

# Identification of chimpanzee subspecies with DNA from hair and allele-specific probes

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## SUMMARY

We describe a non-invasive method of determining the subspecies identity of common chimpanzees (*Pan troglodytes*), based on subspecies-specific sequence differences in the mitochondrial genome. This procedure involves the extraction of DNA from hair, the amplification of a short (410 base pair (b.p.)) segment of the non-coding displacement loop (D-loop) by the polymerase chain reaction (PCR), and subspecies identification based on rapid allele-specific oligonucleotide (ASO) probe dot-blot typing. This approach will contribute to: (i) the colony-level management of captive chimpanzees by enabling managers to recognize hybrids between subspecies and minimize outbreeding depression; (ii) the recognition of inappropriately matched individuals in comparative behavioural and experimental studies; and (iii) forensic questions surrounding the origin of illegally traded animals.

## 1. INTRODUCTION

The maintenance of genetic diversity remains the most fundamental issue in population genetics (Clarke 1979) and has a direct bearing on conservation biology as variability determines a population's ability to evolve (Woodruff 1989*a*). The definition of evolutionarily significant units for *in situ* and *ex situ* conservation management presupposes the availability of information on natural spatial patterns of genetic variation. Even for such well-known species as the common chimpanzee, *Pan troglodytes*, such data are simply unavailable and, despite the long-standing recognition of four subspecies spanning a 5000 km range, >90% of 2200 individuals tracked by the International Species Inventory System are managed as a single 'generic' taxon. Such lumping of often very distantly related individuals is common in captive populations where the geographic provenance of the founders is generally unknown and has resulted in some lamentable mismanagement of orangutans, spider monkeys, ibex and largemouth bass, among others (Woodruff 1989*a*, 1990). In this paper we show how this problem can be overcome in the case of the common chimpanzee: based on a study of geographic variation at a few loci across Africa, it is now possible to trace an individual chimpanzee's genetic roots and incorporate such information in Species Survival Plans (American Association of Zoological Parks and Aquariums) and Global Captive Breeding Plans (Captive Breeding Specialist Group/IUCN). Our approach has considerable generality and can be developed for application to the management of other threatened or biomedically significant species.

Four subspecies of *Pan troglodytes* are currently recognized on morphological and geographical criteria (Hill 1969): West African masked *verus*, Central African black-faced *troglodytes*, East African long-haired *schweinfurthii*, and little-known gorilla-like *koolo-kamba* from Congo. Morphology and our own genetic studies of the first three subspecies suggest that endangered *verus* is the most divergent, but the interrelationships of the subspecies have yet to be established genetically (Seal & Flessness 1986; Groves 1989; Groves *et al.* 1992), and the taxonomy is overdue for reassessment.

The recognition of inter-subspecific hybrids in inadvertently mixed colonies is important because outbreeding depression can be as costly to colony health as inbreeding depression (Templeton 1986; Templeton *et al.* 1986; Lynch 1991). Hybrid individuals may possess aberrant genetic systems of types that have hitherto been ignored by researchers selecting individuals for comparative studies (Woodruff 1989*b*). Hybridization may be a factor contributing to spontaneous abortion and neonate mortality rates which are surprisingly high (>20%) in captive colonies of chimpanzees (Flessness 1986; Seal & Flessness 1986). Obviously, it would be prudent not to interbreed dissimilar groups of apes (different subspecies, for example) unless it is clear that their genetic differences are inconsequential. In the case of chimpanzees, it is not possible to assess quantitatively the relative fitness of inter-subspecific hybrids, as colony inventories rarely record subspecific designations or even the country of origin of founders (Seal & Flessness 1986). Over 2000 of the 2178 *Pan troglodytes* in the International Species Inventory System (ISIS) registry have not been identified to the level of subspecies (N. Flessness, personal communication). To resolve questions sur-

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rounding the identity of some of these apes and facilitate a reassessment of the desirability of managing them as a single generic taxon, as they are in most North American colonies, we have studied nucleotide sequence variation at specific mitochondrial (mtDNA) and nuclear (nDNA) loci in African animals. Our survey of geographic variation in a 340 base pair (b.p.) sequence of the mitochondrial cytochrome *b* gene and a 410 b.p. sequence of the D-loop revealed the subspecies-specific patterns upon which this contribution is based (Morin *et al.* 1992).

