Pour off supernatant. Discard.

6) Resuspend pellet in 1 ml 0.2M NaAcetate pH 5.6 --- vortex.

7) Add 80 ul 20% SDS --- vortex.

8) Allow to sit 5 min. at room temperature.

9) Add 62.5 ul Prot-K (10 mg/ml d H2O).

10) Incubate @ 65°C for 4 - 15 hrs. in waterbath with foil over tubes. (Longer incubation times increase DNA yields.)

11) Transfer fluids to 2 ml Eppendorf tubes.

12) Add 171 ul 8M KOAc --- vortex.

13) Place on ice for 20+ min.

14) Centrifuge for 20 min. @ 14,000 RPM.

15) Decant supernatant. Discard pellet.

== Possible stop - freeze sample.

16) Subsample 900ul of sample for phenol / chloroform.

17) Add equal volume (900ul) phenol --- vortex.
Allow to sit 5 min. Invert. Allow to sit 5 min.
Centrifuge @ 14000 RPM for 15 min.

18) Remove aqueous phase (top). Put in new 2 ml Eppendorf tube.

== Save the phenol and chloroform until the DNA is quantified. (DNA could become trapped in phenol.)

19) Add equal volume of chloroform --- vortex.
Centrifuge @ 14000 RPM for 5 min.

20) Remove aqueous phase (top). Put in new 2 ml Eppendorf tube.

== Possible stop - freeze sample.

21) Add 500ul extract to top of Microcon tube.
Centrifuge @ 13,200 RPM for 15 min.

22) Add remaining extract to top of tube.
Centrifuge @ 13,200 RPM for 5 min.

23) Discard filtrate.

== Reassemble Microcon

24) Add 100 ul 1/10 TE to top of Microcon.
Centrifuge @ 13,200 RPM for 3 min.
Repeat.

25) Invert top over new microcon tube. Centrifuge @ 3500 RPM for 3 min.

Appendix B

RAPD Data

These data are presented as they were entered into the computer programs for statistical analysis. (Column 1 = primer; Column 2 = species; Column 3 = individual; columns 4 - 19 = band scores).

opc1 hyp 620 1 0 1 0 0 0
opc1 hyp 622 1 0 1 0 0 0
opc1 hyp 623 1 0 1 0 0 0
opc1 hyp 626 1 0 1 0 0 0
opc1 hyp 627 1 0 1 0 0 0
opc1 hyp 633 1 0 1 0 0 0
opc1 hyp 635 1 0 1 0 0 0
opc1 hyp 638 1 0 1 0 0 0
opc1 hyp 639 1 0 1 0 0 0
opc1 hyp 645 1 0 1 0 0 0
opc1 hyp 647 1 0 1 0 0 0
opc1 hyp 648 1 0 1 0 0 0
opc1 hyp 649 1 0 1 0 0 0
opc1 hyp 652 1 0 1 0 0 0
opc1 hyp 656 1 0 1 0 0 0
opc1 hyp 667 1 0 1 0 0 0
opc1 hyp 661 1 0 1 0 0 0
opc1 hyp 666 1 0 1 0 0 0
opc1 hyp 684 1 0 1 0 0 0
opc1 hyp 688 1 0 1 0 0 0
opc1 rod r1 1 1 0 1 1 1
opc1 rod r2 1 1 0 1 1 1
opc1 rod r3 1 1 0 1 1 1
opc1 rod r4 1 1 0 1 1 1
opc1 rod r5 1 1 0 1 1 1
opc1 rod r6 1 1 0 1 1 1
opc1 rod r7 1 1 0 1 1 1
opc1 rod r8 1 1 0 1 1 1
opc1 rod r9 1 1 0 1 1 1
opc1 rod r10 1 1 0 1 1 1
opc1 rod r11 1 1 0 1 1 1
opc1 rod r12 1 1 0 1 1 1
opc1 rod r13 1 1 0 1 1 1
opc1 rod r14 1 1 0 1 1 1
opc1 rod r15 1 1 0 1 1 1
opc1 rod r16 1 1 0 1 1 1
opc1 rod r17 1 1 0 1 1 1
opc1 rod r18 1 1 0 1 1 1
opc1 rod r19 1 1 0 1 1 1
opc1 rod r20 1 1 0 1 1 1
opc1 pum p1 1 0 0 1 1 1
opc1 pum p2 1 0 0 1 1 1
opc1 pum p3 1 0 0 1 1 1
opc1 pum p4 1 0 0 1 1 1
opc1 pum p4 1 0 0 1 1 1
opc1 pum p5 1 0 0 1 1 1
opc1 pum p6 1 0 0 1 1 1
opc1 pum p7 1 0 0 1 1 1
opc1 pum p8 1 0 0 1 1 1
opc1 pum p9 1 0 0 1 1 1
opc1 pum p10 1 0 0 1 1 1
opc1 pum p11 1 0 0 1 1 1
opc1 pum p12 1 0 0 1 1 1
opc1 pum p13 1 0 0 1 1 1
opc1 pum p14 1 0 0 1 1 1
opc1 pum p15 1 0 0 1 1 1
opc1 pum p16 1 0 0 1 1 1
opc1 pum p17 1 0 0 1 1 1
opc1 pum p18 1 0 0 1 1 1
opc1 pum p19 1 0 0 1 1 1
opc1 pum p20 1 0 0 1 1 1
opc2 hyp 620 0 0 1 0 1 1 1 0 1 0 1 1 1 1
opc2 hyp 622 0 0 1 0 1 1 1 0 1 1 1 1 0 0
opc2 hyp 623 0 0 1 0 0 0 0 1 1 1 1 1 1 1
opc2 hyp 626 0 0 1 0 0 1 0 1 1 1 1 1 1 1
opc2 hyp 627 0 0 1 0 0 1 0 1 1 1 1 1 1 1
opc2 hyp 633 0 0 1 0 1 1 1 1 1 1 0 1 1 0
opc2 hyp 635 0 0 1 0 1 1 1 1 1 1 1 1 1 1
opc2 hyp 638 0 0 1 0 1 1 1 1 1 0 1 1 1 1
opc2 hyp 639 0 0 1 0 1 1 1 1 1 0 1 1 0 0
opc2 hyp 645 0 0 1 0 1 1 0 1 1 0 1 1 1 1
opc2 hyp 647 0 0 1 0 1 1 1 0 1 0 0 1 1 0
opc2 hyp 648 1 0 1 0 0 1 1 1 1 0 1 1 1 0
opc2 hyp 649 0 0 1 0 1 0 0 1 1 1 0 1 1 0

