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RESEARCH ARTICLE

Subspecies Composition and Founder Contribution of the Captive U.S. Chimpanzee (*Pan troglodytes*) Population

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Chimpanzees are presently classified into three subspecies: *Pan troglodytes verus* from west Africa, *P.t. troglodytes* from central Africa, and *P.t. schweinfurthii* from east Africa. A fourth subspecies (*P.t. vellerosus*), from Cameroon and northern Nigeria, has been proposed. These taxonomic designations are based on geographical origins and are reflected in sequence variation in the first hypervariable region (HVR-I) of the mtDNA D-loop. Although advances have been made in our understanding of chimpanzee phylogenetics, little has been known regarding the subspecies composition of captive chimpanzees. We sequenced part of the mtDNA HVR-I region in 218 African-born population founders and performed a phylogenetic analysis with previously characterized African sequences of known provenance to infer subspecies affiliations. Most founders were *P.t. verus* (95.0%), distantly followed by the *troglodytes*/*schweinfurthii* clade (4.6%), and a single *P.t. vellerosus* (0.4%). Pedigree-based estimates of genomic representation in the descendant population revealed that *troglodytes*/*schweinfurthii* founder representation was reduced in captivity, *vellerosus* representation increased due to prolific breeding by a single male, and reproductive variance resulted in uneven representation among male *P.t.verus* founders. No increase in mortality was evident from between-subspecies interbreeding, indicating a lack of outbreeding depression. Knowledge of subspecies and their genomic representation can form the basis for phylogenetically informed genetic management of extant chimpanzees to preserve rare genetic variation for research,

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INTRODUCTION

Nineteenth- and early 20th-century taxonomists proposed up to a dozen different chimpanzee genera and 10 species [Hill, 1969]. Since the taxonomy was first simplified and clarified by Schwarz [1934], and later standardized by Hill [1969], contemporary taxonomists have recognized three geographically-defined chimpanzee subspecies: *Pan troglodytes verus* from west Africa, *P.t. troglodytes* from central Africa, and *P.t. schweinfurthii* from east Africa. A fourth subspecies, *P.t. vellerosus* from Nigeria and northern Cameroon, has been proposed [Gonder et al., 1997]. Although there is extensive morphological variation [Eckhardt, 1987; Reynolds & Luscombe, 1971; Schwarz, 1934; Shea & Coolidge, 1988; Taylor & Groves, 2003], morphological features do not reliably distinguish one subspecies from another. Traditional subspecies designations were based on transient morphological traits, including juvenile facial pigmentation, the general appearance of the pelage, and the shape of the head hair or beard [Groves, 1986, 2001, in press; Hill, 1969; Schwarz, 1934]. This lack of reliable and informative character traits with which to distinguish subspecies may have led to minor phenotypic characters being given undue importance [Albrecht & Miller, 1993; Groves, 1986; Reynolds & Luscombe, 1971].

Modern phylogenetic studies of chimpanzees began with the discovery of extensive sequence variation in the first hypervariable region (HVR-I) of the noncoding mtDNA control region [Kocher & Wilson, 1991]. Morin et al. [1994] demonstrated that chimpanzee HVR-I sequences from similar geographical regions clustered together phylogenetically, while geographically disjunct populations were separated, with sequence variation patterns attributable to male philopatry and female dispersal. The evolutionary genetics of African *verus*, *vellerosus*, *troglogytes*, and *schweinfurthii* populations were subsequently studied in considerable detail [Gagneux et al., 1999, 2001; Goldberg & Ruvolo, 1997; Gonder, 2000; Gonder et al., 1997]. Phylogenetic evidence revealed two broad clades of chimpanzees: *P.t. verus* from West Africa, and a difficult-to-distinguish *P.t. troglodytes*/*P.t. schweinfurthii* clade from central/eastern Africa [Gagneux et al., 2001; Gonder, 2000]. The significance of this work for captive chimpanzee management lay in the demonstration that one could assign subspecies identities to individuals of unknown provenance by comparing their mtDNA sequences with those of individuals from known geographical regions [Goldberg, 1997; Wise et al., 1997].

Researchers in conservation and comparative biomedicine have been challenged to evaluate the biological significance of chimpanzee subspecies. Once separated by geographical barriers, human encroachment, or habitat loss, gene flow was disrupted [Gonder, 2000; Gonder et al., 1997; Shea & Coolidge, 1988], and reproductively isolated chimpanzee populations diverged genetically over time, leading to subspecies and incipient speciation [Gagneux et al., 2001; Nei, 1987; Stone et al., 2002]. Depending on their evolutionary dynamics, different molecules give different results, and the corresponding divergence time estimates

for the *verus*/non-*verus* split vary considerably, from 2.1 Mya (Xq13.3 [Kaessmann et al., 1999]) to 650 Kya (nine unlinked nuclear intergenic regions [Fischer et al., 2004]) to 720 Kya (NRY [Stone et al., 2002]). Based on mtDNA evidence, west African *verus* diverged from eastern/central *troglodytes*/*schweinfurthii* around 894 Kya [Gonder, 2000], which represents 59,600 generations (15 yr/generation) of independent evolution. Such long divergence times can allow considerable genetic differentiation, due to either selection or different demographic histories. The allele frequencies of transferrin (*Tf*) and phosphoglucomutase (*Pgm*) proteins, the mitochondrial gene cytochrome b (*cyth*), and anonymous single nucleotide polymorphic sites are known to vary among chimpanzee subspecies [Goodman et al., 1967; Goodman & Tashian, 1969; Morin et al., 1994; Smith et al., 2004]. The *troglodytes*/*schweinfurthii* clade also exhibits a unique deletion haplotype for a leukocyte-Ig like receptor gene (LIRa) that is found intact in the *verus* subspecies [Canavez et al., 2001]. The mitochondrial gene ND3 shows evidence of non-neutral variation, due to differential selection of the gene or its relaxation, among western and central/eastern subspecies [Nachman et al., 1996].