## 2. MATERIALS AND METHODS

Hair samples of chimpanzees were collected by field researchers across Africa, mailed or hand carried to our laboratory under ambient conditions, then frozen at  $-80^{\circ}\text{C}$  until DNA was extracted (see Appendix). DNA extraction and amplification procedures have been published elsewhere (Morin & Woodruff 1992, 1993). The section of the mitochondrial D-loop amplified was at the 5' end and corresponded to bases 16019 to 16428 of the human mitochondrial sequence (Anderson *et al.* 1981). The primers used were *D-88* (5'-CTCTGTTCTTTCATGGGAAGC-3') and *D-441* (5'-CGGGATATTGATTTCACGGAGG-3'). Each polymerase chain reaction (PCR) product was added to 210  $\mu\text{l}$  of 0.4 N NaOH, 10 mM EDTA, and heated to  $85^{\circ}\text{C}$  for 5 min, then loaded in triplicate, 100  $\mu\text{l}$  each, such that the blot could be cut into three identical pieces. Probes were synthesized (Operon Technologies, Inc.) to be specific to regions of the D-loop differing by one to three nucleotides between the subspecies. Because of the high levels of variation in the chimpanzee D-loop (Morin *et al.* 1992), probes for the eastern (*P. t. schweinfurthii*) and central (*P. t. troglodytes*) subspecies were made degenerate at two sites each. Probes were endlabeled with either  $\gamma\text{-ATP}^{32}$  or digoxigenin-labelled dideoxyuridine-triphosphate (DIG-ddUTP; Genius<sup>TM</sup> 5 kit, Boehringer Mannheim Corp.), and radioactively labelled probes purified of unincorporated nucleotides using NucTrap push columns (Stratagene). Each replicate blot was pre-hybridized for 2 h at  $42^{\circ}\text{C}$  in 1 M NaCl, 1% SDS, in a volume of about 1 ml  $\text{cm}^{-2}$ . Approximately 10  $\mu\text{g ml}^{-1}$  labelled oligonucleotide probe was added to each blot, and hybridization allowed to occur for 3 h at  $40^{\circ}\text{C}$ . Blots were combined and washed twice for 15 min each at room temperature in  $2\times\text{SSPE}$ , 0.1% SDS, once for 10 min at  $52^{\circ}\text{C}$  in  $2\times\text{SSPE}$ , 0.1% SDS, and rinsed with room temperature  $2\times\text{SSPE}$  twice. Blots labelled with  $^{32}\text{P}$  were wrapped in plastic and exposed to autoradiography film for 4–16 h. Blots labelled with DIG-ddUTP were activated according to the Genius 5 kit manufacturer's instructions and exposed to autoradiography film for 2–15 min.

## 3. RESULTS

One non-degenerate and two degenerate probes were designed based on D-loop sequences of 60 chimpanzees from 14 localities across Africa, representing at least seven distinct populations of *P. t. schweinfurthii*, four of *P. t. troglodytes*, and two of *P. t. verus* (Morin *et al.* 1992). Six additional sequences of chimpanzees of unknown provenance (three from Kocher & Wilson (1991) and three from P. A. Morin, unpublished results) were also included to increase the probability of obtaining all variation at these sites. Although individual nucleotide variation is high in the

D-loop segment we sequenced, the regions chosen for probe hybridization had limited sequence polymorphism within each subspecies (figure 1). Our sample size for each subspecies ranged from 13 to 35, including six samples probed but not sequenced; we may not have detected all the variants at these loci, but, assuming random sampling, we can be 95% confident, based on a binomial distribution, that any allele with a frequency of greater than 0.08, 0.19 and 0.21 has been detected in the subspecies, *P. t. schweinfurthii*, *P. t. troglodytes* and *P. t. verus*, respectively. Because the probe for *P. t. verus* differs by at least two nucleotides from each individual of the other subspecies, the probability of exclusion of those subspecies by this method is much greater than suggested by simple allele frequency-based statistical probability. Also, the wide geographic distribution of populations sampled within the eastern and central subspecies, and successful application of probes to captive *P. t. verus* individuals of unknown and probably diverse origins (described below), suggests that only rarer alleles will interfere with the efficiency of this first-order attempt at subspecies genetic identification.

PTS-2	C	G	T	A	C	A	C/T	C	A	T	A	C	A	T	A/G	G	C
1	.	.	.	.	.	.	T	.	.	.	.	.	.	.	A	.	.
2	.	.	.	.	.	.	T	.	.	.	.	.	.	.	A	.	.
3	.	.	.	.	.	.	C	.	.	.	.	.	.	.	A	.	.
4	.	.	.	.	.	.	C	.	.	.	.	.	.	.	G	.	.
5	.	.	.	.	.	.	C	.	.	.	.	.	.	.	G	.	.
6	.	.	C	.	.	.	C	.	G	.	.	.	.	.	A	.	.
7	.	.	C	.	.	.	C	.	G	.	.	.	.	.	A	.	.
8	.	.	.	.	.	.	T	.	G	C	.	.	.	.	A	.	.
9	.	.	C	.	.	.	C	.	G	.	.	.	.	.	A	.	.
10	.	.	.	.	.	.	T	.	G	C	.	.	.	.	A	.	.
11	.	A	C	.	.	.	C	.	G	.	.	.	.	.	A	.	.
12	.	A	.	.	.	.	C	.	G	.	.	.	.	.	A	.	.
13	.	A	.	.	.	.	C	.	G	.	.	.	.	.	A	.	.
14	.	A	.	.	.	.	C	.	G	.	.	.	.	.	A	.	.
588	T	A	C	.	.	.	C	.	G	.	.	.	.	.	A	.	.