opc2 hyp 652 0 0 1 0 1 0 0 1 1 0 1 1 1 1
opc2 hyp 656 0 0 1 0 1 1 1 0 1 0 1 1 1 1
opc2 hyp 667 0 0 1 0 0 1 1 1 1 1 0 1 1 0
opc2 hyp 661 0 0 1 0 1 1 1 1 1 0 1 1 1 1
opc2 hyp 666 0 0 1 0 1 1 1 1 1 1 1 1 1 1
opc2 hyp 684 0 0 1 0 1 0 1 1 1 0 0 1 0 1
opc2 hyp 688 0 0 1 0 0 1 1 1 1 1 0 1 0 1
opc2 rod r1 1 1 1 0 1 0 0 1 1 0 1 0 0 0
opc2 rod r2 1 1 1 0 1 0 0 1 1 0 1 0 0 0
opc2 rod r3 1 1 1 0 1 0 0 1 1 0 1 0 0 0
opc2 rod r4 1 1 1 0 1 0 0 1 0 0 1 0 0 0
opc2 rod r5 1 1 1 0 1 0 0 1 1 0 1 0 0 0
opc2 rod r6 1 1 1 0 1 0 0 1 1 0 1 0 0 0
opc2 rod r7 1 1 1 0 1 0 0 1 1 0 1 0 0 0
opc2 rod r8 1 1 1 0 1 0 0 1 1 0 1 1 0 0
opc2 rod r9 1 1 1 0 1 0 0 1 1 0 1 0 0 0
opc2 rod r10 1 1 1 0 1 0 0 1 1 0 1 0 0 0
opc2 rod r11 1 1 1 0 1 0 0 1 1 0 1 0 0 0
opc2 rod r12 1 1 1 0 1 0 0 1 1 0 1 0 0 0
opc2 rod r13 1 1 1 0 1 0 0 1 1 0 1 0 0 0
opc2 rod r14 1 1 1 0 1 0 0 1 0 0 1 0 0 0
opc2 rod r15 1 1 1 0 1 0 0 1 1 0 1 0 0 0
opc2 rod r16 1 1 1 0 1 0 0 1 1 0 1 0 0 0
opc2 rod r17 1 1 1 0 1 0 0 1 1 0 1 0 0 0
opc2 rod r18 1 1 1 0 1 0 0 1 1 0 1 0 0 0
opc2 rod r19 1 1 1 0 1 0 0 1 1 0 1 0 0 0
opc2 rod r20 1 1 1 0 1 0 0 1 1 0 1 0 0 0
opc2 pum p1 1 1 0 1 1 1 0 1 0 1 0 1 0 0
opc2 pum p2 1 1 1 1 1 1 0 1 0 1 0 1 0 0
opc2 pum p3 1 1 1 1 1 1 0 1 0 1 0 1 0 0
opc2 pum p4 1 1 1 1 1 1 0 1 0 1 0 1 0 0
opc2 pum p5 0 1 0 1 0 1 0 1 0 1 0 0 0 0
opc2 pum p6 1 1 1 1 1 1 0 1 0 1 0 1 0 0
opc2 pum p7 1 1 0 1 0 1 0 1 0 1 0 1 0 0
opc2 pum p8 1 1 1 1 1 1 0 1 0 1 0 1 0 0
opc2 pum p9 1 1 0 1 1 1 0 1 0 1 0 1 0 0
opc2 pum p10 1 1 0 1 1 1 0 1 0 1 0 1 0 0
opc2 pum p11 1 1 1 1 1 1 0 1 0 1 0 1 0 0
opc2 pum p12 1 1 0 1 0 1 0 1 0 1 0 1 0 0
opc2 pum p13 1 1 0 1 1 1 0 1 0 1 0 1 0 0
opc2 pum p14 1 1 0 1 1 0 0 1 0 1 0 1 0 0
opc2 pum p15 1 1 0 1 1 1 0 1 0 1 0 1 0 0
opc2 pum p16 1 1 0 1 0 0 0 1 0 1 0 1 0 0
opc2 pum p17 1 1 1 0 1 1 1 1 0 1 0 1 0 0
opc2 pum p18 1 1 1 1 1 1 0 1 0 1 0 1 0 0
opc2 pum p19 1 1 1 1 0 0 0 1 0 1 0 1 0 0
opc2 pum p20 1 1 1 1 1 0 0 1 0 1 0 1 0 0
opc5 hyp 620 0 1 0 0 1 0 1 1 1 0 1 0
opc5 hyp 622 1 1 0 0 1 1 1 1 1 0 1 0
opc5 hyp 623 0 1 0 0 1 1 1 1 1 0 1 0
opc5 hyp 626 0 0 0 0 1 1 0 1 0 0 1 0
opc5 hyp 627 0 0 0 0 1 1 0 1 0 0 1 1
opc5 hyp 633 0 1 0 0 1 0 1 1 1 0 1 0
opc5 hyp 638 0 1 0 0 1 1 1 1 1 0 1 0
opc5 hyp 639 0 1 0 0 1 0 1 1 1 0 1 0
opc5 hyp 645 0 1 0 1 1 1 1 1 1 0 1 0
opc5 hyp 647 0 1 0 0 1 0 1 1 1 0 1 0
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opa2 hyp 648 0 1 0 0 1 0 1 1 0 1
opa2 hyp 649 0 1 0 0 1 1 1 1 0 0
opa2 hyp 652 0 1 0 0 1 1 1 1 0 0
opa2 hyp 656 0 1 0 0 1 1 1 1 0 1
opa2 hyp 667 0 1 0 0 1 0 1 1 0 0
opa2 hyp 661 0 1 0 0 1 1 1 1 0 1
opa2 hyp 666 0 1 0 0 1 1 1 1 0 0
opa2 hyp 684 0 1 0 0 1 0 1 1 0 0
opa2 hyp 688 0 1 0 0 1 0 1 1 0 1
opa2 rod r1 1 0 1 0 1 1 1 0 1 1
opa2 rod r2 1 0 1 0 1 1 1 0 1 1
opa2 rod r3 1 0 1 0 1 1 1 0 1 1
opa2 rod r4 1 0 1 0 1 1 1 0 1 1
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opa2 rod r6 1 0 1 0 1 1 1 0 1 1

