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Non-invasive Sampling and DNA Amplification for Paternity Exclusion, Community Structure, and Phylogeography in Wild Chimpanzees

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ABSTRACT. Genetic studies of free-ranging primates have been seriously impeded by difficulties of sampling tissues, including the undesirability of bleeding habituated animals, of transporting frozen samples to the laboratory, and of the inherent inadequacies of accessible variation including allozymes, mtDNA RFLP patterns and DNA fingerprints. We have developed methods of non-invasive DNA sampling and DNA-level genotyping which, when combined with a hierarchical analysis of mtDNA sequences and hypervariable nDNA simple sequence repeat (microsatellite) loci size length polymorphisms, facilitate the resolution of most questions at the individual, social group (community), population, and species (phylogenetic) levels. This approach, based on DNA amplified from shed hair, represents an important new tool for the acquisition of genetic information and will facilitate the study and management of both captive and free-ranging chimpanzees (*Pan troglodytes*). Our hierarchical analysis of population genetics of chimpanzees has revealed high historical levels of gene flow and large effective population sizes, as well as substantial divergence between the West African subspecies and chimpanzees from central and East Africa. At the community level, closer relatedness among philopatric males than among females supports the view that kin selection has been an evolutionary force shaping male-male cooperation in this species. Results from our study of the now relatively isolated Gombe community suggest that habitat fragmentation affects population genetic structure and possibly population viability.

Key Words: *Pan*; Mitochondrial DNA; Microsatellites; D-loop; Cytochrome b.

INTRODUCTION

Traditionally, geneticists have approached questions of primate evolution at two discrete levels. At one extreme, nuclear and mitochondrial gene genealogies are employed in studies of phylogenetics and phylogeography of species and higher taxa to reveal evolutionary history (AVISE, 1989). At the other extreme, variable nuclear loci are used to clarify pedigree relationships and population structure. This dichotomy is, like many in biology, false. As AVISE (1989) has shown, phylogenetic principles and reasoning are usefully applied to lower levels of biotic organization including populations, and as EDWARDS et al. (1992) have shown, individual genotype data can be analyzed to reveal patterns of population structure and microevolution operating at higher levels of organization. Recent advances in molecular genetics permit the generation of DNA-level data which can be used to address questions at several different levels of this organizational hierarchy. To illustrate

this new approach we describe patterns of genetic variation at social community, geographic population, and subspecies levels in free-ranging chimpanzees. The goals of this report are to demonstrate the great power of such hierarchical analyses, to address some chimpanzee-specific questions, and to illustrate the utility of the non-invasive DNA sampling methods we have developed (see MORIN, 1992a, for data and methods).

The hierarchical interpretation of genetic data presupposes the availability of information on a species geographic range, dispersion, dispersal, and mating behavior. For the so-called common chimpanzee (*Pan troglodytes*), much of what is known is summarized by GOODALL (1986) and NISHIDA (1990). Chimpanzees live in a fission-fusion community in which individuals associate with others in their community on an irregular basis. Males are typically philopatric and cooperate to defend the community range. Females may emigrate as adolescents, or migrate temporarily to other communities as reproductively active adults. As little is known about chimpanzee dispersal patterns or population structure beyond the social community, we have sampled chimpanzees from within a single social community, as well as at geographic distances ranging from tens to hundreds of kilometers within each subspecies range. Until a clear genetic definition becomes available, we will consider individuals sampled within roughly 100 km of each other as members of one population, and animals identified with the Kasakela social group (Gombe National Park, Tanzania) as members of one community. For now, we have also accepted the vaguely defined geographic limits on the ranges of the three widely recognized subspecies: West African *P. t. verus*, central African *P. t. troglodytes*, and East African *P. t. schweinfurthii* (HILL, 1969).

Before any study of free-ranging chimpanzees could be undertaken, methods had to be developed that were non-invasive and did not require frozen storage and shipment of tissues from equatorial Africa to laboratory facilities in the U.S.A. We have developed protocols that permit the recovery of DNA from shed or plucked hair and circumvent the costs and logistical difficulties associated with what is, in effect, the reverse of the vaccine delivery "cold train." These techniques, based on DNA amplification and direct sequencing are described by MORIN and WOODRUFF (1992) and MORIN et al. (1992a) and reviewed elsewhere in this volume (WOODRUFF, 1993). In this paper, we report the use of non-invasively obtained simple sequence repeat (SSR) nuclear loci and more and less variable mitochondrial loci, in a hierarchical analysis of chimpanzee genetic variation at the levels of the family, social community, population, and subspecies.