PTT-2	C	A	T	A	A	A/G	A	C/T	G	C	A	A	C	T	C	C	A	A	
1	.	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.
2	.	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.
3	.	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.
4	.	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.
5	.	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.
6	.	.	.	.	.	G	.	C	.	.	.	.	.	.	.	.	.	.	.
7	.	.	.	.	.	A	.	C	.	.	.	.	.	.	.	.	.	.	.
8	.	.	.	.	.	A	.	T	.	.	.	.	.	.	.	.	.	.	.
9	.	.	.	.	.	A	.	T	.	.	.	.	.	.	.	.	.	.	.
10	.	.	.	.	.	A	.	T	.	.	.	.	.	.	.	.	.	.	.
11	.	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.
12	.	.	.	.	.	G	.	C	A	.	.	.	.	.	.	.	.	.	.
13	.	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.
14	.	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.
588	.	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.

PTV-1	A	C	A	T	A	C	A	A	T	C	A	T	A	C	A	C	C	G
1	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	A
2	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	A
3	.	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	A
4	.	.	.	.	.	T	.	.	C	G	.	.	.	.	.	.	.	A
5	.	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	A
6	.	.	.	.	.	.	.	.	C	G	C	.	.	.	.	.	.	A
7	.	.	.	.	.	.	.	.	C	G	C	.	.	.	.	.	.	A
8	.	.	.	.	.	.	.	.	C	G	.	.	.	T	.	.	.	.
9	.	.	.	.	.	.	.	.	C	G	C	.	.	.	.	.	.	A
10	.	.	.	.	.	.	.	.	C	G	.	.	.	T	.	.	.	.
11	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.
12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
588	.	.	.	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.

Figure 1. DNA sequences of 15 individual *Pan troglodytes* selected to represent all variation found in 66 animals examined at each probe locus (see Appendix). Individuals and subspecies identified by each probe are shaded: *PTV*, *P. t. verus*; *PTT*, *P. t. troglodytes*; *PTS*, *P. t. schweinfurthii*. Specific identities of individual chimpanzees (1–14, 588) are given in the Appendix.

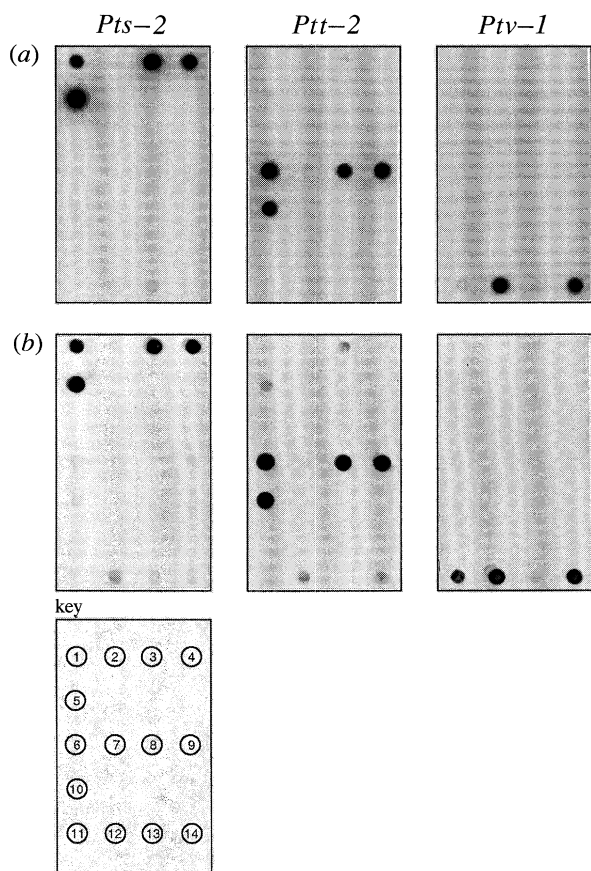


Figure 2. Dot-blot of amplified chimpanzee mtDNA (as in figure 1) representing all three chimpanzee subspecies, and probed with the subspecies-specific probes *Pts-2*, *Ptt-2* and *Ptv-1*. (a) Probes labelled with  $\gamma$ -ATP<sup>32</sup>, and (b) labelled with DIG-ddUTP and treated for fluorescence by Genius 5 kit protocols.