opa2 rod r7 1 0 1 0 1 1 1 0 1 1
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opa2 rod r9 1 0 1 0 1 1 1 0 1 1
opa2 rod r10 1 0 1 0 1 1 1 0 1 0
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opa2 rod r17 1 0 1 0 1 1 1 0 1 0
opa2 rod r18 1 0 1 0 1 1 1 0 1 1
opa2 rod r19 1 0 1 0 1 1 1 0 1 1
opa2 rod r20 1 0 1 0 1 1 1 0 1 1
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opa2 pum p3 0 0 0 0 0 1 0 1 1 0
opa2 pum p4 1 0 0 0 0 1 0 1 0 0
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opa2 pum p13 0 0 0 0 0 1 0 1 0 0
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opa2 pum p15 0 0 1 0 0 1 0 0 1 0
opa2 pum p16 0 0 1 0 0 1 0 0 1 1
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opa5 hyp 620 1 0 1 0 0 1 0 1 0 1 0 1 1 1
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opa5 rod r19 1 1 0 0 1 1 0 1 1 0 0 1 0 1
opa5 rod r20 1 1 0 0 1 1 0 1 1 0 0 1 0 1
opa5 pum p1 1 1 1 0 0 1 1 1 1 0 0 1 0 1
opa5 pum p2 1 1 1 0 0 1 1 1 1 0 1 1 0 1
opa5 pum p3 1 1 1 0 0 1 1 1 1 0 0 1 0 1
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opa5 pum p5 1 1 0 0 0 1 1 1 1 0 1 1 0 1
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opa5 pum p7 1 1 1 0 0 1 1 1 0 0 0 1 0 1
opa5 pum p8 1 1 1 0 0 1 1 1 1 0 0 1 0 1
opa5 pum p9 1 1 1 0 0 1 1 1 1 0 1 1 0 1
opa5 pum p10 1 1 1 0 0 1 1 1 1 0 0 1 0 1
opa5 pum p11 1 1 1 0 0 1 1 1 1 0 0 1 0 1
opa5 pum p12 1 1 1 0 0 1 1 0 1 0 0 1 0 1

