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Identification of subspecies and parentage relationship by means of DNA fingerprinting in two exemplary of *Pan troglodytes* (Blumenbach, 1775) (Mammalia Hominidae)

Viviana Giangreco[1], Claudio Provito[1], Luca Sineo[2], Tiziana Lupo[1], Floriana Bonanno[1] & Stefano Reale[1*]

[1]Istituto Zooprofilattico Sperimentale della Sicilia "A. Mirri", Via G. Marinuzzi 3, 90129 Palermo, Italy
[2]Biologia animale e Antropologia, Dip. STEBICEF, Via Archirafi 18, 90123 Palermo, Italy
[*]Corresponding author, e-mail: stefano.reale@izssicilia.it

ABSTRACT Four chimpanzee subspecies (Mammalia Hominidae) are commonly recognised: the Western Chimpanzee, *P. troglodytes verus* (Schwarz, 1934), the Nigeria-Cameroon Chimpanzee, *P. troglodytes ellioti,* the Central Chimpanzee, *P. troglodytes troglodytes* (Blumenbach, 1799), and the Eastern Chimpanzee, *P. troglodytes schweinfurthii* (Giglioli, 1872). Recent studies on mitochondrial DNA show the incorporation of *P. troglodytes schweinfurthii* in *P. troglodytes troglodytes*, suggesting the existence of only two subspecies: *P. troglodytes troglodytes* in Central and Eastern Africa and *P. troglodytes verus*-*P. troglodytes ellioti* in West Africa. The aim of the present study is twofold: first, to identify the correct subspecies of two chimpanzee samples collected in a Biopark structure in Carini (Sicily, Italy), and second, to verify whether there was a kinship relationship between the two samples through techniques such as DNA barcoding and microsatellite analysis. DNA was extracted from apes' buccal swabs, the cytochrome oxidase subunit 1 (COI) gene was amplified using universal primers, then purified and injected into capillary electrophoresis Genetic Analyzer ABI 3130 for sequencing. The sequence was searched on the NCBI Blast database. In addiction, the microsatellite analysis was performed on the same machine for parentage detection among samples, and data were analyzed with GenMapper software. Our results show that both samples were *P. troglodytes troglodytes*, while the analysis of the microsatellite results in an unclear relationship between two chimpanzee samples.

KEY WORDS Hominidae; *Pan*; Africa; DNA; cytochrome oxidase; evolution.

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INTRODUCTION

Cellular Biology and Molecular Genetics have assumed over time a greater role in species identification. The identification was based on the assumption that there are no individuals (except homozygous twins) who have exactly the same genome. The sequence of mitochondrial cytochrome C oxidase subunit 1 (COI), often referred to as a "DNA barcode" (Hebert et al., 2003), contain approximately 648 base-pair in almost all the species and can serve as the standard barcode for almost all animals.

DNA barcode amplicons are typically obtained by PCR using standardized and universal primer sets; there are approximately five million COI barcode sequences in GenBank and/or BOLD (Barcode of Life) databases in about 280,000 species.

In addition to "DNA barcode", the study of genetic diversity among species or subspecies can be obtained analyzing the combination of a group of microsatellites loci with relative alleles frequencies extracted from genomic sequences.

The possibility of carrying out genetic traceability analysis on biological samples in the framework of surveillance programs, represents without doubt a strong deterrent for illegal commercial procedures, illegal hunting, and protected species commercialization. Conducting this type of investigation requires a technique that combines sensitivity and high discriminating power, so as to allow researchers to use it even on minimum sample quantities and to trace or identify an individual in a univocal way and with a low margin of error.

By means of microsatellites, we can detect parentage relationship among samples, but also carry out population studies and shed light on migration and evolutionary processes.

Knowing the genetic profile of a single animal in relation to certain polymorphisms allows to:

• ascertain the pedigree by genetic investigation of paternity and / or maternity;
• tackle suspected cases of poaching, acts of cruelty, and illegal imports of protected animals;
• identify the species and / or determine the sex, helping to safeguard biodiversity.