With degenerate probes and side-by-side comparisons of identical blots hybridized with the three different probes, it was possible to unambiguously identify the three well-known subspecies of chimpanzees in all cases but one (discussed below) (figure 2). Hair samples freshly plucked from captive animals and stored dry at room temperature or in an ultracold freezer all provided enough DNA for 25–30 separate amplifications. Hair collected at field sites across Africa, some from nests several weeks old, and stored in ambient conditions for months also usually provided adequate DNA for multiple amplifications (Morin & Woodruff 1992). Amplification products loaded on an agarose gel and stained with ethidium bromide (figure 3) were usually clearly visible, but even some that were so faint as to be undetectable using this standard assay method yielded sufficient product for dot-blot typing with longer exposure times (e.g. samples 2, 7 and 13 in figures 2–3). Although the allele-specific oligonucleotide (ASO) probe *Ptt-2* sequence differed by only one nucleotide from the most common sequence in the eastern subspecies (*P. t. schweinfurthii*) (figure 1), sufficiently stringent post-hybridization washes allowed unambiguous discrimination of these taxa.

The first test of these probes on animals of unknown origin was done on seven animals (five known to be wild born) in the North Carolina Zoological Park

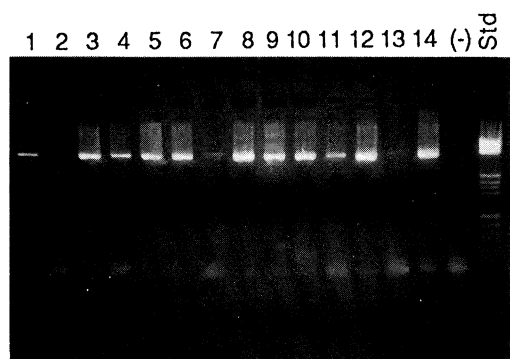


Figure 3. Ethidium bromide stained 3% Nusieve / 1% agarose minigel showing mtDNA D-loop PCR products. Standard (Std) = pBR322/Hae-III; lanes 1–14 are as in figure 1; lane 15 is a negative control.

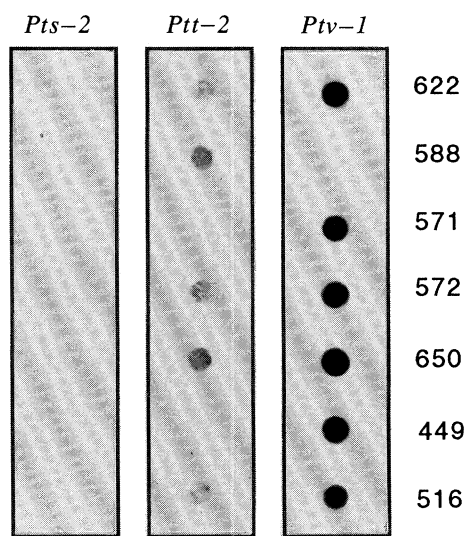


Figure 4. Dot blot of amplified DNA from the North Carolina Zoological Park chimpanzee colony; individuals identified by NARS numbers. Three subspecies probes as in figure 2.

colony. Results showed unambiguously that six of the seven chimpanzees were of the western subspecies (*P. t. verus*). The remaining animal, North American Regional Studbook (NARS) No. 588, was not identified by the *Ptv-1* probe, but neither did it hybridize strongly with the other probes. Subsequent sequencing of the sample revealed a single base change which prevented hybridization of the *Ptv-1* probe, suggesting polymorphism within *P. t. verus* which could be taken into account by designing a new probe with degenerate sites.

#### 4. DISCUSSION

Although aso probes have found widespread use in studies of human population genetic structure, the detection of genetic diseases (see, for example, Saiki *et al.* 1986; Bugawan *et al.* 1988; Kazazian 1989) and for forensic identification in paternity suits and criminal proceedings (von Beroldingen *et al.* 1989), this is their first application to the problems of genetic management of threatened and endangered species or

captive colonies of biomedically significant non-human primates. This first step will allow subspecific identification of wild-born individuals, maternal lineages, and captive-born individuals when the father's subspecies designation can be determined (Morin & Woodruff 1992). The next step will be the development of ASO probes for nuclear loci for the identification of hybrids where the father is not known.

Given the small sample size ( $n = 66$ ) used for development of the probes, it is possible that some rare alleles have been missed and some individuals may be unclassifiable or even misclassified. For example, subsequent to designing and testing the probes on available samples, we discovered another variant at position 16320 in a published sequence (Kocher & Wilson 1991) which may interfere with the *Ptv-1* probe hybridization. As unclassifiable individuals are discovered, the probes will have to be modified to accommodate additional within-subspecies sequence polymorphism.

The development of ASO probes obviates the need to repeatedly sequence the diagnostic loci. Furthermore, the adoption of a non-isotopic ASO detection system is a safer, more cost-effective, and more sensitive way of visualising hybridized probes on a dot-blot (figure 2) than traditional methods based on radioactive labels (Bugawan *et al.* 1988). Of the available methods, we chose the Genius 5 kit with luminescent detection because the probes did not have to be synthesized with biotin tags, they can be used for multiple hybridizations, and because of the sensitivity and ease of creating permanent records with autoradiography film. The use of such non-radioactive methods will make it easier for laboratories associated with zoos or primate colonies to sort animals.