Because of their close phylogenetic relationship to humans, chimpanzees have been used extensively in research on HIV, hepatitis B, kuru, Creutzfeldt-Jakob disease, respiratory syncytial virus, and other infectious pathogens with strong host specificity [Bukh, 2004; Lu, 1997; Maynard et al., 1972; Purcell, 1992; Yu et al., 1974]. The problem is that subspecies genetic variation could have far-reaching implications for both conservation and biomedicine, potentially including outbreeding depression and the confounding of experimental results [Morin et al., 1994; Gagneux et al., 1999, 2001]. Until now, reliable data on the subspecies composition of captive chimpanzees were lacking. We report here on the subspecies composition of the African-born chimpanzee founders and their genetic representation in the extant, captive-born population.

MATERIALS AND METHODS

DNA Isolation and Sequencing

Whole blood samples were collected and shipped overnight to our laboratory for DNA extraction, PCR, and DNA sequence analysis [Ely et al., 1998a]. Oligonucleotide primers were designed from a chimpanzee mtDNA sequence (forward: 5′-CAACCGCTATGTATTTCGTA-3′=bases 55–74; reverse: 5′-GCGGGATATTGATTTCAC-3′=bases 388–405 (after Kocher and Wilson [1991]); and used to amplify and sequence a 350-bp segment of chimpanzee HVR-I [Ely et al., 1998b]. This region contains numerous phylogeographically informative nucleotide sites [Gagneux et al., 1999; Kocher & Wilson, 1991; Morin et al., 1994]. PCR cocktails consisted of 16 pmol of each primer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 1.0 U Taq polymerase (PGC, Frederick, MD), 1 × manufacturer-supplied PCR buffer, and 25 ng template DNA quantified by spectrophotometry, in a total reaction volume of 25 μl. PCR amplifications (30 × 94°/45″, 55°/45″, 72°/60″) were performed on an MWG thermal cycler (MWG Biotech, Highpoint, NC), and the amplification products were separated in 1% LMP agarose gels and purified with QIAquick columns (Qiagen, Valencia, CA). For the sequencing reactions we used ThermoSequenase (USB, Cleveland, OH) or SequiTherm Excel II (Epicentre Technologies, Madison, WI) kits, following the manufacturer's recommendations. Sequencing was performed on a Licor DNA4200, and the images analyzed using AlignIR© software (Licor, Lincoln, NE). All templates had ≥2 × coverage, with 100% overlap between the forward and reverse sequences.

Identification of African-Born Founders

Colony and demographic records from the International Species Inventory System, ISIS, 14 August 1999 version) were inspected to identify African-born founders [Flesness, 1986; Seal & Flesness, 1986]. Colonies from the former Chimpanzee Biomedical Research Program (CBRP [Swyers, 1990]) included the U.T.M.D. Anderson Cancer Center, Bastrop, TX (Bas); New Iberia Research Center (NIRC); Primate Foundation of Arizona (PFA); The Coulston Foundation, Alamogordo, NM (TCF); and Yerkes Regional Primate Research Center (Yks).

Three criteria were used to identify African-born founders. First, the date of birth (DOB) must have preceded 1 July 1975, the date on which the CITES convention took effect in the U.S. and ended the legal importation of African chimpanzees. Since the DOBs for true African-borns were frequently estimated (e.g., 1 January 1960 or simply 1960), individuals with specific DOBs (e.g., 17 March 1954) were treated as captive-born. Second, the founders must have had no known parents. Listings with a dam but no sire reflected either unknown paternity or possible capture of partial family groups (mother plus immature offspring) in Africa [Kortlandt, 1966]. Third, ISIS classifications of true founders listed either ''wild born'' or ''unknown'' birth origins, the difference being that actual import/export certificates could not be located for ''unknowns'' (Nate Flesness, personal communication). These criteria identified 429 African-born founders. DNA samples were available for 218 of these founders for mtDNA sequencing (Genbank accession numbers AY918496–AY918713). This sample represents 50.8% (218/429) of all African-born founders of the NIH-supported captive chimpanzee population.

mtDNA Sequence Comparisons and Subspecies Designations

The PAUP 4.0b10 package [Swofford, 1990] was used for phylogenetic analyses of a 313-bp segment of HVR-I (350-bp amplicon minus primer binding sites [Ely et al., 1998b] from the 218 NIH founders. The broader issue of African chimpanzee phylogeny has been addressed elsewhere [Gagneux et al., 1999, 2001]. The primary criterion for inclusion of mtDNA sequences here was the availability of information on the precise locality of origin in Africa. Subspecies identities of NIH founders were inferred by comparison with 272 previously characterized African chimpanzees (66 *verus*, 18 *vellerosus*, 44 *troglodytes*, and 144 *schweinfurthii* mtDNA sequences), using two bonobo sequences as the outgroup [Gagneux et al., 1999, 2001; Goldberg & Ruvolo, 1997; Hu et al., 2001; Morin et al., 1994; Gonder, 2000; Gonder et al., 1997; Nerrienet et al., 2005]. Both distance (neighbor-joining (NJ)) and cladistic (maximum likelihood (ML)) methods were used for phylogenetic analysis. We inferred the subspecies identities of the founders by clustering them with African individuals of known provenance [Goldberg, 1997; Wise et al., 1997]. The Modeltest program [Posada & Crandall, 1998] identified the HKY85 model as the most appropriate model of evolution [Hasegawa et al., 1985]. Bootstrap resampling with replacement was used to generate 1,000 replicate samples, and NJ trees recalculated from each replicate were used to evaluate the statistical support for the major branches in the phylogenetic tree [Felsenstein, 1985].

Erroneous phylogenetic inferences may be made if numts, or mtDNA fragments that have been transferred to the nuclear genome [Zhang & Hewitt, 1996; Zischler et al., 1998], are preferentially amplified instead of mtDNA. Numts are particularly problematic in gorillas [Jensen-Seamen et al., 2004; Thalmann et al., 2004]. In humans, only 2.8% of 612 known numts include portions of the

non-transcribed D-loop [Woischnik & Moraes, 2002]. A comparison of sequences from dilutions recommended for human forensic identity testing with mtDNA (0.5 ng, 5 ng, and 20 ng, quantified by spectrophotometry) revealed no sequence differences, indicating that we had truly sampled mtDNA and not numts [Budowle et al., 2002; Hirano et al., 1997; Holland & Huffine, 2000].