Knowledge of population relationships might also facilitate the use of the limited resources available for conservation efforts (Schonewald-Cox et al., 1983; Avise, 1996), and might help in guiding breeding programs of chimpanzees kept in captivity (see Witzenberger & Hochkirch, 2011; Hvilsom et al., 2013).

Genetic data on mitochondrial DNA (Gonder et al., 2006), analyses of complete genomes (Prado-Martinez et al., 2013), and on autosomal microsatellites (Fünfstück et al., 2015) suggest that the subspecies form two distinctive groups: one group includes *P. troglodytes verus* and *P. troglodytes ellioti* in West Africa and the other group includes *P. troglodytes troglodytes* and *P. troglodytes schweinfurthii* in Central and Eastern Africa.

Fischer et al. (2006) argue that, based on their work on nuclear DNA and considerations on morphological and behavioral similarity, the difference between chimpanzees is too small to justify the distinction in subspecies.

Later studies, including more mtDNA haplotypes, once more did not find consistent support for monophyly of *P. troglodytes troglodytes* and *P. troglodytes schweinfurthii* (Gagneux et al., 2001; Gonder et al., 2006). In addition, in the study by Gonder et al. (2006), no fixed nucleotide differences distinguishing the haplotypes of Central and Eastern chimpanzees were detected.

Other bibliographies consulted are: Boesch & Boesch, 1993; Sakura, 1994; Bard, 1995; Jones et al., 1996; Goldberg & Wrangham, 1997; Gagneux et al., 1999; Mitani et al., 2000; Butynski, 2003; Marsh, 2003; Poulsen & Clark, 2004; Prufer et al., 2012.

MATERIAL AND METHODS

Two samples of chimpanzee (*Pan troglodytes*) from the Bioparco di Sicilia, Carini (Sicily, Italy), both coming from a Belgian circus, were analyzed in April 2017.

Eight DNA samples were collected from buccal swabs, four samples for each chimpanzee. The two specimens have been identified as: first individual, named Mango (MN), born in 2000; second individual, named Whiskey (WY), born in 1998.

The samples, numbered and subdivided into different plastic bags, were deposited in a transport box and placed in a cooler bag, and the following day brought to the "Istituto Zooprofilattico Sperimentale della Sicilia", Palermo (Italy). DNA extraction was performed through the kit E.Z.N.A. Tissue DNA Kit Protocol - Whole Blood and Body Fluids. Quantification of DNA extracted was performed using NANODROP 1000 spectrophotometer from THERMO SCIENTIFIC. The COI gene was amplified by PCR using the AmpliTaq Gold™ DNA Polymerase Kit (Applied Biosystems) and the specific primers Cyt-1 (F) CCAATGATATGAAAAACATCGTT and Cyt-2 (R) GCCCCTCAGAATGATATTTGTCCTC for a final size of amplificate of 474 base pairs. The mixture was optimized as follows: 1X PCR Buffer 5 µl, 2 mM MgCl2 4 µl, 10 mM dNTP mixture 2 µl, 0.6 pmol/µl cyt B1 0.5 µl, 0.6 µl cyt B1 0.5 µl , 0,6 µl cyt B2 0.5 µl, 0.03 U/µl taq Polymerase 0.3 µl and water to 50 µl final volume. The amplification was

optimized in accord to the manufacturer (Thermo), in a 9700 thermal cycler (Applied Biosystems) with an initial denaturation step at 94 °C for 8 min, followed by 40 cycles, primer annealing at 53 °C for 50 s, and elongation at 72 °C for 1 minute and a final extension step at 72 °C for 7 minutes. All gene amplification reactions were visualized on 2% agarose gel (GellyPhor Euroclone), prepared by dissolving the 0.5X TBE agar and the DNA bands of interest displayed through an UV image acquisition system, ChemiDoc BioRad (Biotec 206). The samples of amplified DNA were subjected to purification through the "GFX PCR DNA and gel band purification kit". Once the purified ones were obtained, the sequence PCR was performed, with the "Bigdye Terminator Cycle Sequencing Kit" (Applied Biosystems), considering a reaction volume of 20 µl for each sample. The sequence products were then purified through the "Big Dye Xterminator Purification KIT" kit and injected into the ABI Prism 3130 DNA sequencer (Life technologies).