It now becomes possible to sort captive chimpanzees into their three well-established subspecies and reassess the significance of these taxa as evolutionarily significant units. That such reassessment may be necessary is shown by recent findings that *Pan troglodytes*, as a taxon, has more genetic variability than all other primate species studied, including man (Kocher & Wilson 1991; Ruano *et al.* 1992; M. Ruvolo, personal communication). Such genetic characterizations will facilitate retrospective analyses of colony birth and fitness records for evidence of outbreeding depression and other effects of hybridization. It will allow the establishment of genetically defined maternal lines in some closed colonies (Morin & Ryder 1991) and enable researchers to select appropriately matched individuals for comparative studies. Additional studies of D-loop variation (and other variable mtDNA and nDNA sequences) may provide more information about an individual's geographic place of origin and allow forensic identification of illegally traded chimpanzees. That such genotyping can be based on non-invasive DNA sampling will greatly facilitate field studies of population structure and sociobiology (Woodruff 1992*a, b*).

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#### APPENDIX. Chimpanzee sample list

sample number	sample identification/subspecies	country of origin	collected by
1	Gigi / <i>P.t.s.</i>	Tanzania (Gombe)	Phil Morin
2	Skosha/ <i>P.t.s.</i>	Tanzania (Gombe)	Jim Moore
3	Pts6/ <i>P.t.s.</i>	Tanzania (Tongwe)	Jim Moore
4	Judy/ <i>P.t.s.</i>	Gabon/Zaire	Jean Wickings
5	Mgbadolite/ <i>P.t.s.</i>	Zaire	Jean Wickings
6	Ptt59/ <i>P.t.t.</i>	Gabon (Estuaire)	Jean Wickings
7	Ponia/ <i>P.t.t.</i>	Gabon (Ogooue-Maritime)	Jean Wickings
8	Ptt36/ <i>P.t.t.</i>	Congo	Jim Moore
9	Ptt37/ <i>P.t.t.</i>	Congo	Jim Moore
10	Bertha/ <i>P.t.t.</i>	Cameroon	Jean Wickings
11	Ptv4/ <i>P.t.v.</i>	Ivory Coast (Tai forest)	C. & H. Boesch
12	Ptv5/ <i>P.t.v.</i>	Ivory Coast (Tai forest)	C. & H. Boesch
13	Ptv15/ <i>P.t.v.</i>	Ivory Coast (Tai forest)	C. & H. Boesch
14	Edgar/ <i>P.t.v.</i>	Gabon/unknown	Jean Wickings
588	NARS 588/ <i>P.t.v.</i>	Unknown/North Carolina Zoological Park	Randy Fulk

These samples represent all sequence variations found in 63 sequences produced in our lab. We choose two African individuals, Judy and Edgar, despite uncertain origins, to test the effectiveness of the assay on all available sequence variants. Samples from Gabon are from the Centre International de Recherches Médicales de Franceville Primate colony, where most individuals are identified by the village where they were caught. Several animals at CIRMF are from other countries, and the sample labelled Judy (number 4) is probably from Zaire, although she was bought in a market in Port Gentile, Gabon (S. Evans, personal communication); the DNA sequence from this individual most closely matches that of Mgbadolite, known to be from north-central Zaire, and is quite different from other Gabonese sequences. Edgar (number 14) is designated as *P. t. verus* by CIRMF and has a D-loop sequence very similar to *P. t. verus* of known provenance.

PTS-2	C	G	T	A	C	A	C/T	C	A	T	A	C	A	T	A/G	G	C
1	.	.	.	.	.	.	T	.	.	.	.	.	.	.	A	.	.
2	.	.	.	.	.	.	T	.	.	.	.	.	.	.	A	.	.
3	.	.	.	.	.	.	C	.	.	.	.	.	.	.	A	.	.
4	.	.	.	.	.	.	C	.	.	.	.	.	.	.	G	.	.
5	.	.	.	.	.	.	C	.	.	.	.	.	.	.	G	.	.
6	.	.	C	.	.	.	C	.	.	G	.	.	.	.	A	.	.
7	.	.	C	.	.	.	C	.	.	G	.	.	.	.	G	.	.
8	.	.	.	.	.	.	T	.	G	C	.	.	.	.	A	.	.
9	.	.	C	.	.	.	C	.	.	G	.	.	.	.	G	.	.
10	.	.	.	.	.	.	T	.	G	C	.	.	.	.	A	.	.
11	.	A	C	.	.	.	C	.	G	.	.	.	.	.	A	.	.
12	.	A	.	.	.	.	C	.	G	.	.	.	.	.	A	.	.
13	.	A	.	.	.	.	C	.	G	.	.	.	.	.	A	.	.
14	.	A	.	.	.	.	C	.	G	.	.	.	.	.	A	.	.
588	T	A	C	.	.	.	C	.	G	.	.	.	.	.	A	.	.