opa5 pum p13 1 1 0 0 0 1 1 1 1 0 1 1 0 1
opa5 pum p14 1 1 1 0 0 1 1 1 1 0 0 1 0 1
opa5 pum p15 1 1 1 0 0 1 1 1 1 0 0 1 0 1
opa5 pum p16 1 1 1 0 0 1 1 1 1 0 0 1 0 1
opa5 pum p17 1 1 1 0 0 1 1 1 1 0 0 1 0 1
opa5 pum p18 1 1 1 0 0 1 1 1 1 0 0 1 0 1
opa5 pum p19 1 1 0 0 0 1 1 1 1 0 0 1 0 1
opa5 pum p20 1 1 1 0 0 1 1 0 1 0 0 1 0 1
opa7 hyp 620 1 0 1 0 1 0 0 1 1 1
opa7 hyp 622 1 0 0 1 0 0 0 1 1 0
opa7 hyp 623 1 0 1 0 1 0 0 1 0 1
opa7 hyp 626 1 0 1 0 1 0 0 1 1 0
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opa7 hyp 633 1 0 1 1 1 0 0 1 1 1
opa7 hyp 635 1 0 1 1 1 0 0 1 1 0
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opa7 hyp 639 1 0 1 0 1 0 0 1 1 1
opa7 hyp 645 1 0 1 0 1 0 0 1 0 1
opa7 hyp 647 1 0 1 0 1 0 0 1 1 1
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opa7 hyp 667 1 0 1 0 1 0 0 1 1 1
opa7 hyp 661 1 0 1 0 1 0 0 1 1 1
opa7 hyp 666 1 0 1 1 1 0 0 1 1 0
opa7 hyp 684 1 0 1 0 1 0 0 1 1 1
opa7 hyp 688 1 0 1 0 1 0 0 1 1 1
opa7 rod r1 0 1 0 0 0 1 0 1 1 0
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opa7 rod r3 0 1 0 0 0 1 0 1 1 0
opa7 rod r4 0 1 0 0 0 1 0 1 1 0
opa7 rod r5 0 1 0 0 0 1 0 1 1 0
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opa7 rod r9 0 1 0 0 0 1 0 1 1 0
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opa7 rod r18 0 1 0 0 0 1 0 1 1 0
opa7 rod r19 0 1 0 0 0 1 0 1 1 0
opa7 rod r20 0 1 0 0 0 1 0 1 1 0
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opa7 pum p4 0 1 1 0 0 1 1 1 1 0
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opa7 pum p19 0 1 1 0 0 1 1 1 1 0
opa7 pum p20 0 1 1 0 0 1 0 1 1 0
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opa8 hyp 622 0 0 0 1 1
opa8 hyp 623 0 1 0 0 1
opa8 hyp 626 0 1 1 0 1
opa8 hyp 627 0 1 1 1 1
opa8 hyp 633 0 1 0 0 1
opa8 hyp 635 0 1 0 1 1
opa8 hyp 638 0 1 0 1 1
opa8 hyp 639 0 1 0 1 1
opa8 hyp 645 0 1 0 1 1
opa8 hyp 647 0 1 0 1 1
opa8 hyp 648 0 1 0 1 1
opa8 hyp 649 0 1 0 1 1
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opa8 hyp 656 0 1 0 0 1
opa8 hyp 667 0 1 0 0 1
opa8 hyp 661 0 0 0 1 1
opa8 hyp 666 0 1 0 1 1