The sequences obtained were searched on the Basic Local Alignment Search Tool (BLAST) Database for species identification. Only sequences with a low e-value and high degree of identity were retained. Further analyzes for the identification of microsatellites were carried out. Initially, after the DNA extraction from buccal swabs, samples were adjusted for their concentration in ng/µl after dilution with the corresponding TE at 0.1%. The mix was constituted in according to Kit "AMPF1 STR Identifiler PCR Amplification". The amplification program includes an initial 95 °C incubation step for 11 minutes, a denaturation phase at 94 °C for 1 minute, an annealing step at 59 °C for 1 minute, an initial extension at 72 °C for 1 minute, an extension final at 60 °C for 60 minutes. Genetic profiles obtained from the microsatellites analysis were analyzed using the GeneMapper ID v4.0 software. Applied Biosystems multicolour fluorescent dye technology enables the analysis of multiple loci, including loci that have alleles with overlapping size ranges. The alleles for the superimposed loci are distinguished by the labeling of specific primers for locus with different colored dyes. Multi component analysis is the process that separates the different colors of fluorescent dye into distinct spectral components. The four dyes used in the Identifiler kit for labeling the samples are shown in Table 1.

RESULTS AND DISCUSSION

The amplification of the COI gene in eight isolates taken from two samples of *P. troglodytes* gave a specific band on agarose gel. The size of amplified fragment was 508 bp (Fig. 1).

NCBI BLAST database search of the sequence gave a similarity with species *Pan troglodytes troglodytes* for both Wishy and Mango samples with 99% of identity.

The microsatellites fragment were analyzed using the GeneMapper ID software. All electropherograms derived from the fragment analysis of samples of *Pan troglodytes* for each locus are reported in figures 2–7. We excluded the fluorescent dye PET® because it did not give any peak.

With the VIC® dye, in the two figures (Figs. 4, 5), the electropherograms of each locus, respectively of Whisky and Mango, were represented.

With the NED™ dye, in the two figures (Figs. 6, 7), the electropherograms of each locus, respectively of Whisky and Mango, were represented.

In Table 2, the alleles for each locus are reported for all samples. Sample 3 of Whisky didn't present

Dye	Locus
6-FAM	D8S1179 D21S11 D7S820 CSF1PO
VIC	D3S1358 TH01 D13S317 D16S539 D2S1338
NED	D19S433 vWA TPOX D18S51
PET	Amelogenina D5S818 FGA

Table 1. Loci list and relative dye employed.