PTT-2	C	A	T	A	A	A/G	A	C/T	G	C	A	A	C	T	C	C	A	A	
1	.	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.
2	.	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.
3	.	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.
4	.	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.
5	.	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.
6	.	.	.	.	.	G	.	C	.	.	.	.	.	.	.	.	.	.	.
7	.	.	.	.	.	A	.	C	.	.	.	.	.	.	.	.	.	.	.
8	.	.	.	.	.	A	.	T	.	.	.	.	.	.	.	.	.	.	.
9	.	.	.	.	.	A	.	T	.	.	.	.	.	.	.	.	.	.	.
10	.	.	.	.	.	A	.	T	.	.	.	.	.	.	.	.	.	.	.
11	.	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.
12	.	.	.	.	.	G	.	C	A	.	.	.	.	.	.	.	.	.	.
13	.	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.
14	.	.	.	.	.	C	.	C	A	.	.	.	.	.	.	.	.	.	.
588	.	.	.	.	.	A	.	C	A	.	.	.	.	.	.	.	.	.	.

PTV-1	A	C	A	T	A	C	A	A	T	C	A	T	A	C	A	C	C	G
1	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	A
2	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	A
3	.	.	.	.	.	.	.	.	C	.	G	.	.	.	.	.	.	A
4	.	.	.	.	.	T	.	.	C	.	G	.	.	.	.	.	.	A
5	.	.	.	.	.	.	.	.	C	.	G	.	.	.	.	.	.	A
6	.	.	.	.	.	.	.	.	C	.	G	C	.	.	.	.	.	A
7	.	.	.	.	.	.	.	.	C	.	G	C	.	.	.	.	.	A
8	.	.	.	.	.	.	.	.	C	.	G	.	.	.	.	T	.	.
9	.	.	.	.	.	.	.	.	C	.	G	C	.	.	.	.	.	A
10	.	.	.	.	.	.	.	.	C	.	G	.	.	.	.	T	.	.
11	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
588	.	.	.	.	.	.	.	.	T	.	C	.	.	.	.	.	.	.

Figure 1. DNA sequences of 15 individual *Pan troglodytes* selected to represent all variation found in 66 animals examined at each probe locus (see Appendix). Individuals and subspecies identified by each probe are shaded: *PTV*, *P. t. verus*; *PTT*, *P. t. troglodytes*; *PTS*, *P. t. schweinfurthii*. Specific identities of individual chimpanzees (1–14, 588) are given in the Appendix.