opa8 hyp 684 0 1 0 0 1
opa8 hyp 688 0 0 0 1 1
opa8 rod r1 1 1 1 0 1
opa8 rod r2 1 1 1 0 1
opa8 rod r3 1 1 1 0 1
opa8 rod r4 1 1 1 0 1
opa8 rod r5 1 1 1 0 1
opa8 rod r6 1 1 1 0 1
opa8 rod r7 1 1 1 0 1
opa8 rod r8 1 1 1 0 1
opa8 rod r9 1 1 1 0 1
opa8 rod r10 1 1 1 0 1
opa8 rod r11 1 1 1 0 1
opa8 rod r12 1 1 1 0 1
opa8 rod r13 1 1 1 0 1
opa8 rod r14 1 1 1 0 1
opa8 rod r15 1 1 1 0 1
opa8 rod r16 1 1 1 0 1
opa8 rod r17 1 1 1 0 1
opa8 rod r18 1 1 1 0 1
opa8 rod r19 1 1 1 0 1
opa8 rod r20 1 1 1 0 1
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opa8 pum p2 1 1 0 0 1
opa8 pum p3 1 1 0 0 1
opa8 pum p4 1 1 1 0 1
opa8 pum p5 0 1 1 0 1
opa8 pum p6 1 1 1 0 1
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opa9 rod r1 0 1 1 1 1 1 0
opa9 rod r2 0 1 1 1 1 1 0
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opa9 rod r5 0 1 1 1 1 1 0
opa9 rod r6 0 1 1 1 1 1 0
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opa9 rod r16 0 1 1 1 1 1 0
opa9 rod r17 0 1 1 1 1 1 0
opa9 rod r18 0 1 1 1 1 1 0
opa9 rod r19 0 1 1 1 1 1 0
opa9 rod r20 0 1 1 1 1 1 0
opa9 pum p1 1 1 1 1 1 1 1
opa9 pum p2 1 1 1 1 1 1 1
opa9 pum p3 1 1 1 1 1 1 1
opa9 pum p4 0 1 1 1 0 1 1

opa9 pum p5 1 1 1 1 1 1 0
opa9 pum p6 1 1 1 1 0 1 1
opa9 pum p7 1 1 0 1 1 1 1
opa9 pum p8 1 1 1 1 1 1 1
opa9 pum p9 1 1 1 1 1 1 1
opa9 pum p10 1 1 0 1 1 1 0
opa9 pum p11 1 1 1 1 1 1 1
opa9 pum p12 1 1 1 1 1 1 1
opa9 pum p13 1 1 1 1 0 1 1
opa9 pum p14 1 1 1 1 0 1 1
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opa9 pum p16 1 1 1 1 1 1 1
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opa9 pum p18 1 1 1 1 1 1 1
opa9 pum p19 1 1 0 1 1 1 0
opa9 pum p20 1 1 1 1 0 1 1
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opa11 hyp 622 1 0 1 0 0 1
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opa11 rod r2 1 0 1 1 0 1
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opa11 rod r14 1 0 1 1 0 1
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opa11 rod r18 1 0 1 1 0 1
opa11 rod r19 1 0 1 1 0 1
opa11 rod r20 1 0 1 1 0 1
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opa11 pum p3 1 0 1 1 0 0
opa11 pum p4 1 0 1 0 0 1
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opa11 pum p6 1 0 1 1 0 1
opa11 pum p7 1 0 1 1 0 1
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opa11 pum p9 1 0 1 1 0 0
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opa11 pum p19 1 0 1 1 0 1
opa11 pum p20 1 0 1 1 0 0
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opa14 rod r2 0 1 1 1 0 1
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opa14 rod r7 0 1 1 1 0 0
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opa14 rod r10 0 1 1 1 0 0
opa14 rod r11 0 1 1 1 0 1
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opa14 rod r17 0 1 1 1 0 1
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opa14 rod r19 0 1 1 1 0 1
opa14 rod r20 0 1 1 1 0 1
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opa14 pum p3 0 1 1 1 0 1
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opa14 pum p5 0 1 1 1 0 1
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opa14 pum p19 0 1 1 1 0 1
opa14 pum p20 0 1 1 1 0 0