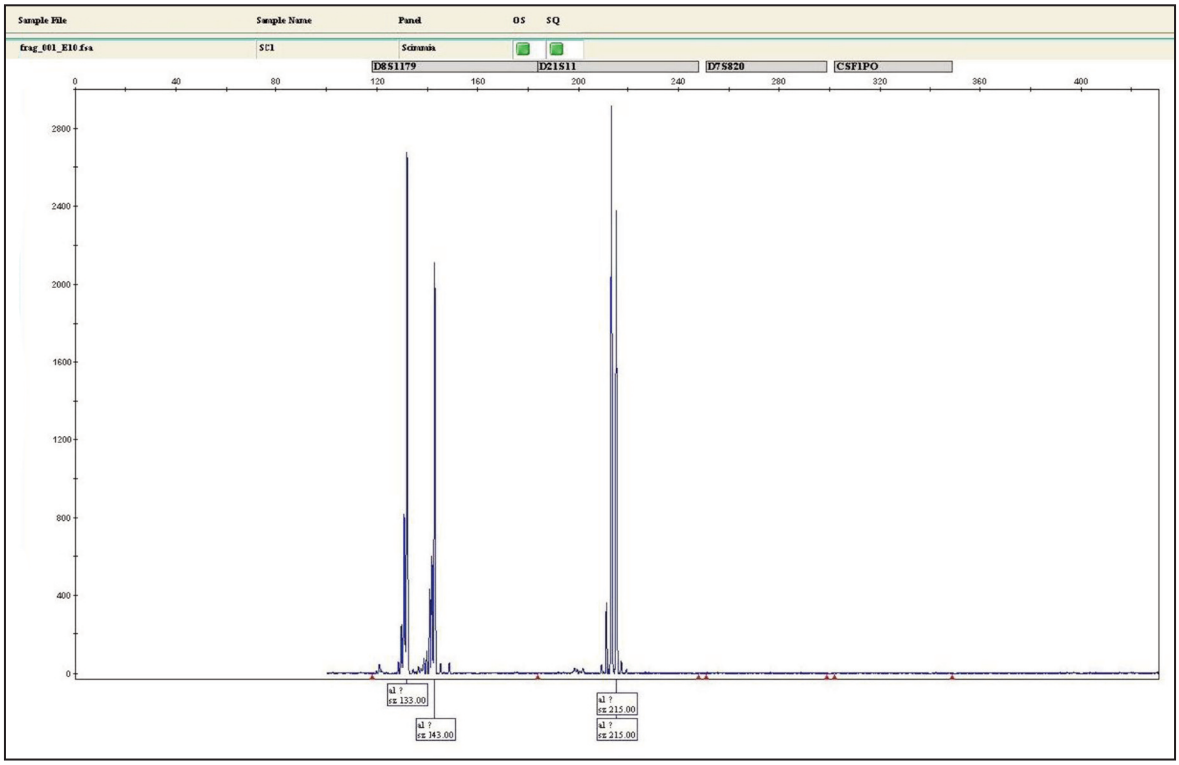

Figure 1. Electropherograms of the Whisky sample with 6- FAM ™ dye.