Effective Population Size

The effective population size, N_e (a mathematical abstraction that is useful for predicting the effects of genetic drift on neutral variation in small populations), has been a key component of conservation genetics since the 1980s [Schonewald-Cox, 1983]. Standard formulas for estimating the inbreeding effective size ($N_{e(I)}$), which controls for the effects of inbreeding, and for the variance effective size ($N_{e(V)}$), which controls for reproductive variance, were used [Crow & Kimura, 1970]. Reproductive data from the earliest birth in March 1926 to the breeding moratorium in July 1995 [Commission on Life Sciences of the National Research Council, 1997] included 1,402 births to 429 founders. Of these, 952 captive-born offspring that were still alive at the time of the moratorium were used to estimate N_e by sex and subspecies.

Pedigree Analysis

Pedigrees assembled from colony demographic and housing records were up to date through mid-1995, the year of the breeding moratorium. Unknown parentage was resolved with the use of short tandem repeat (STR) markers [Ely et al., 1998a]. The PEDSYS ''FoundRep'' algorithm was used to trace lines of descent from each founder to all living descendants, and to estimate each founder's genetic representation as the number of founder equivalents (FEs) among living, captive-born descendants [Dyke, 1993]. The FE is defined as the number of hypothetical founders that would produce a population with the same allelic diversity as observed in the descendant population, if all founders had contributed equally [Lacy, 1989]. Unequal reproduction results in the FE being smaller than the actual number of founders, which may generally be true in real populations. Individual estimates of the FE represent the allelic contribution of each founder to a descendant population. For purposes of comparison, the FEs were also averaged over sex, colony, and subspecies.

Statistical Analyses

Statistical analyses were performed with the SYSTAT package (v. 9.0) using standard statistical tests [Sokal & Rohlf, 1981]. Molecular evolutionary analyses were conducted using MEGA v. 2.1 [Kumar et al., 2004].

RESULTS

Subspecies Composition and Phylogenetic Analysis

Of the 218 founder mtDNA sequences examined, there were 109 different haplotypes, which consisted of 80 unique HVR-I haplotypes and 29 repeated haplotypes that jointly accounted for 138 founder sequences. The most common haplotype found in 24 founders was identical to a previously published sequence from west Africa (Genbank accession #AF137420 [Gagneux et al., 1999]. At the other extreme, four founder sequences (three from west Africa and one from east Africa) each matched a single known African haplotype (Fig. 1). The remaining

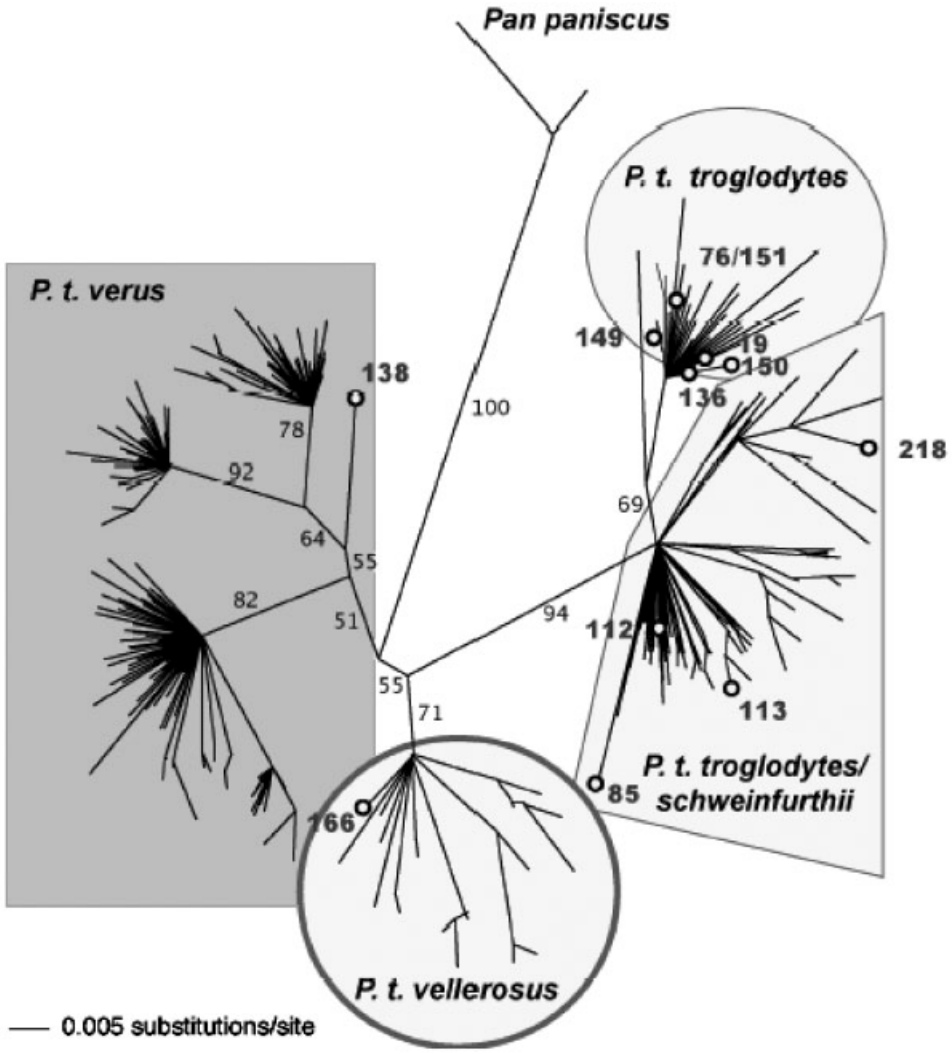

Fig. 1. NJ tree of 218 African-born NIH chimpanzee founders clustered with 272 African chimpanzees of known geographical provenance. Numbers along the interior branches indicate the percentage of 1,000 bootstrap replicates that yield the same branching order depicted here. Open dots with numeric labels on terminal branches represent consecutive IDs of the 11 non-*verus* and one oddly-placed *verus* chimpanzees inferred from the phylogenetic analysis (see Table I).

190 sequences, representing 104 haplotypes, were unique in that they were not observed in the sample of African chimpanzee haplotypes (although they may be represented by other mtDNA sequences in Genbank). For example, the second most common CBRP founder haplotype, represented by 14 founder sequences, was not observed in our African sample. The distribution is highly overdispersed relative to the Poisson expectations ($t=24.5$, $P<0.001$), indicative of a nonrandom clumping process [Sokal & Rohlf, 1981]. This may have derived from the capture of multiple members of a single matriline [Kortlandt, 1966] or it may reflect an unequal distribution of haplotypes in African chimpanzee populations due to coalescence [von Haeseler et al., 1996].

