

Table 2 | Probability of exact allelic matching at 16 microsatellite loci in a population of Afghan hounds

Scenario	Probability
Hardy–Weinberg*	7×10^{-14}
Full siblings†	9×10^{-6}
Inbred $F = 0.25$ ‡	3×10^{-8}
Pedigree§	4×10^{-4}

*A random sampling of the population, assuming Hardy–Weinberg conditions.
†Assuming the clone is a full sibling of the donor.
‡Assuming everyone in the population is related with an inbreeding coefficient of 0.25.
§An assumed pedigree in which the donor is the product of a mother–son mating, and the putative clone is the product of crossing the donor back to the mother.

The probability that the putative clone should have precisely the same genotype as the donor was computed for different assumptions regarding the relatedness of the sample and the donor⁷ (Table 2). In all cases, the allele frequencies were computed from a sample of 11 AKC-registered Afghan hounds plus the donor. According to genetic maps of the canine chromosomes, the 14 mapped markers were unlinked to one another, so each microsatellite was treated as an independent locus^{8,9}.

The match probabilities ranged from 7×10^{-14} for unrelated dogs to 4×10^{-4} for

those with a specific inbred pedigree. A higher degree of inbreeding would increase the match probability further, but the donor does not seem to be extremely inbred; both the donor and Snuppy are heterozygous at 8 of the 16 markers, which is not significantly different from the number of heterozygous markers expected, given the observed allele frequencies and no inbreeding.

Mitochondrial D-loop sequencing revealed 26 variable bases within the 614 analysed (Table 3). Snuppy and the donor dog differed at 12 of the 26 sites. Nine of the Afghan hound sequences disagreed with each other at only one base (position 548) and differed from the donor by only three to four bases. Also, the two Labrador retrievers had identical mitochondrial sequences that differed from the donor by only three bases. The sequence from Snuppy differed from that of any other dog at 9–14 sites.

These data are consistent with Snuppy being a genetic clone of the donor dog Tai. Our analysis rules out most feasible alternatives to a true clone, such as the production of a delayed twin, which would have produced dogs with the same mitochondrial D-loop sequence, or an animal resulting from extreme inbreeding, which would have yielded dogs

that were homozygous at more than the observed eight loci.

Conclusions drawn from these results are subject to caveats. First, we did not witness the drawing of the blood samples, which was done under the supervision of a third party. However, no obvious hypothetical manipulation of the samples would have generated the results described here — perfect matching of the nuclear markers, and distinct differences between the mitochondrial sequences for the donor and Snuppy. Second, we were not provided with samples from the oocyte donor, although tissue samples from this dog have been tested by investigators at Seoul National University¹⁰. Without this sample, we are unable to confirm the original experimental details¹, or to say with certainty that the mitochondrial variants observed were those that were expected.

Finally, our statistical analysis is based on a limited number of Afghan hounds. A larger number of unrelated individuals might have provided a more precise estimate of population allele frequencies. However, given that the dogs tested are representative of this relatively restricted breed and that many share a partial heritage with the donor, the statistical conclusions are conservative.

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Table 3 | Mitochondrial genotypes at 26 polymorphic loci within the canine mitochondrial D-loop

Loci*	49	76	110	118	135	143	148	150	153	155	159	162	166	175	304	323	334	338	371	435	478	482	526	548	561	562
Boxer	C	A	T	C	T	C	T	G	C	C	T	A	A	G	C	T	A	T	G	C	T	C	A	T	C	T
Labrador	C	A	T	C	T	C	T	G	C	C	T	A	A	G	C	T	A	T	G	C	C	C	A	T	C	T
Afghan-1	C	A	T	C	T	T	T	A	C	C	T	A	A	G	T	T	A	T	G	C	C	C	A	C	C	T
Afghan-2	C	A	T	C	T	T	C	A	T	T	C	A	A	G	C	C	A	C	A	T	C	T	G	T	C	C
Afghan-3	C	A	T	C	T	T	T	A	C	C	T	A	A	G	T	T	A	T	G	C	C	C	A	T	C	T
Afghan-4	T	A	T	T	C	T	T	A	C	T	T	G	G	A	C	C	A	C	G	T	T	C	G	T	C	T
Afghan-5	C	A	T	C	T	T	T	A	C	C	T	A	A	G	T	T	A	T	G	C	C	C	A	T	C	T
Afghan-6	C	A	T	C	T	T	T	A	C	C	T	A	A	G	T	T	A	T	G	C	C	C	A	C	C	T
Afghan-7	C	A	T	C	T	T	T	A	C	C	T	A	A	G	T	T	A	T	G	C	C	C	A	T	C	T
Afghan-8	C	A	T	C	T	T	T	A	C	C	T	A	A	G	T	T	A	T	G	C	C	C	A	C	C	T
Afghan-9	C	A	T	C	T	T	T	A	C	C	T	A	A	G	T	T	A	T	G	C	C	C	A	C	C	T
Afghan-10	C	A	T	C	T	T	T	A	C	C	T	A	A	G	T	T	A	T	G	C	C	C	A	C	C	T
Afghan-11	C	A	T	C	T	T	T	A	C	C	T	A	A	G	T	T	A	T	G	C	C	C	A	C	C	T
Donor	C	A	T	C	T	T	T	G	C	C	T	A	A	A	C	T	A	T	G	C	C	C	A	C	C	T
Snuppy	T	G	C	C	T	T	T	A	C	T	T	A	A	A	C	C	G	T	G	T	T	C	G	T	T	T
Surrogate	C	A	T	C	T	C	T	G	C	C	T	A	A	G	C	T	A	T	G	C	C	C	A	T	C	T

*The locations of the variable bases are given relative to the 5' end of the forward primer used to amplify and sequence the region. The entire length of the segment is 614 bases.