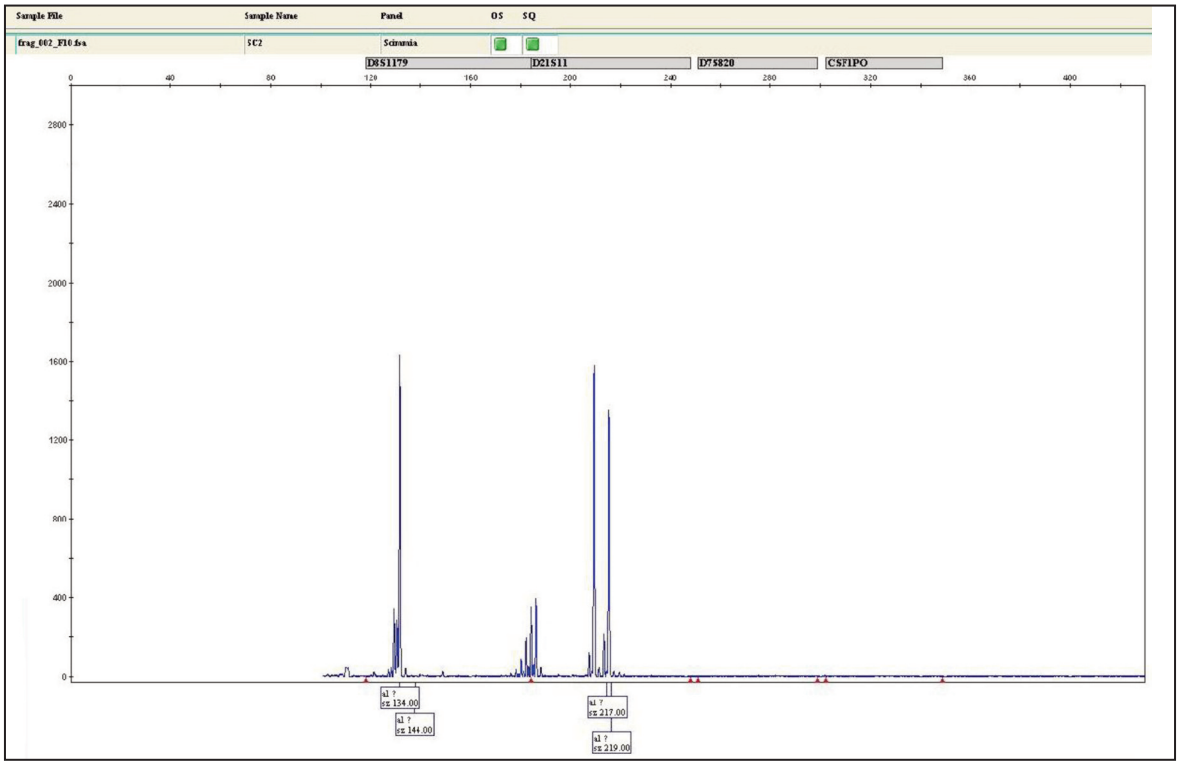

Figure 2. Electropherograms of the Mango sample with 6- FAM ™ dye.

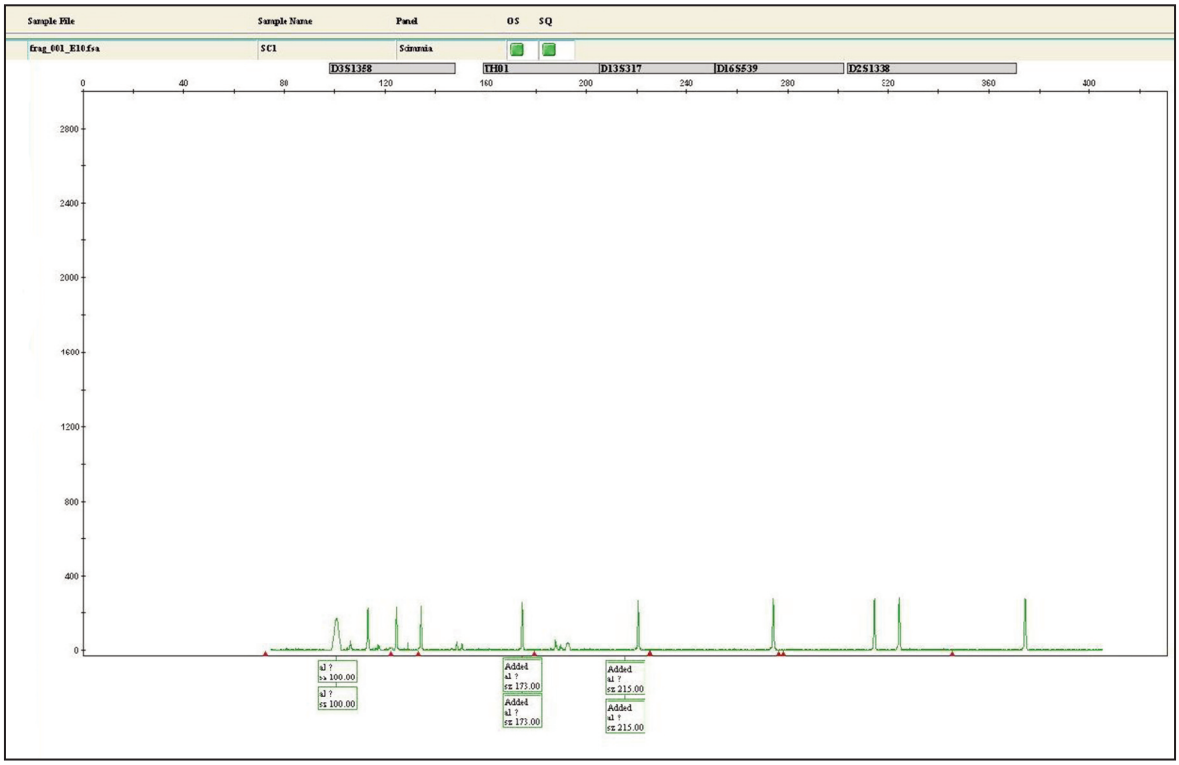

Figure 3. Electropherogram of the Whisky sample with VIC® dye.