Appendix C

SAS Program Used to Perform Similarity Index Analysis

(Written by Dr. Arnold Saxton, Tennessee Agricultural Experiment Station)

```
options ls=72 ps=54 pageno=1;
data one;
  infile 'primer.dat' missover;
  input primer $ pop$ ind$ b1-b15;
  if sum (of b1-b15) = . then delete;
run;
proc sort; by primer pop;
data one; set one; by primer pop;
 if first.pop and last.pop then delete;
run;

proc iml workspace=300; reset nolog nocenter;
  use one;
  read all into ddd;
  chk=ddd[+,];
  ddd=ddd[,loc(chk ^= 0)];

  read all var {primer pop}  into lab;
  nobs=nrow(lab);
 do obs=1 to nobs;
  free  sim  index numind ;
  pid = lab[obs,1];  ** current primer;
  chk=lab[obs,2];   ** current population;

  ***** sent up index of obs numbers ******;
  start=obs;
  do ii=obs to nobs while (lab[ii,1] = pid);
   if lab[ii,2] ^= chk then do;
     index=index//(start||ii-1);
     chk=lab[ii,2]; start=ii;
   end;
  end; index=index//(start||ii-1);
  obs=ii-1;   ** advance counter while convenient;

********* Within Similarity ******;
  npop= nrow(index);
  do pp=1 to npop;
   start=index[pp,1]; end=index[pp,2];
   numind=numind//(end-start+1);
   num=0;
```

```
   sxy=0;
    do ii = start+1 to end;
       bandi = ddd[ii, ];
       nx = bandi[+,+];
        do jj = start to ii-1;
          bandj = ddd[jj, ];
          ny = bandj[+,+];
            nxy = bandi # bandj;
            nxy = nxy[+,+];
            sxy = sxy+ 2#nxy/(nx+ny);
            num=num+1;
      end;
    end;
  sim=sim//(sxy/num );
 end;
 cumsim=cumsim//sim;
********** between population similarity ******;
 dmatrix=j(npop,npop,0);
  do ii=1 to npop;
   starti=index[ii,1]; endi=index[ii,2];
   id=id//(lab[starti,1]||lab[starti,2]);
  do jj=ii+1 to npop;
   startj=index[jj,1]; endj=index[jj,2];
   num=0;
   sumsij=0;
    do kk = starti to endi;
       bandi = ddd[kk, ];
       nx = bandi[+,+];
        do ll = startj to endj;
          bandj = ddd[ll, ];
          ny = bandj[+,+];
            nxy = bandi # bandj;
            nxy = nxy[+,+];
            sumsij = sumsij+ 2#nxy/(nx+ny);
            num=num+1;
        end;
    end;

  corsimi=(2#numind[ii,1]#sim[ii,1]-1)/(2#numind[ii,1]-1);
  corsimj=(2#numind[jj,1]#sim[jj,1]-1)/(2#numind[jj,1]-1);

  sij= 1 + sumsij/num - .5#(sim[ii,1]+sim[jj,1]);
** dij =  (sumsij/num) / sqrt(corsimi#corsimj) ;
```

```
  dij =  (sumsij/num) / sqrt(sim[ii,1]#sim[jj,1]) ;
  if dij < 0 then print 'ERROR negative distance' dij;
  if dij > 0 then dij = -log( dij  );
  ans=ans//(sumsij/num || sij||dij );
  dmatrix[jj,ii]= dij;
  poplab=poplab//(pid||lab[kk-1,2]||lab[ll-1,2]);
 end;end;

** reset noname;
** print  'Within Similarity Values';
** print  'Primer Population Similarity';
** print  id cumsim ;
** print  'Between Population Results';
** print 'Primer   Pop 1    Pop 2   Sij(corrected)  Sij  Distance';
**print  poplab ans;

end;
 create dist from ans [colname={'btwnsim' 'corbsim' 'distance'}];
 append from ans;
 create id from id [colname={'primer' 'pop'}];
 append from id;
 create simm from cumsim [colname='wthnsim'];
 append from cumsim;
 create plab from poplab [colname={'primer' 'pop1' 'pop2'}];
 append from poplab;
quit;

data dist; merge dist plab;
proc print;
data simm; merge simm id;
proc print;
proc sort data=dist; by pop1 pop2;
proc means noprint; by pop1 pop2;
 var distance;
 output out=ddd mean=mdist;
run;
proc print data=ddd;
run;

data ddd; set ddd; if mdist<0 then mdist=0;
drop _type_ _freq_;
run;
proc iml; use ddd;
```

```
read all into data;
read all var{pop1 pop2} into lab;
nn=nrow(lab);
index=j(nn,2,0);
list=union(lab);  num=ncol(list);
dist=j(num,num,0);
do ii=1 to num;
  chk=list[1,ii];
  do jj=1 to nn;
  if lab[jj,1]=chk then index[jj,1]=ii;
  if lab[jj,2]=chk then index[jj,2]=ii;
  end;
end;
do jj=1 to nn;
 dist[index[jj,1],index[jj,2]]=data[jj,1];
end;
create dist from dist;
append from dist;
list=list`;
create id from list [colname='pop'];
append from list;
quit;
data dist; merge dist id;
proc print;
proc cluster data=dist method=average pseudo nonorm nosquare;
 id pop;
run;
proc tree;
 id pop;
run;
```