GENETIC VARIATION AT THE FAMILY OR PEDIGREE LEVEL

We have used simple sequence length polymorphism (SSLP) at eight SSR loci for paternity exclusion in a wild community of chimpanzees (*P. t. schweinfurthii*) in the Gombe National Park, Tanzania (MORIN, MOORE, WALLIS, & WOODRUFF, 1992). In 1991, we obtained hair samples from all of the living members of the Kasakela social community as well as two unidentified males from a neighboring community to establish relationships among as many individuals as possible, and calculated probabilities of exclusion of non-fathers based on SSR allele frequencies according to CHAKRAVARTI and LI (1983), SMOUSE and CHAKRABORTY (1986), and CHAKRABORTY et al. (1988). Paternity data were then compared to behavioral and observational records collected by the Gombe Stream Research Centre staff over the past 32 years. This technique is quite powerful in providing high probabilities of paternity exclusion in wild communities even in less than ideal situations with missing individuals and incomplete sampling.

Table 1. Paternity exclusions for selected Gombe chimpanzees.*

	Offspring		
Potential fathers	*Sherehe*	*Flossie*	*Pax*
Evered	*Evered*†	*Evered*†	Evered**
Freud	*Freud*†	*Freud*†	
Frodo	*Frodo***		
Wilkie	*Wilkie*†	*Wilkie*†	
Prof	*Prof*†	*Prof*†	
Goblin	*Goblin*†	*Goblin*†	
Atlas	*Atlas*†	*Atlas*†	
Beethoven	*Beethoven*†	*Beethoven*†	
Apollo	*Apollo*†		
Spindle	*Spindle*†		
Mitumba 1	*Mitumba 1*†	*Mitumba 1*†	*Mitumba 1*†
Mitumba 2	*Mitumba 2*†	*Mitumba 2*†	*Mitumba 2*†
Satan		*Satan*	*Satan*
Jomeo		*Jomeo*	*Jomeo*
Mustard		*Mustard*	
Jageli		*Jageli*	
Humphrey			*Humphrey*
Figan			*Figan*
Sherry			*Sherry*
Willy Wally			*Willy Wally*
Charlie			*Charlie*

*Based on seven or eight SSR loci sampled in the mother, offspring, and most potential fathers (*Sherehe* and *Flossie*), or just in the offspring and potential fathers in the case of *Pax* whose deceased mother, *Passion*, could not be tested. † Males that have been excluded; **males that were tested and could not be excluded; all other samples were not available. Only males that were potential fathers were included in the analyses. Data from MORIN, MOORE, WALLIS, & WOODRUFF (1992).

We performed 140 paternity exclusions for 26 individuals, resulting in all sampled males being excluded as potential fathers in 18 cases, two or more males not being excluded in 3 cases, and 5 cases in which only one male could not be excluded. Three sample cases are provided in Table 1. The genotypes of the selected individuals in Table 1 are given in Table 2.

The probability of exclusion of non-fathers ranged from greater than 99% in cases in which all of the loci were scored for the mother, offspring, and all sampled males, to about 80% if the mother was not available for sampling and calculations involved the offspring and potential fathers only. Even when only three loci were scored and the mother was not sampled, probabilities of exclusion of non-fathers were greater than 60%. Although one would expect positive identification of a greater proportion of fathers with such powerful

Table 2. Allele designations at eight SSR loci.

	SSR locus															
Name	Mfd18		Mfd3		Mfd32		FABP		Pla2a		Rena4		Mfd23		LL	
Sandi (M)	z+18	z+6	z	z+8	z+2	z−6	z+3	z+12	z	z	z	z+4	z+42	z+46	z−10	z−12
Sherehe (O)	z+6	z+18	z+8	z+8	z−6	z+2	z+12	z+12	z	z			z+42	z+42	z−12	z−12
Frodo (F)	z+6	z+18	z	z+8	z+2	z−6	z+3	z+12	z	z	z+4	z	z	z+42	z−10	z−12
Fifi (M)	z+10	z+18	z	z+8	z−6	z+2	z+3	z+12	z	z	z+4	z+4	z	z	z−4	z−10
Flossi (O)	z+6	z+10	z+8	z+8	z−6	z−14	z+3	z+12	z	z	z+4	z+4	z	z+6	z−4	z−10
Pax (O)	z	z+6	z	z+8	z−4	z−6	z+3	z+3	z	z+6	z+4	z+4	z+46	z+6	z−10	z−12
Evered (F)	z	z+6	z	z+8	z+2	z−4	z+3	z+3	z	z	z+4	z+4	z+46	z+26	z−4	z−10

M: Mother; O: offspring; F: putative father, the same individuals as in Table 1. Sources of primers and locus descriptions are given from MORIN, MOORE, WALLIS, & WOODRUFF (1992). One allele has been arbitrarily designated as the 'z' allele, and all others in each locus are sized (in base pairs) relative to that allele.

paternity exclusion data, the loss of over half of the Kasakela community males to a pneumonia-like epidemic in 1988 prevented sampling of most potential fathers. Paternity identification with a very high level of confidence was only possible for two infants (*Faustino* and *Sherehe*) who were conceived after the epidemic, during episodes of promiscuous mating, and for whom all loci could be scored.