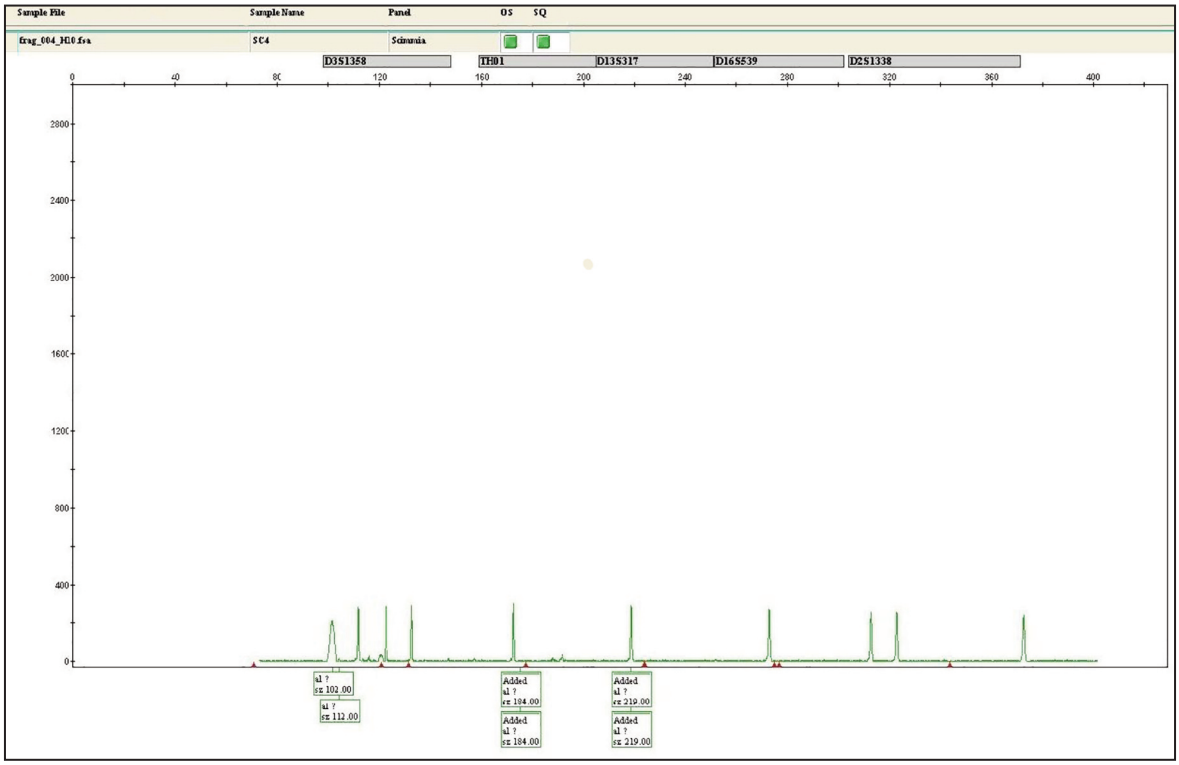

Figure 4. Electropherogram of Mango sample with VIC® dye.