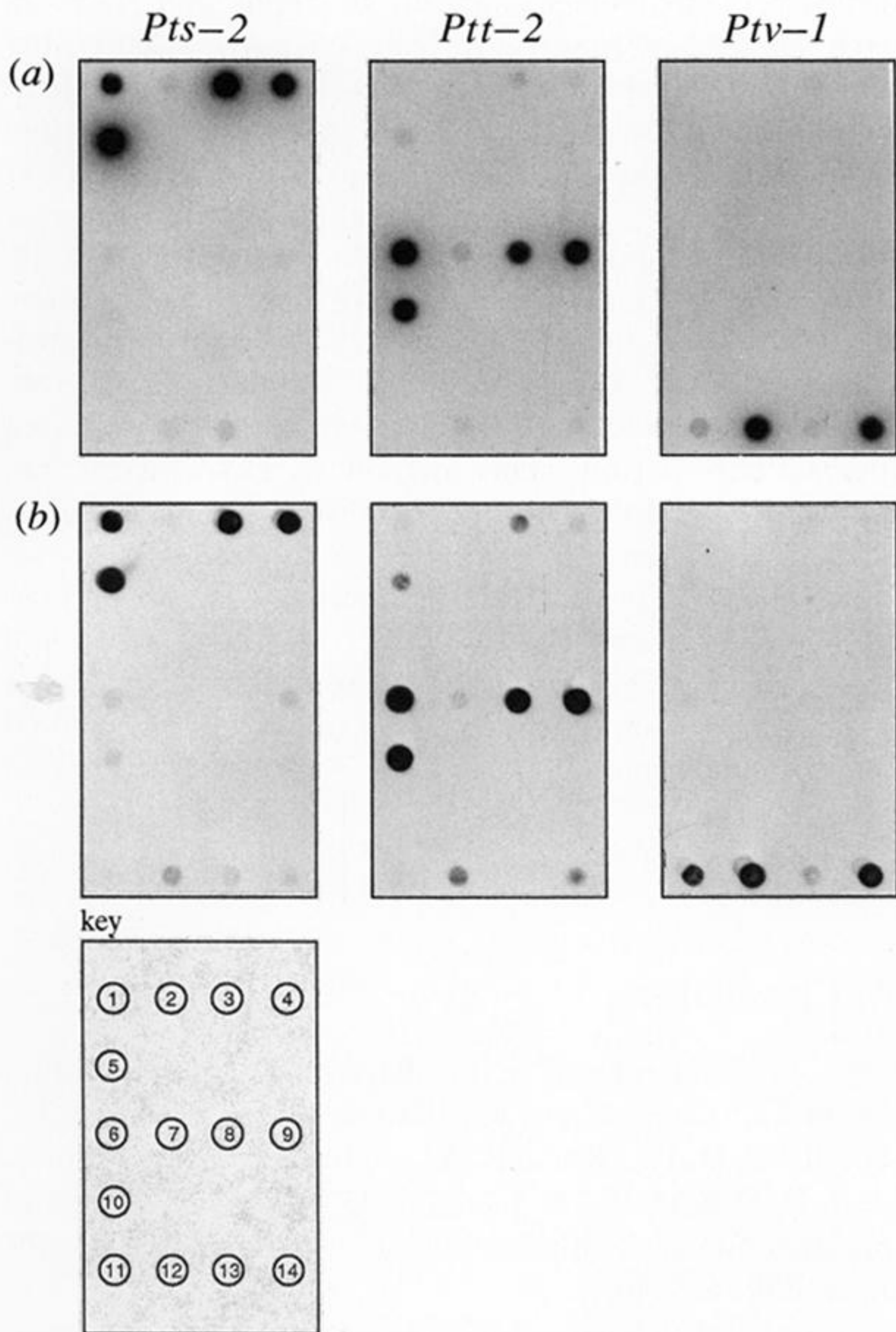


Figure 2. Dot-blot of amplified chimpanzee mtDNA (as in figure 1) representing all three chimpanzee subspecies, and probed with the subspecies-specific probes *Pts-2*, *Ptt-2* and *Ptv-1*. (a) Probes labelled with  $\gamma$ -ATP<sup>32</sup>, and (b) labelled with DIG-ddUTP and treated for fluorescence by Genius 5 kit protocols.