It is clear from our analyses that the power for paternity exclusion in a community of wild chimpanzees is very high using these eight SSR loci. Further, when reproductive behavior or other observational data were available to exclude or implicate potential fathers [e.g. as in the case of *Pax*, whose mother, *Passion*, was observed in consort with *Evered* during the conception cycle (GOODALL, 1986); Table 1], greater confidence could be placed in the genetic data, or the pool of potential fathers could be narrowed.

Non-invasive DNA-level methods are a great improvement over prior methods which required the capture of animals for blood or tissue extraction, sterile lab conditions and/or liquid nitrogen storage of samples for transport to laboratory facilities, and often resulted in genetically ambiguous results (LANDER, 1989, 1991; LYNCH, 1988; MORIN & RYDER, 1991). Furthermore, the resulting multiple-locus genotype data set is permanently expandable. It may be added to as new individuals are sampled, by researchers in any laboratory, without the need to replicate any previous sampling or gene amplification. As technology allows use of other tissue types such as bone, tooth, feces, buccal cells, and museum skins (ORREGO et al., in prep), genetic data from unhabituated and dead animals may also be easier to collect and add to the database.

GENETIC VARIATION AND COMMUNITY STRUCTURE

At the second level of study, the social community, the SSR SSLP variation data used for paternity exclusion were re-analyzed to characterize the population's genetic structure (MORIN, MOORE, CHAKRABORTY, & WOODRUFF, 1992). Features such as basic variation (polymorphism and heterozygosity), breeding system, effective population size, demographic history, gene flow, and the genetic relatedness of nearby populations (genetic distance) are all important determinants of a population's viability and evolutionary potential. The SSLP data used in this study were analyzed for departure from Hardy-Weinberg equilibrium (HWE), for linkage disequilibrium, for non-random association of alleles, and for levels of relatedness among individuals and groups. This allowed us to characterize the Gombe community to an extent not previously possible, even if allozyme electrophoresis, mtDNA RFLP patterns or DNA fingerprinting had been attempted — which, of course, they had not.

Statistical methods for calculating deviations from Hardy-Weinberg equilibrium and non-random association of alleles are described in MORIN, MOORE, CHAKRABORTY, & WOODRUFF (1992) and EDWARDS et al. (1992). Methods for calculating relatedness are described in QUELLER and GOODNIGHT (1989), and were performed using the program Relatedness 4.2 developed by QUELLER and GOODNIGHT. Relatedness values were calculated on the complete Gombe data set as well as on a subset including only individuals thought to be maternally unrelated on the basis of 32 years of behavioral observations and birth records.

Mitochondrial DNA sequence data analysis (see below) suggested that high levels of gene flow have occurred historically among chimpanzee populations. If female dispersal was high enough to maintain a large effectively panmictic population over the subspecies range, we would expect samples from both dispersed "populations" (as defined earlier) and a

Table 3. Levels of relatedness based on seven-eight SSR loci (from program Relatedness 4.2*) among subgroups of chimpanzees from the Gombe and non-Gombe sample sets.**

Subgroup	*N*	R
Non-Gombe	28	0.05
Gombe, all	39	0.28
Gombe males, all	20	0.20
Gombe males, unrelated	10	0.21
Gombe females, all	19	0.12
Gombe females, unrelated	11	0.10

*QUELLER & GOODNIGHT (1989). **The non-Gombe sample set includes animals from 15 populations and 3 subspecies. *N*: Number of individuals; R: relative relatedness (higher values reflect closer relationships). Data from MORIN, MOORE, CHAKRABORTY, & WOODRUFF (1992).

single community to be in HWE. Our analysis showed that assumptions for HWE in the Gombe community sample set were not satisfied according to the likelihood-ratio test for three of the eight loci. Several factors, including nonrandom mating, distortions in the contributions of gametes to offspring, nonrandom sampling, Wahlund effect, strong selection, and allele mis-scoring, can all lead to deviations from the predictions of HWE (SPIESS, 1989; STAUB et al., 1990). If departures from panmixia were due to population substructuring, we would have expected a larger number of deviations in dispersed population samples than from within a single community. These data showed, however, more significant deviations in the Gombe sample set than the dispersed sample set, suggesting inbreeding in the former. In addition, all significant deviations in the Gombe sample were toward deficiencies in the number of heterozygotes, as would be expected if inbreeding were the cause.