SAS Program Used to Calculate Gene Frequency and Heterozygosity
(Written by Dr. Arnold Saxton, Tennessee Agricultural Experiment Station)

```
************************************************************************
******
 Program for "Analysis of population genetic structure with RAPD
markers"
      by M Lynch and B G Milligan. 1994. Molecular Ecology 3:
91-99.

 Input file:  One line per individual-primer combination that was
scored.  Each
   line has values for <primer  population  individualid  band1
band2 .....>.
   Current limit is 15 bands, less is fine, but more would be
ignored.
   This is easily changed by switching all 15's in the program to
the new max
   band number.  Band values are 1 if present, 0 if absent.

 Limitations - the between heterozygousity section is coded for 3
populations
          only.  More pops would require major additions - but
just copies
          of what is there.

************************************************************************
******

** read raw data of bands ;
data one;
  infile 'primer.dat' missover;
  input primer $ pop$ ind$ b1-b15;
  if sum (of b1-b15) = . then delete;
  **** delete parents from  locally inbred populations;
  **** parent data not used at all if you do not need FIS;
  ****if pop='rod' and ind in('r1','r2','r3','r4','r5','r6','r7','r8')
  ****then delete;
run;
proc sort; by pop primer;

*** get xhat, then qhat;
proc means noprint data=one; by pop primer;
```

```
 var b1-b15;
 *** n1-n15 will be number of individuals scored for each pop primer
combination;
 *** s1-s15 will be the number of 1 band values scored;
 output out=nnn n=n1-n15 sum=s1-s15;
run;
data nnn; set nnn; by pop;
 drop n1-n15 s1-s15;
 array nnn n1-n15;
 array sss s1-s15;
 do over nnn;
   if nnn gt 0 then do;

     *** xhat is the fraction of individuals with null bands;
     xhat=(nnn-sss)/nnn;

     *** qhat is from equation 2a of publication;
     if xhat=0 then qhat=0;
     else qhat = sqrt(xhat) * (1 -  xhat*(1-xhat)/
(nnn*8*xhat*xhat))**-1;

     *** variance of estimate is equation 2b;
     varqhat = (1-xhat)/(4*nnn);

     ***within pop heterozygousity for ith locus - equation 4a;
     whet_i = 2*qhat*(1-qhat) + 2*varqhat;
     *** variance formula 4b;
     vwhet_i=varqhat*4*(1-2*qhat)**2;

     *** save counts and band id for possible later use;
     num=nnn;
     num1=sss;
     band=_i_;

     *** indicate those with too few zeros;
     if xhat*nnn lt 3 then cutoff=0; else cutoff=1;

     *** output results only for bands with data;
     output;
   end;
 end;
run;
```

```
*** the mincut variable produced here is 0 if number of null bands
was less than
   3. Take mincut and merge back onto all populations so that if
ANY population
   has too few nulls, then ALL pops can have that locus deleted;
proc sort; by primer band;
proc means noprint; by primer band;
 var cutoff;
 output out=cut min=mincut;
run;
data nnn; merge nnn cut; by primer band;
run;


*** some descriptive output to check what was done;
proc print;
title2 'Data summed over individuals with qhats, hets and cutoffs for
each locus';
run;
proc freq; tables pop*band;
title2 'This is loci counts if all data used';
run;

*** now average over loci to get other measures of within
heterozygousity;
proc sort data=nnn; by pop;
proc means mean stderr n var min max prt; by pop;
 *** by taking the means, get equation 5 plus std. error described
below equation;
 title2 'Average Within Heterozygousity by population - all data';
 var whet_i ;
run;

**** probably not of interest, but this gets equation 6 results;
**** but also need whet means for next section;
proc means noprint; by pop;
 var whet_i vwhet_i;
 output out=hhh mean=whet vwhet stderr=sewhet n=nloci;
run;
data hhh; set hhh;
  eq6a=vwhet/nloci;
  eq6b=sewhet*sewhet - eq6a;
run;
```

```
proc print; title2 'Variance contributions to Within heterozygousity
- all data';
run;

*** take average of pop means for equation 7;
proc means data=hhh mean stderr n var min max prt;
 title2 'Average over populations - Within heterozygousity - all
data';
 var whet ;
run;


**********************************************************************
*
repeat calcs, but only for loci which have