TABLE I. Consecutive IDs, ISIS IDs, Genbank Accession Numbers and inferred Subspecies of 11 Non-*verus* and 1 Oddly-Placed *verus* Chimpanzee Founders Inferred From the Phylogenetic Analysis

Consecutive ID (from Fig. 1)	ISIS ID	Genbank accession number	Inferred subspecies
19	2105	AY918514	*troglodytes*
76	1436	AY918571	*troglodytes*
85	804	AY918580	*troglodytes/schweinfurthii*
112	925	AY918606	*troglodytes/schweinfurthii*
113	3020	AY918607	*troglodytes/schweinfurthii*
136	2367	AY918630	*troglodytes*
138	1126	AY918632	*verus*
149	1033	AY918642	*troglodytes*
150	348	AY918643	*troglodytes*
151	9681	AY918644	*troglodytes*
166	2412	AY918658	*vellerosus*
218	1103	AY918709	*troglodytes*

Three major clusters of chimpanzee haplotypes were evident in the inferred NJ phylogenetic tree, representing the west African *verus*, the proposed *vellerosus* from Nigeria and Cameroon, and the weakly differentiated central and east African *troglodytes*/*schweinfurthii* clades (Fig. 1). Open dots in Figure 1 represent the 11 non-*verus* CBRP founders, along with a single oddly-placed *verus* founder (#138), labeled by consecutive ID (the corresponding ISIS IDs and Genbank accession numbers appear in Table I). The topology of the NJ tree was identical to the ML tree (not shown). The first major clade included animals from the west African *P.t. verus*, with 99 haplotypes representing 210 CBRP founders. The three subclades evident in this *verus* clade do not represent geographic clustering among *P.t. verus*. This lack of geographical structuring across west African *P.t. verus* was noted previously, with only one *verus* population (Comoe National Park, northeastern Ivory Coast) showing a slight signature of isolation from the otherwise panmictic *verus* population, due to the Comoe chimpanzees' longer isolation time and their consequent genetic depauperation [Gagneux, 1998]. The second clade consisted of *P.t. vellerosus* sequences from Nigeria and Cameroon, and included the sequence of a single CBRP founder. The third clade was comprised of a poorly distinguished mixture of central African *P.t. troglodytes* and east African *P.t. schweinfurthii*., as observed previously [Gagneux et al., 1999, 2001]. It contained a subcluster of sequences found only in central Africa, which included five sequences from six founders likely drawn from central Africa. A second, basally branching cluster included mostly east African sequences, but also several sequences from animals sampled in Cameroon [Gagneux et al., 2001]. The four CBRP founder haplotypes that fall into this cluster cannot be safely assigned, with the exception of chimpanzee #113, which was known to be an east African *P.t. schweinfurthii* captured in Kisangia in northeast Zaire in the 1970s.

The Cameroonian sequences branching from the same point as the east African sequences might be more ancestral, reflecting the likely founder populations of the east African *schweinfurthii* chimpanzees. As expected, the most strongly supported clade was the species-level separation between bonobo and common chimpanzees (100%). The central/east African clade was supported by 94%, and the Nigerian clade was supported by 71%–just above the nominal

level for statistical significance (70%) [Hillis & Bull, 1993]. The three deep west African clades had stronger bootstrap support (82%, 92%, and 78%, respectively), even though *verus* subclades lack geographical structuring [Gagneux, 1998]. CBRP founder haplotypes were found in each of the three major clades (Fig. 1). Of the 218 NIH founders, 95% belonged to the west African *P.t. verus* subspecies, 4.6% belonged to the *troglodytes*/*schweinfurthii* clade, and 0.4% belonged to the *vellerosus* clade.

Two sequences appeared oddly positioned in the phylogram. Sequence #138 branched off basally between the *P.t. verus* clades, while #85 fell on its own relatively long branch from the root of the *troglodytes*/*schweinfurtii* cluster (Fig. 1). The inability to clearly resolve the phylogenetic distinction between *troglodytes* and *schweinfurthii* was noted previously [Gagneux et al., 1999, 2001; Gonder, 2000]. This is likely due to the recent (117 Kya) separation between these putative subspecies, when the central African *troglodytes* population expanded into eastern Africa and thereby gave rise to the contemporary *schweinfurthii* population [Gagneux et al., 1999; Goldberg & Ruvolo, 1997; Gonder, 2000]. Their odd placements suggest that these are rare haplotypes with intermediate phylogenetic traits that may be resolved after more thorough sampling of African chimpanzee mtDNA sequences [e.g., Wise et al., 1997].

Genetic Diversity: Pairwise Differences and Nucleotide Diversity

We used pairwise differences to calculate the mismatch distributions [Rogers & Harpending, 1992] for the complete collection of sequences and for each well-defined subspecies separately. Apart from the *schweinfurthii* samples, which showed a symmetrical and unimodal distribution, all of the groups showed multimodal distributions of pairwise mismatches (not shown). There are indications based on mitochondrial and nuclear loci that central African *troglodytes* chimpanzees harbor the highest genetic diversity [Fischer et al., 2004; Gagneux et al., 1999; Yu et al., 2003]. Our results indicating a mismatch distribution support this. Given that the number of individuals sampled from the *troglodytes* range is still relatively small compared to *verus* and *schweinfurthii*, the estimate of variability in this subspecies may still be an underestimate.

The mean number of pairwise differences among HVR-I sequences from the 218 CBRP founder chimpanzees was 20.2 (standard deviation (SD)=11.2), with a range of 0–49.0 nucleotide differences and equivalent to 6% mean difference. This mean is almost identical to the expectation derived from previously estimated mean pairwise differences among African chimpanzee HVR-I sequences [Gagneux et al., 1999]. For the *verus* founder sequences, we observed a multimodal mismatch distribution (Fig. 2A), which is probably indicative of a mixture distribution composed of an artificial combination of haplotypes that are not found in African chimpanzee populations (Fig. 2B). *P.t. verus* founders had a mean mismatch of 19.2 (SD=9.6) compared to the sample of sequences from known locales in West Africa, with a mean of 17.7 (SD=8.1). This indicates that the founder population contained a degree of genetic variability (as measured by mismatch distribution) comparable to that of the panel of wild animals used for comparison. A recent comparative study on mtDNA variability reported 21.8 differences in 312 bp of HVR-I sequences within the *verus* subspecies, 14.6 differences within *troglodytes*, and 7.9 differences within *schweinfurthii*, as well as a *verus*/*troglodytes* difference of 36.2, a *verus*/*schweinfurthii* difference of 33.0, and a *troglodytes*/*schweinfurthii* difference of 19.7 [Krings et al., 1999].