We further tested for population substructuring within the Gombe community by testing for non-random association of alleles from genetically unlinked loci. Results indicated that the extent of inbreeding at Gombe, if it is occurring, is not yet sufficient to cause significant co-segregation of alleles at these particular loci.

Finally, levels of relatedness among males and females in the Gombe community were calculated to determine whether philopatric males were more closely related to one another than were the typically dispersing females. Primatologists have invoked such a close kinship among males as an explanation for the evolution of male cooperation in defense of the community territory (PUSEY, 1979; WRANGHAM, 1979, 1982). As expected, males were more related to each other than were the females (Table 3). Furthermore, Gombe females had higher levels of relatedness among themselves than did the mixed-sex samples of individuals from geographically widely dispersed communities. These data thus provide the first formal evidence that the evolution of the chimpanzee social community may be at least partially explained by kin selection theory.

GEOGRAPHIC AND SUBSPECIFIC LEVELS OF GENETIC VARIATION

Although three geographically characterized subspecies have been recognized by taxonomists for several decades (HILL, 1969), they cannot be distinguished morphologically in captivity (GROVES, 1989; GROVES et al., 1992; SEAL & FLESSNESS, 1986). Previous attempts to identify subspecies based on mtDNA haplotypes (FERRIS et al., 1981) were compromised by the use of captive animals of questionable provenance, so we developed a hierarchical approach to the study of chimpanzee population genetics using animals of known provenance from 20 geographic locations across Africa (MORIN et al., 1992b). We

had three goals: 1) to characterize the genetic differences between the subspecies, and to establish a method for discerning the subspecific identities of chimpanzees in captivity (MORIN et al., 1992a); 2) to characterize levels of genetic variation and phylogeographic patterns within each subspecies at selected loci; and 3) to determine whether social structure, gene flow patterns, or nonrandom mating result in population substructuring of genetic variation.

Toward these ends, we sequenced a portion of the cytochrome *b* locus to examine deeper branches of chimpanzee phylogenetics, and variation in the D-loop to characterize phylogeographic patterns and population structure at the local level: 178 base pairs (bp) of the cyt *b* region of 35 animals representing all three subspecies, and 345 bp of the D-loop of 63 individuals representing all 19 maternal lineages in the Kasakela community, Gombe, and 6 – 21 additional individuals of each subspecies. Additionally, three published chimpanzee, two bonobo (one sequenced by us), and one human D-loop sequence were also included for subspecies characterization and as outgroups (DI RIENZO & WILSON, 1991; FORAN et al., 1988; KOCHER & WILSON, 1991). These data allowed some inferences about the historical levels of gene flow within two of the subspecies, and new age estimates for divergence between the western subspecies and the other two. The data can also be used to recognize evolutionarily significant units among chimpanzee populations and so better manage and conserve both wild and captive animals.

The cyt *b* sequence was highly conserved within subspecies, and no fixed differences were found between the central (*troglodytes*) and eastern (*schweinfurthii*) subspecies. The western (*verus*) subspecies was significantly different, however, showing four transitions between *P. t. verus* individuals and the central and eastern individuals combined. The D-loop sequence was highly variable (35% variable sites) both within and between subspecies. Fixed differences were found for each subspecies, allowing confident assignment of subspecies for captive individuals based on mtDNA sequence variation. We used this information to develop a rapid and relatively inexpensive method for genetic typing of captive chimpanzees for subspecies identification based on subspecies-specific oligonucleotide probes (MORIN et al., 1992a).

The branch lengths and structure of phylogenetic trees based on D-loop sequence data, when compared to other published estimates of divergence times (HASEGAWA et al., 1990; PESOLE et al., 1992; WILSON et al., 1987; WILSON & SARICH, 1967), suggest an origin for *P. troglodytes* in West Africa. The western subspecies *P. t. verus*, has both larger genetic distances within the subspecies, and a much deeper split between it and the other two subspecies (Table 4). Although the D-loop sequence fragment used here was too short to apply current models of evolutionary rates of genetic change, we estimate that a surprisingly long period of approximately 1.4 (range: 0.68 – 3.99) million years has elapsed since divergence between *P. t. verus* and the two more eastern subspecies based on the observed genetic distances. Interpretation of taxonomic and phylogenetic implications of these genetic distances, however, would be premature until more West African samples and longer DNA sequences are available (HOELZEL et al., 1991; PESOLE et al., 1992).