     enough data in all pops;
data makecut; set nnn; if mincut ne 0;
title2 'This output has only loci with more than 2 null bands in ALL
pops';

*** some descriptive output to check what was done;
proc print;
title3 'Data summed over individuals with qhats, hets and cutoffs for
each locus';
run;
proc freq; tables pop*band;
title3 'This is loci counts if selected data used';
run;

*** now average over loci to get other measures of within
heterozygousity;
proc sort data=nnn; by pop;
proc means mean stderr n var min max prt; by pop;
 *** by taking the means, get equation 5 plus std. error described
below equation;
 title3 'Average Within Heterozygousity by population ';
 var whet_i ;
run;

**** probably not of interest, but this gets equation 6 results;
**** but also need whet means for next section;
proc means noprint; by pop;
 var whet_i vwhet_i;
```

```
 output out=hhh mean=whet vwhet stderr=sewhet n=nloci;
run;
data hhh; set hhh;
  eq6a=vwhet/nloci;
  eq6b=sewhet*sewhet - eq6a;
run;
proc print;
title3 'Variance contributions to Within heterozygousity';
run;

*** take average of pop means for equation 7;
proc means data=hhh mean stderr n var min max prt;
 title3 'Average over populations - Within heterozygousity';
 var whet ;
run;

********** heterozygousity between pops ***************;

** stack 3 pops qhats side by side;
proc sort data=makecut; by primer band;
data pop1; set nnn;
 if pop='rod';
 rename whet_i=whet1 vwhet_i=vwhet1 qhat=qhat1 varqhat=varqhat1;
run;
data pop2; set nnn;
 if pop='hyp';
 rename whet_i=whet2 vwhet_i=vwhet2 qhat=qhat2 varqhat=varqhat2;
run;
data pop3; set nnn;
 if pop='pum';
 rename whet_i=whet3 vwhet_i=vwhet3 qhat=qhat3 varqhat=varqhat3;
run;
data final; merge pop1 pop2 pop3; by primer band;
  het12=qhat1+qhat2-2*qhat1*qhat2;
  het13=qhat1+qhat3-2*qhat1*qhat3;
  het23=qhat2+qhat3-2*qhat2*qhat3;
   chet12=het12 - (whet1+whet2)/2;
   chet13=het13 - (whet1+whet3)/2;
   chet23=het23 - (whet2+whet3)/2;


run;
proc means mean stderr var n min max prt;
```

```
 var het12 het13 het23 chet12 chet13 chet23;
run;

**** genetic distance;
proc means noprint;
 var het12 het13 het23 whet1 whet2 whet3 vwhet1 vwhet2 vwhet3;
 output out=ddd mean=
  mhet12 mhet13 mhet23 mwhet1 mwhet2 mwhet3 mvwhet1 mvwhet2 mvwhet3
  stderr=sehet12 sehet13 sehet23
  n=nloci;
run;
data ddd; set ddd;
 mvwhet1=mvwhet1/nloci;
 mvwhet2=mvwhet2/nloci;
 mvwhet3=mvwhet3/nloci;
 J1=1-mwhet1; J2=1-mwhet2; J3=1-mwhet3;
 J12=1-mhet12; J13=1-mhet13;   J23=1-mhet23;
 dist12= -log( (J12)/sqrt(J1*J2) ) - sehet12*sehet12/(2*J12*J12)
        + mvwhet1/(4*J1*J1) + mvwhet2/(4*J2*J2);
 dist13= -log( (J13)/sqrt(J1*J3) ) - sehet13*sehet13/(2*J13*J13)
        + mvwhet1/(4*J1*J1) + mvwhet3/(4*J3*J3);
 dist23= -log( (J23)/sqrt(J2*J3) ) - sehet23*sehet23/(2*J23*J23)
        + mvwhet2/(4*J2*J2) + mvwhet3/(4*J3*J3);
 run;
proc print;
 title2 'Genetic Distances';
run;
```

Appendix D

Demonstration of Paternity Exclusion

Band	Sire 1	Sire 2	Sire 3	Pup	Dam
396	____	____	____	____	____
500	____	____			____
760	____			_____	
1060	____	____			
2000	____	____		_____	____

Fig. 4. Demonstration of paternity exclusion.

According to the above gel representation, only locus 760 is suitable for paternity exclusion according to the criteria suggested by Scott *et al.* (1992). At this locus, sires 2 and 3 can be excluded, and sire 1 is identified as the sire. Locus 500 is not useful for exclusion because any of the sires could have donated a null allele (i.e. sires 1 and 2 could be heterozygotes).

Vita

Lisa Bailey Comeaux was born in Norton, Virginia on January 22, 1967. She graduated from J.J. Kelly High School in Wise, Virginia in June, 1985. She attended Clinch Valley College in Wise, Virginia from 1985 - 1987. In 1987 she transferred to Virginia Polytechnic Institute and State University where, after an absence, she earned a Bachelor of Science degree with a major in zoology in 1994. She entered graduate school at the University of Tennessee, Knoxville in August, 1994. She received a Master of Science degree with a major in zoology in August, 1997.

She is presently pursuing a doctoral degree in ecology at the University of Tennessee, Knoxville.