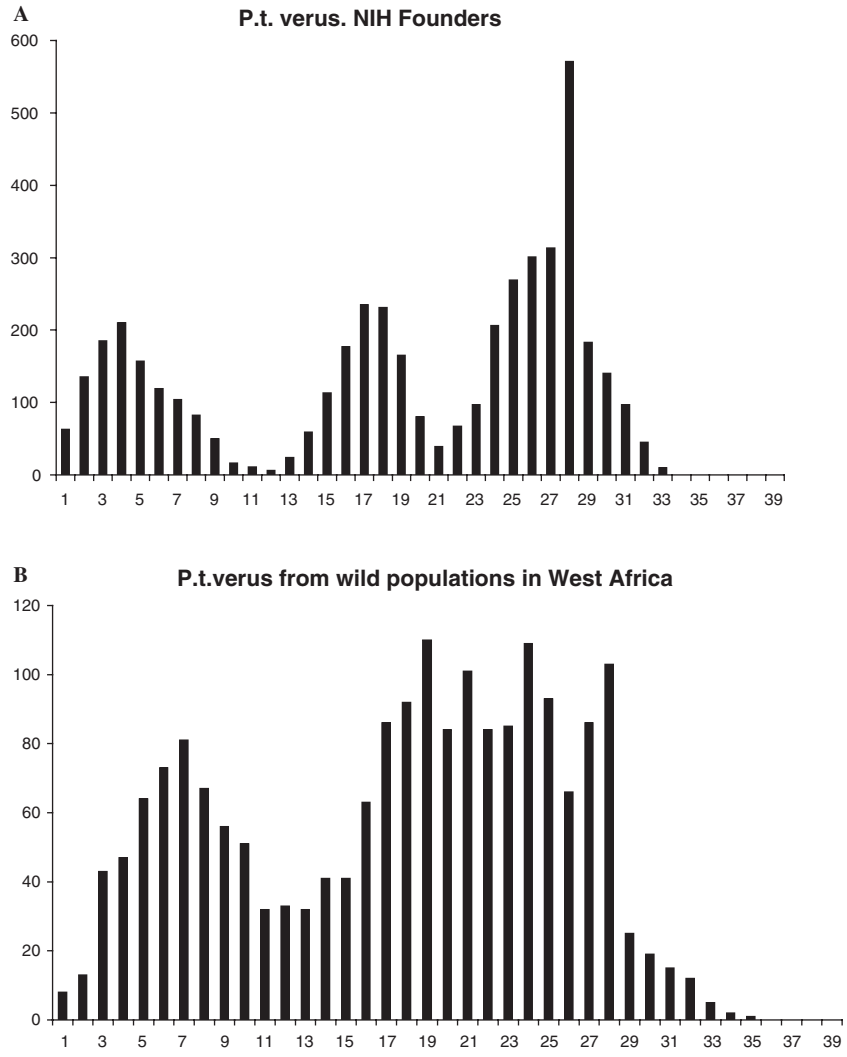

Fig. 2. Mean pairwise differences (mismatch distribution) in HVR-I. **A:** Captive NIH founder chimpanzees. **B:** African chimpanzees of known geographical provenance.

Nucleotide diversity, π, measures the average pairwise difference between two sequences [Nei, 1987]. West African chimpanzees typically have substantially lower genetic diversity than eastern/central African chimpanzees [Fischer et al., 2004; Yu et al., 2003]. Among the NIH founders, the *verus* sample had a nucleotide diversity, π_v, of 0.05882, and the combined *troglodytes*/*schweinfurthii* sample had π_{ts}=0.07013. By comparison, the sample of African HVR-I sequences used here had a π_v=0.05186 and π_{ts}=0.04821. The NIH founders (perhaps because they represent a broadly mixed distribution of haplotypes that do not naturally occur together in African chimpanzee populations) represented 113% (*verus*) to 145% (*troglodytes*/*schweinfurthii*) of the genetic diversity found among African chimpanzee populations, as measured by nucleotide diversity. The odd phylogenetic positions of #85 and #138 indicate that there are specimens with

HVR-I haplotypes in captivity that are as yet unknown in Africa. These haplotypes may still exist in Africa but have simply not yet been sampled in recent (1994 or later) collections. Alternately, given the historically recent decimation of African chimpanzee populations (a 50% decline from 1983–2000 [Walsh et al., 2003]) in the 45+ years since founders #85 and #138 were trapped, these lineages may have become extinct in Africa before they could be resampled.