Both parsimony and distance-based methods of phylogenetic tree construction created trees with identical clades within subspecies. Analysis of these clades for phylogeographic patterns showed that, within the two subspecies (*troglodytes* and *schweinfurthii*) for which we have adequate geographic samples, there is little or no isolation by distance over approximately 500 – 800 km in both subspecies. This, together with very high levels of genetic variation within the subspecies, indicates that these subspecies have historically had large population sizes and high levels of gene flow. Even at the community level the data support

Table 4. Average genetic distances within and between subspecies*

Subspecies	Average genetic distances
Pts — Pts	0.029
Ptt — Ptt	0.055
Ptv — Ptv	0.081
Pts — Ptt	0.072
Ptt — Ptv	0.155
Ptv — Pts	0.192

*Based on 345bp of the mitochondrial D-loop, calculated using the DNADIST program from Felsenstein's PHYLIP DNA analysis software package. Pts: *P. t. schweinfurthii*; Ptt: *P. t. troglodytes*; Ptv: *P. t. verus*. Data from MORIN et al. (1992b).

high levels of historical gene flow. Of the 24 adults at Gombe in July 1991, we sequenced 19 individuals known or suspected to be maternally unrelated, and found 15 different haplotypes in the D-loop region. This high level of genetic variation is consistent with observations of female dispersal leading to high levels of gene flow (GOODALL, 1986; NISHIDA, 1979; PUSEY, 1979, 1980).

DISCUSSION

Chimpanzees are of special interest to primatologists and population geneticists because of their close relationship to humans, and their complex social and mating structure. The complexity of the chimpanzee mating system has become apparent through several studies (e.g. BYGOTT, 1979; GHIGLIERI, 1984; GOODALL, 1986; HASEGAWA, 1990; NISHIDA, 1979; SUGIYAMA & KOMAN, 1979; TUTIN et al., 1983; WRANGHAM, 1979). Mating behaviors include promiscuous mating in a group, consortships lasting from hours to months, and possessive matings by dominant males (NISHIDA, 1979; TUTIN, 1979; reviewed in MORIN, 1992b). Our study is the first to establish paternity in wild communities of chimpanzees, demonstrating the potential usefulness of the non-invasive genetic approach to the study of mating structure and reproductive success in wild populations.

Our analyses of SSR data indicate that chimpanzee populations are not panmictic, despite high levels of historical gene flow suggested by patterns of mitochondrial DNA variation, possibly as a result of social community structure, nonrandom mating behavior, and/or patterns of female dispersal. The higher levels of relatedness among males in a social community support arguments for the evolution of the chimpanzee's multimale social system, but do not account for the overall higher level of relatedness found in the Gombe community. It is possible that fewer females are dispersing, or that most female dispersal is between neighboring communities (NISHIDA, 1979; see CALEY, 1991; WASER, 1987), and habitat fragmentation or other effects of human contact are enough to reduce the levels of gene flow and cause increased inbreeding among the Gombe chimpanzees.

Our results indicate that *Pan troglodytes* has a long evolutionary history, and has historically had large effective population sizes and high levels of gene flow. Today, habitat fragmentation has limited both the population sizes and levels of gene flow in natural populations of all three subspecies. Without natural genetic exchange with other populations, genetic drift and inbreeding may begin to change the genetic structure of populations in protected areas, such as the Gombe National Park, and may significantly increase the probability of extinction of this and other populations (LYNCH & GABRIEL, 1990; WOODRUFF, 1990, 1992).

The techniques developed for non-invasive DNA sampling based on shed hair can be applied to other species of primates, as can the molecular methods of PCR amplification of nuclear and mitochondrial DNA for phylogenetic, phylogeographic, population structure, and paternity studies. This first molecular genetic survey of variation throughout the range of the chimpanzee demonstrates the tremendous wealth of basic information that can be obtained from a hierarchical study. Future genetic studies can add to the data already collected and can be combined with ecological, behavioral, and theoretical studies for increased understanding of the evolutionary history, population genetic structure, and current changes in populations due to habitat fragmentation. Anticipated advances in tissue acquisition methods and biotechnology and decreasing costs of DNA-level research will make non-invasive field genotyping routine in the near future. The genetic data will permit the first rigorous tests of numerous hypotheses and lay the foundations for the genetic management of the conservation or future evolution of these increasingly endangered species.

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