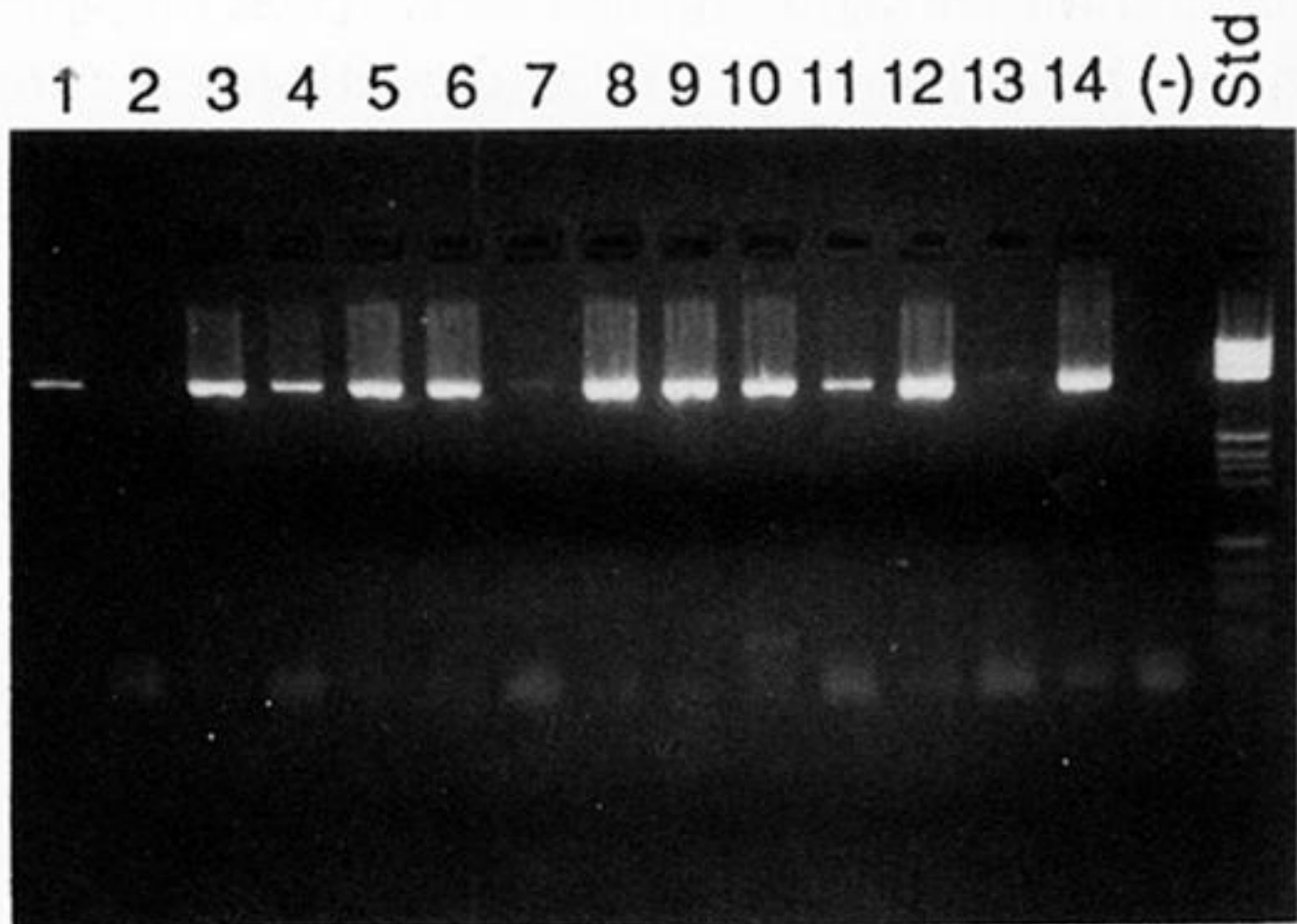


Figure 3. Ethidium bromide stained 3% Nusieve / 1% agarose minigel showing mtDNA D-loop PCR products. Standard (Std) = pBR322/Hae-III; lanes 1-14 are as in figure 1; lane 15 is a negative control.



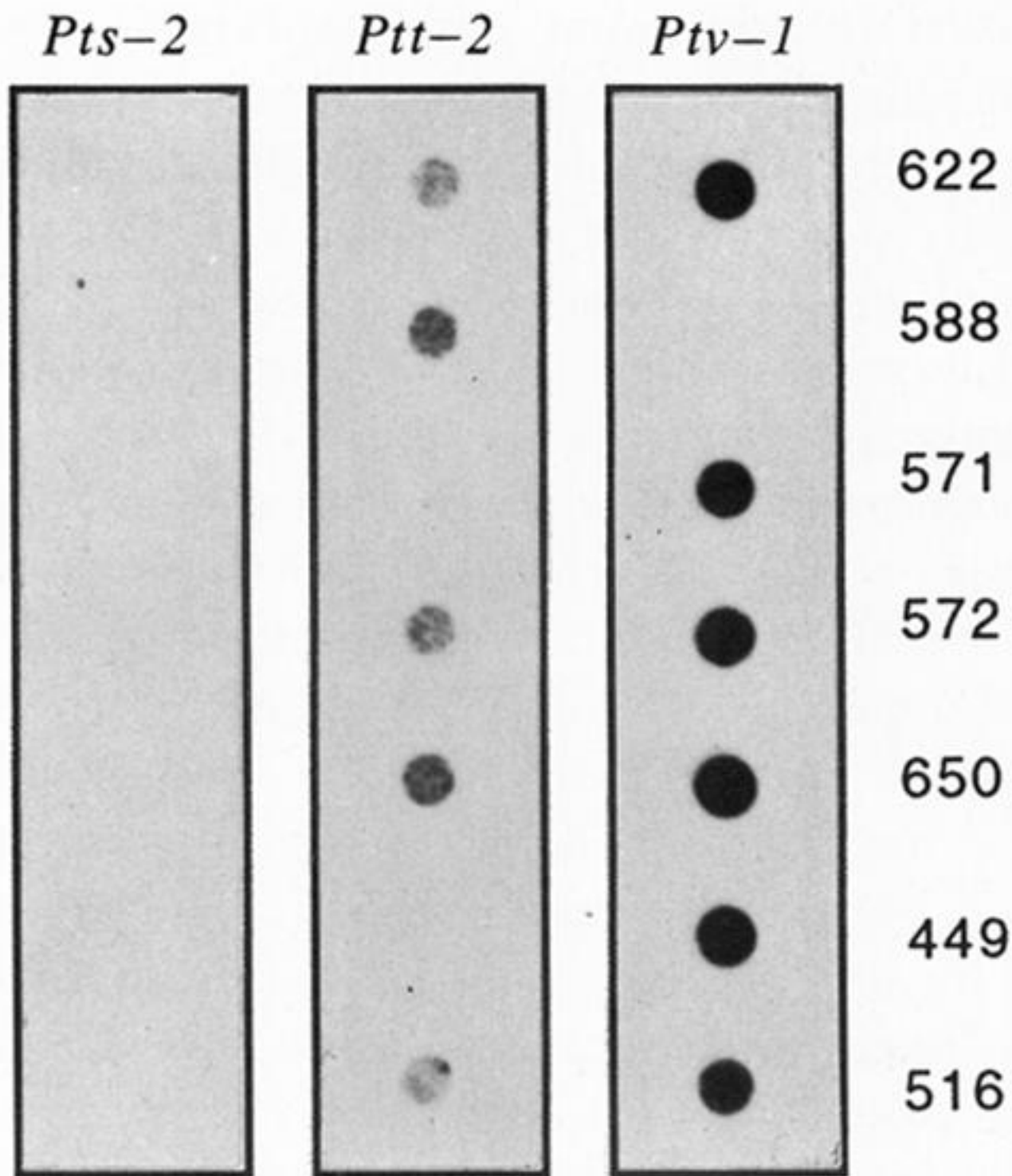


Figure 4. Dot blot of amplified DNA from the North Carolina Zoological Park chimpanzee colony; individuals identified by NARS numbers. Three subspecies probes as in figure 2.