Subspecies Effective Population Sizes

Previous estimates of N_e and reproductive variance among African-born chimpanzee founders were limited by unknown subspecies identities [Ely et al., 1991]. Here we analyzed 1,402 captive births, including 952 offspring that were still alive at the time of the 1995 moratorium, to estimate N_e (the effective population size) by sex and subspecies. With only one *vellerosus* founder and one male among the 10 *troglodytes*/*schweinfurthii* founders, only the *verus* subspecies had sufficient numbers of both sexes to allow numerical estimation of N_e. There were 94.1 variance- and 103.2 inbreeding-effective *verus* founder females, representing a reduction to 76–83% of the *verus* female census (n=124). There were 18.0 variance- and 37.6 inbreeding-effective *verus* male founders, corresponding to a reduction to 22–45% from the *verus* male census (n=83). Combining these sex-specific effective numbers yielded N_e size estimates (males and females combined) of $N_{e(V)}=60.5$ and $N_{e(I)}=110.2$. Both estimates represented very large reductions from the observed census size of 207 breeding-eligible *verus* adults (124 females and 83 males). This reduction was primarily due to large reproductive variance, especially among males. Although the *verus* subspecies as a whole reproduced well during the captive breeding phase of the former CBRP, reproduction among both sexes violated the Poisson expectations ($t_{Female}=5.0$, $P<0.001$; $t_{Male}=46.1$, $P<0.001$), with significantly greater reproductive variance among males ($F_{82,123}=9.38$, $P<0.001$). That is, breeding success was nonrandomly distributed in both sexes relative to Poisson expectations. The mean number of offspring per *verus* female was 3.20 (variance=5.24), compared to the mean number of offspring per male *verus* founder of 5.99 (variance=49.13). The offspring distributions of both sexes were highly overdispersed relative to Poisson expectations, with female reproductive variance 1.6 times its mean, and male variance more than 8 times its mean. Male *verus* reproduction ranged from zero (n=28 male founders, or 33.8%) to a high of 28 offspring sired by a single male. Female reproduction ranged from zero offspring (n=17 female founders, or 13.8%), to a high of 11 offspring born to a single female. By analogy to LD_{50} (the lethal drug dose for 50% of the individuals tested), we counted the number of male and female founders that were responsible for ~50% of the progeny. Among founders of known subspecies, only 14 males (all but one of which were *verus*; 15.3% of the total) sired 250 babies (47.8%), compared to 37 females (all but one *verus*; 27.8% of the total) that gave birth to 235 babies (54.8%). This comparison reveals how overrepresented the males are relative to the female founders: 2.6 times as many female founders were needed to produce half of all the offspring. Sex differences in reproductive variance among captive chimpanzees were noted previously [Ely et al., 1991]. Since female *verus* reproduction was also significantly overdispersed, the present sex differences were merely a matter of degree. Overdispersed reproduction among females was not an artifact of captivity, since the reproductive variance observed among NIH females was similar to that found among female chimpanzees at Mahale, Tanzania [Nishida et al., 2003].

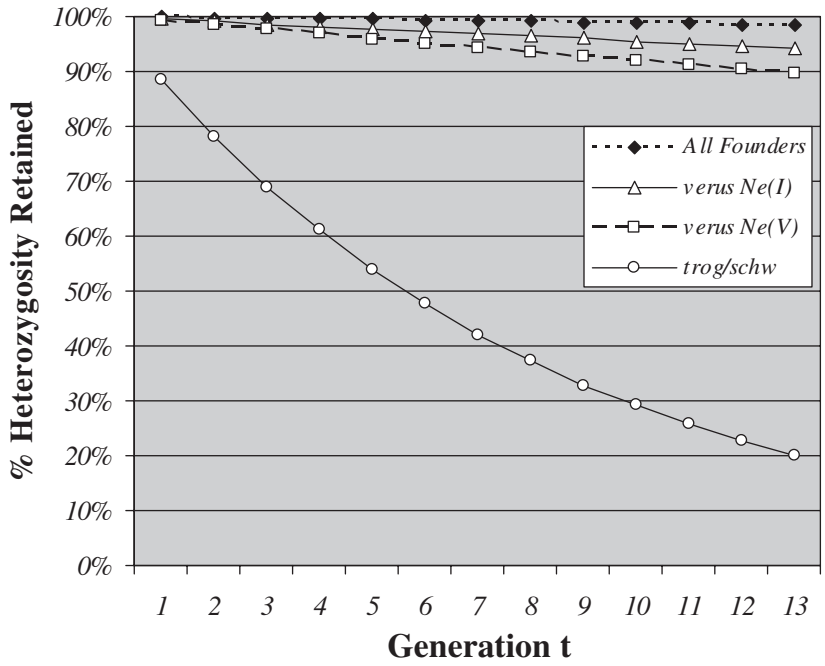

Fig. 3. Loss of heterozygosity over time (in 15-year generations) by subspecies.

Equivalent estimates of non-*verus* effective population sizes were determined only for females. The *troglodytes*/*schweinfurthii* clade had nine females but only one male founder, which mathematically precluded the estimation of male N_e. There were 3.2 variance and 4.3 inbreeding effective *troglodytes* females, with an average of 3.6 offspring and a variance of 16.5, again violating Poisson expectations ($t=7.7$, $P<0.001$]. With only one male and no female founders, it was impossible to estimate the N_es for *vellerosus*. The estimated values of N_e indicated that only the *verus* subspecies had a large enough population size to retain the desired 90+% of founder genetic variation during captivity [Ballou & Foose, 1996]. The small numbers of non-*verus* (*troglodytes*/*schweinfurthii* and *vellerosus*) founders, and their meager representation among captive-born descendants makes the long-term captive preservation of non-*verus* subspecies very unlikely (Fig. 3).

FE by Sex and Subspecies

The results of the pedigree analysis are first described for all 429 African-born founders, and then restricted to the 218 founders with subspecies ascertainments. Founder genetic representation in the descendant population showed considerable variation (Fig. 4). There were a total of 786.6 FEs, with an average of 1.83 FE per founder (range 0–14.25). Female founders had an average of 1.68 FEs (range=0–7.5), compared to the male average of 2.10 (range=0–14.25). Only 42 female (15.5%) and 26 male (16.5%) founders were represented in the descendant population at exactly the ideal replacement value of 1.0 FE per

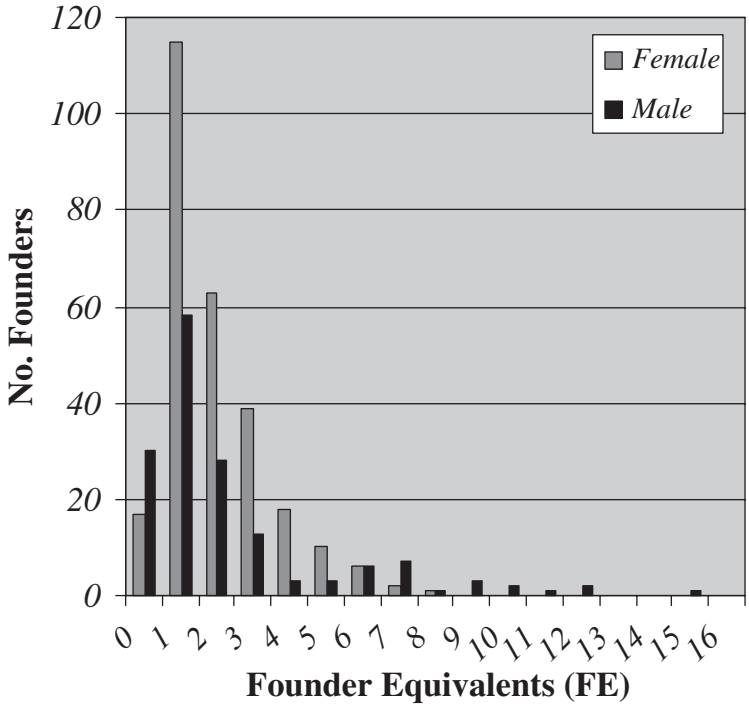

Fig. 4. Number of founder equivalents (FEs) by sex for all African-born founders.

founder. Genetically underrepresented (FE < 1.0) individuals included 90 female (33%) and 62 male (39%) founders, including 17 females (6.3%) and 30 males (19%) with no descendants at all (FE=0). Genetically overrepresented founders (FE > 1.0) included 139 females (51%) and 70 males (44%) .