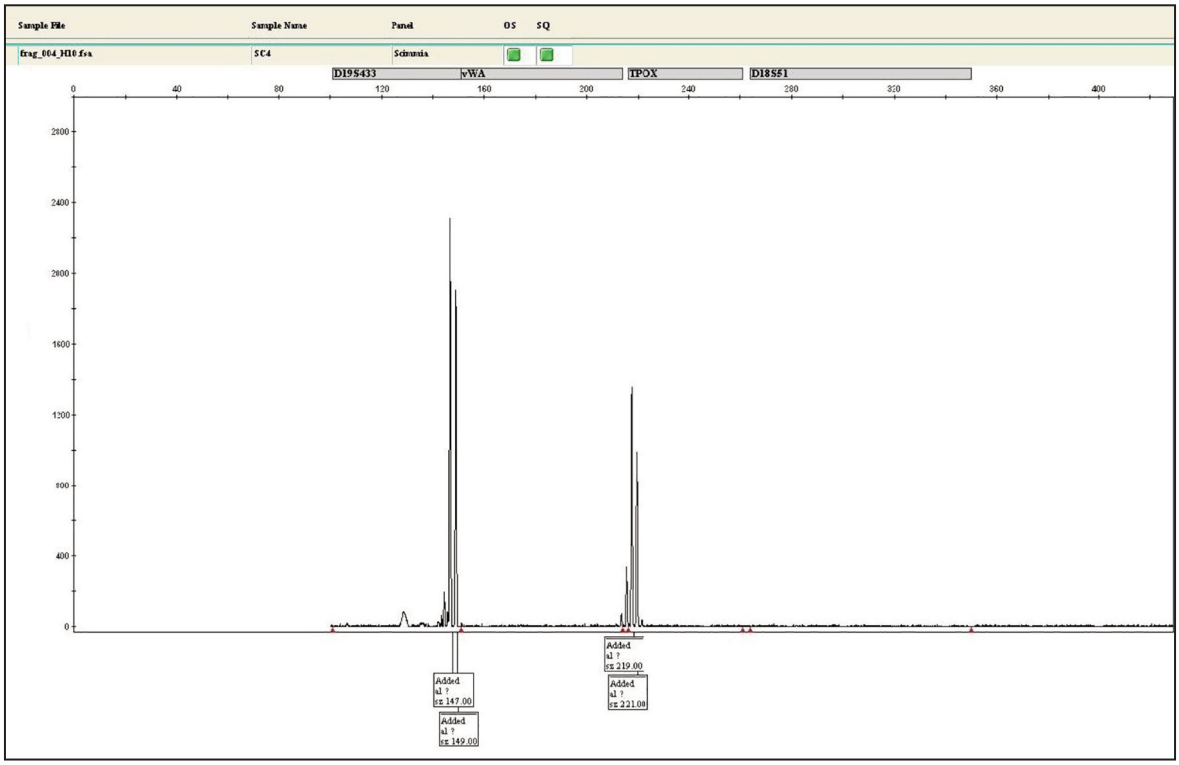

Figure 5. Electropherogram of Whisky samples with NED™ dye.