The average FE was greater among males (2.10 $\pm$ 0.22) than females (1.68 $\pm$ 0.09), but varied among colonies. By colony, the average male FE varied fourfold, ranging from 1.30 to 2.07 in the four youngest colonies, with the highest value (FE=5.33) found at Yerkes, the oldest colony. The range for average female FE among colonies was much narrower: from 1.16 (Bastrop) to 2.04 (Yerkes). Both sexes had larger mean FEs at Yerkes, reflecting the deeper pedigrees in the oldest chimpanzee colony, as compared to the younger colonies with shallower pedigrees (i.e., Bastrop, NIRC, PFA, and TCF). Interestingly, the mean FE was about equal between the sexes everywhere except at Yerkes, where the average male FE (5.33) was 2.6 times larger than the average female FE (2.04). This sex difference found only at Yerkes, and other between-colony variations may reflect a historical preference for ''proven breeder'' males during breeding efforts, and other cumulative results of breeding practices and management decisions that differed among the colonies. Finally, by analogy to the LD_{50} used to measure chemical toxicity, we counted the number of founders of each sex that were responsible for 50% of the descendant FEs. A total of 59 females (21.8% of all female founders) were responsible for half the descendant FEs, compared to only 20 males (12.7% of all male founders). This comparison underscores the larger male reproductive variance relative to females, and further emphasizes how much of the genetic

variation originally present among the male founders was lost from the descendant generation.

Of 786.6 total FEs, 405.6 (51.6%) were attributable to 211 founders that were unavailable for sampling and therefore were of indeterminate subspecies. The number of FEs due to founders of indeterminate subspecies was proportional to their numbers: 49.2% of all founders were of undetermined subspecies, and they produced 51.6% of the FEs. The similarity of FE distributions between the founders of known subspecies and the wild/unknown founders further indicated that FE and breeding success were random with respect to our ability to ascertain the subspecies. Since the wild/unknown founders and their descendants were unsampled, and were thus genetically inscrutable, they were not further analyzed.

The remaining 381 FEs were attributed to the 218 founders of known subspecies. Founders of known subspecies represented 50.8% (218/429) of all African-born founders and were jointly responsible for 48.4% (381/786.6) of the FE represented in the descendant population. By subspecies, 95.1% (362.5/381) of the FE descended from the *verus* clade, 3.4% (13.0/381) descended from the *troglodytes*/*schweinfurthii* clade, and 1.4% (5.5/381) descended from the *vellerosus* clade (Table II). We estimated changes in the genetic representation of each subspecies over time by comparing the subspecies composition of founders with the total number of surviving offspring and the total FE in the descendant population (Table II). Overrepresentation of *P.t. verus* was virtually unchanged over time. The 207 *verus* founders (95.0% of all founders of known subspecies) bore 894 surviving offspring, representing 93.9% of the offspring and 95.1% of FE to founders of known subspecies (Table II). Representation of the *troglodytes*/*schweinfurthii* and *vellerosus* clades changed somewhat more noticeably. The 10 *troglogdytes*/*schweinfurthii* founders (4.6% of founders of known subspecies) were prolific breeders, and gave birth to 43 surviving offspring (4.3 offspring per founder). Nevertheless, the number of *troglodytes*/*schweinfurthii* offpring in the descendant population actually decreased slightly to 4.5%, while *troglodytes*/*schweinfurthii* representation fell to 3.4% of descendant FE (Table II). Finally, the single *vellerosus* founder sired 15 living offspring, which tripled *vellerosus* representation from 0.4% of founders to 1.4% of offspring and 1.4% of descendant FE. This *vellerosus* increase almost exactly balanced the *troglodytes*/*schweinfurthii* decrease. Overall, the initial bias toward *verus* founders changed very little after breeding, while the *troglodytes*/*schweinfurthii* representation decreased and *vellerosus* increased by about 1% each.

TABLE II. Subspecies Composition of Founders, Offspring and Founder Equivalents (FE)

Subspecies	All African-born founders		% All founders	% Founders of known subspecies	% Offspring of known subspecies	% FE of known subspecies
	n(male)	*n*(female)				
verus	83	124	47.5	95.0	93.9	95.1
troglodytes/ *schweinfurthii*	1	9	2.5	4.6	4.5	3.4
vellerosus	1	0	0.2	0.4	1.6	1.4
Wild/*unknown*	73	138	49.8	–	–	–
Total	158	271	100%	100%	100%	100%

Mortality Among Progeny of Between-Subspecies Mating

Matings between genetically different animals can result in outbreeding depression, characterized by elevated levels of infant morbidity and mortality [Schonewald-Cox, 1983]. We tested for outbreeding depression by comparing total mortality among progeny born to same- or between-subspecies matings. Same-subspecies matings included 1,323 *verus–verus* births (894 living offspring and 429 deaths) plus twin neonatal deaths from the single *troglodytes*/*schweinfurthii*–*troglodytes*/*schweinfurthii* mating. The 77 between-subspecies matings included 21 *vellerosus* crosses (six deaths and 15 living offspring) and 56 *troglodytes*/*schweinfurthii* crosses (13 deaths and 43 living offspring). Overall survival was 67.9% and (excluding the *troglodytes*/*schweinfurthii* twins) varied from 67.6% (*verus–verus* matings) to 76.8% (*troglodytes*/*schweinfurthii*–other). The outcomes did not differ between same-subspecies to between-subspecies matings ($X^2_{(1)}=2.059$, $P=0.151$, $\alpha=0.679$; Fisher's exact test, $P=0.168$). A more detailed analysis of these data is needed. However, contrary to earlier speculation, overall mortality was not elevated by between-subspecies outbreeding depression [Morin et al., 1994; Gagneux et al., 1999]. In fact, the trend was exactly opposite to that speculated, since same-subspecies matings had higher overall mortality (33%) compared to the presumably problematic between-subspecies matings (25%).