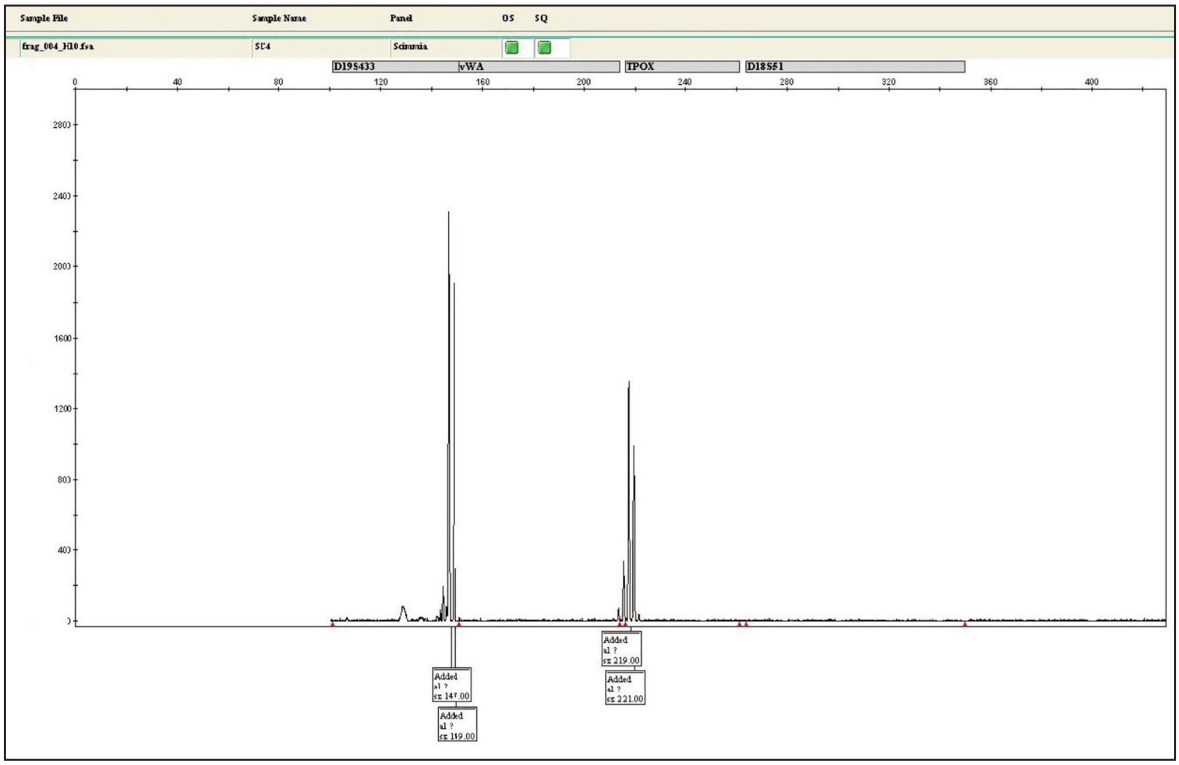

Figure 6. Electropherogram of Mango samples with NED™ dye.

LOCUS	DYE	DYE	1 WHISKY	3 WHISKY	5 WHISKY	7 WHISKY	2 MANGO	4 MANGO	6 MANGO	8 MANGO
D8S1179	Fam	Blue	133-143	N	133-143	133-143	134-144	134-144	134-144	134-144
D21S11	Fam	Blue	215-215	N	215-215	215-215	217-219	217-219	217-219	217-219
D7S820	Fam	Blue	N	N	N	N	N	N	N	N
CSF1PO	Fam	Blue	N	N	N	N	N	N	N	N
D3S1358	Vic	Green	100-100	N	100-100	100-100	102-112	102-112	102-112	102-112
TH01	Vic	Green	173-173	N	173-173	173-173	184-184	184-184	184-184	184-184
D13S317	Vic	Green	215-215	N	215-215	215-215	219-219	219-219	219-219	219-219
D16S539	Vic	Green	N	N	N	N	N	N	N	N
D2S1338	Vic	Green	N	N	N	N	N	N	N	N
D19S433	Ned	Yellow	147-149	N	147-149	147-149	147-149	147-149	147-149	147-149
vWA	Ned	Yellow	N	N	N	N	N	N	N	N
TPOX	Ned	Yellow	217-221	N	217-221	217-221	219-221	219-221	219-221	219-221
D18S51	Ned	Yellow	N	N	N	N	N	N	N	N

Table 2. List of alleles for each locus in the two examined chimpanzees.

any peak. For the remaining seven samples, the two Mango and Whisky groups of replicates presented the same size for each locus and so we grouped it in two samples. We noted also that six loci (D7S820, CSF1PO, D16S539, D2S1338, vWA, and D18S51) do not give any result for all the samples, probably due to non specificity of the human locus primer used for the *P. troglodytes* species.

We observed that locus D19S433 share the same pair of allele between the two replicates (147 and 149) and that locus TPOX share one allele between the two groups of samples (221).

Despite the fact that Mango and Whisky had some locus allele size in common and in consideration of the fact that six loci didn't give any results, we can not assert a parentage relationship among two chimpanzees only based on these data.

CONCLUSIONS

Pan troglodytes s.l. is the most abundant, protected, and widespread of the great apes, the declines that have occurred are expected to continue, satisfying the criteria for an Endangered listing (Oates, 2006). Due to high levels of poaching, infectious diseases, and loss of habitat and habitat quality caused by expanding human activities, this species is estimated to have experienced a significant population reduction in the past 20–30 years and it is suspected that this reduction will continue for the next 30–40 years. Furthermore, zoonosis and disease outbreaks present significant risks; there is, for example, evidence that *Ebolavirus* will continue to spread in some parts of the Chimpanzee's geographic range (Walsh et al., 2005).

Actually, it is considered an Endangered species (EN) (Humle et al., 2016).

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