DISCUSSION

This is the first large-scale study of the subspecies composition of chimpanzee founders using objective genetic criterion. Earlier estimates of subspecies composition were grossly inaccurate. Reynolds and Luscombe [1971], using Hill's [1969] morphological criteria, found that the Holloman AFB population was composed of 56.7% *verus*, 41.8% *troglodytes*/*schweinfurthii*, and 1.4% *koolakamba*. Teleki [1989] argued that historical patterns of the chimpanzee trade resulted in a domestic chimpanzee population composed predominantly of west African *P.t. verus* from Guinea, Liberia, and Sierra Leone, but presented no data. ISIS listed a total of 7,473 chimpanzees, including 1,259 African-born, 4,359 captive-born, and 1,855 of unknown origin. Of the 1,259 African-borns, 780 (62%) had no country of origin; the remaining 479 were listed as Sierra Leone (252), Cameroon (39), Guinea (24), Liberia (21), Congo (7), Ivory Coast (6), Senegal (3), west Africa (25), Africa (52), and the South Africa/Africa region (50). While these figures yield a broadly correct founder composition of 87.8% *verus* and 12.2% non-*verus*, importation records often recorded inaccurate geographical origins that were uncorroborated by molecular or other data. Corroboration of inferred subspecies composition was demonstrated by closely similar mtDNA results from zoological garden chimpanzees indicating that eight of nine African founders (88.9%) were *P.t. verus*, which does not differ from the NIH composition (Fisher's exact test, $P=0.42$).

Our results represent an important advance in knowledge of the subspecies composition of captive chimpanzees, which is specifically geared toward management concerns. A pronounced bias favored the *P.t. verus* subspecies (95.0%), which was followed distantly by the *P.t. troglodytes*/*schweinfurthii* clade (4.6%) and *P.t. vellerosus* (0.4%). The founder population contained a large proportion of African mtDNA variation and by some measures actually exceeded it. The pedigree analysis revealed that the bias toward *P.t. verus* founders persisted among captive-born descendants, with only minor changes in representation of the non-*verus* subspecies. Genetic management reduced some genetic inequalities among founders during the captive breeding phase. However, subspecies could

not be accurately ascertained prior to Morin et al.'s [1994] seminal study, and there was no opportunity to rectify the skewed composition after the 1995 breeding moratorium. Only the *verus* subspecies and *troglodytes*/*schweinfurthii* females had large enough census data to allow estimation of N_es, and both were reduced to 40–50% of the adult census. Since 1995, many underrepresented founders (including four of the 12 non-*verus* founders) have died or have been permanently transferred into privately owned retirement sanctuaries or vasectomized. These are permanent genetic losses that will hinder any future attempts at breeding, conservation, or phylogenetic population management.

Unrecognized genetic differences among chimpanzee subspecies could impact research and management practices. In terms of management, matings between genetically different animals can result in outbreeding depression, which is characterized by elevated levels of infant morbidity and mortality [Schonewald-Cox, 1983]. It has been suggested that *verus* and non-*verus* subspecies are so genetically different that separate captive maintenance is required to avoid outbreeding depression [Deinard & Kidd, 2000; Morin et al., 1994]. In contrast, lack of demographic evidence for outbreeding depression indicated that subspecies identities were of no concern [Commission on Life Sciences of the National Research Council, 1997]. Until now, key data were lacking on both sides of this debate. Having resolved subspecies identities, we found no evidence that hypothetical genetic incompatibilities [Morin et al., 1994; Gagneux et al., 1999] led to higher mortality in between-subspecies matings. However, subspecies genetic variation could still influence research results. The combination of long subspecies divergence times [Gagneux et al., 1999; Gonder, 2000; Morin et al., 1994], differences in endogenous viral pathogens [Hu et al., 2001; Switzer et al., 2004], variation in mitochondrial and nuclear genes (including the MHC) [de Groot et al., 2002; Wise et al., 1997], noncoding sequence variation [Fischer et al., 2004; Kaessmann et al., 1999], and genetic variation in other primates [Champoux et al., 1996; Joag et al., 1994; Williams-Blangero et al., 1990] suggest that chimpanzee subspecies are likely to differ genetically in clinically important ways. Given a selection coefficient of 0.16 [Hoekstra et al., 2001] and N_e=55,000 [Stone et al., 2002], an advantageous dominant mutation could be driven to fixation in less time than it took for *schweinfurthii*/*troglodytes* to expand into east Africa 7,328 generations ago. Human populations separated less than 171,000 years ago (or 8,575 years, for 20-year generations), which makes them only 20% as old as the 894,000-year-old chimpanzee subspecies [Ingman et al., 2000; Gonder, 2000]. Yet human populations differ at loci that influence susceptibility and disease progression for AIDS, hepatitis, and cardiovascular heart disease [Berger et al., 1999; Powell et al., 2000; Stengard et al., 2002]. Viewed comparatively, it would be surprising if chimpanzee subspecies did not exhibit similar genetic variation.

One disagreement over the potential impact of subspecies genetic divergence may arise from the notion that neutral genetic variation, which is so informative in evolutionary studies, has no direct relevance to clinical medicine [Commission on Life Sciences of the National Research Council, 1997; Deinard & Kidd, 2000; Morin et al., 1994]. The relationship of mitochondrial to nuclear genetic diversity varies considerably among species, without demarcating consistent nuclear genetic differences [Avise, 1994; Moore, 1995]. Given the limited space in zoological gardens and sanctuaries, informed judgment regarding the significance of chimpanzee subspecies will ultimately impact chimpanzee conservation efforts [Barrowclough & Flesness, 1996]. Viewing subspecies as potential reservoirs of nuclear genetic diversity can allow the identification and preservation of rare

genetic variants without requiring separate management. The fact that African chimpanzee populations are critically endangered [Walsh et al., 2003] makes phylogenetic guidance for captive management and propagation even more important for chimpanzee conservation.

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