MOLECULAR GENETICS

Verification that Snuppy is a clone

Arising from: B. C. Lee et al. Nature 436, 641 (2005)

Somatic-cell nuclear-transfer technology has been used to clone a variety of animal species^{1–3}, but the overall efficiency of the cloning process and the viability of embryos has remained low⁴. Until Lee et al. described the cloning of two Afghan hounds by nuclear transfer from adult skin fibroblasts into oocytes that had matured

*in vivo*⁵, dog cloning had been unsuccessful because of the difficulty of collecting canine oocytes matured *in vivo* at metaphase II (ref. 6). Here we provide independent evidence from the Seoul National University Investigation Committee that Snuppy, the one of the pair to survive, is a genuine clone.

To investigate whether the cloned dog was genetically identical to the donor Afghan, we obtained blood samples from Snuppy, from the male Afghan hound that provided the somatic cell, and from the surrogate mother. In addition, autopsy samples from the since-deceased mixed-breed dog that originally provided the egg used to create Snuppy were obtained from the research team who generated the cloned dog. DNA was extracted from the blood and autopsy samples and used for microsatellite analysis of genomic DNA and nucleotide sequences of mitochondrial DNA.

Table 1 | Analysis of canine-specific microsatellite loci

Canine markers*	Snuppy (blood leukocytes)		Donor Afghan (blood leukocytes)		Surrogate (blood leukocytes)		Egg donor* (autopsy lung tissue)	
	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1	Peak 2
AHT111	72	78	72	78	78	80	76	80
AHT137	130	148	130	148	130	148	130	142
C01.424	186	188	186	188	186	188	182	188
C07.620	106	114	106	114	112	114	114	124
Wilms-TF	291		291		291		297	299
FH2274	278	286	278	286	278		278	282
FH2289	284	328	284	328	296		288	292
REN210K18	216		216		212	217	202	217

*Eight of 27 canine-specific markers used for microsatellite assay are shown.

Table 2 | Sequence alignments within D-loop region, cytochrome *b*, and 16S rRNA of mtDNA

	Nucleotide positions											
	D-loop region (409 bp)								Cytochrome <i>b</i> (380 bp)		16S rRNA (440 bp)	
	170	175	195	343	354	455	498	546	568	426	1117	1145
Snuppy	A	T	A	C	G	T	T	G	T	G	C	G
Donor Afghan	G	C	A	T	A	C	C	A	C	A	T	A
Surrogate	G	C	G	T	A	C	C	A	T	A	C	A
Egg donor	A	T	A	C	G	T	T	G	T	G	C	G

*The nucleotide positions were counted from the start, the initiation codon, and the transcriptional start of the D-loop, cytochrome *b*, and 16S rRNA sequences, respectively (GenBank accession number AY729880). bp, Base pairs.

The microsatellite analyses were performed with genomic DNA from four dogs (Snuppy, the donor Afghan, the surrogate mother and the egg donor) using 27 canine-specific markers that allow extremely inbred animals to be distinguished. As shown in Table 1, which shows only 8 of 27 loci used for microsatellite analysis, Snuppy and the donor Afghan have identical microsatellite patterns for all loci, whereas the surrogate mother evidently has a different genetic origin.

For 18 of the microsatellite loci analysed (including those shown in Table 1), the egg donor and Snuppy showed distinct microsatellite patterns. The microsatellite loci used for the analyses show enough variation among Afghan hound individuals to allow them to be distinguished (results not shown). Thus, the Committee's microsatellite analysis of genomic

DNA demonstrates that the cloned dog Snuppy is genetically identical to its fibroblast-donor Afghan hound.

If Snuppy is a cloned dog, his mitochondrial DNA (mtDNA) should be identical to that of the egg donor, but not to that of the fibroblast-donor Afghan. To test this, we determined partial sequences of four regions of the dog mtDNA. Based on the complete nucleotide sequence (GenBank accession number U96639), four primer sets were synthesized and used for polymerase chain reaction (PCR)⁷: the cytochrome *b* gene (L14,252–L14,631), the 16S ribosomal RNA gene (L2,033–L2,472), and the two overlapping hypervariable D-loop regions (L15,622–L16,030 and L15,485–L16,090). PCR products were sequenced and a BLAST search confirmed their identity as mtDNA.

The sequence alignments of 409 base pairs

of the hypervariable D-loop region revealed eight mismatches between the mtDNA of Snuppy and the donor Afghan (Table 2). A non-match was observed even in more conserved regions (one mismatch in the cytochrome *b* gene and two mismatches in the 16S rRNA gene). These results indicate that Snuppy could not have been produced by splitting the early blastomere from which the donor Afghan originated. The sequence alignments also revealed a perfect match between Snuppy and the egg donor, and a non-match between Snuppy and the surrogate mother (Table 2). This is consistent with Snuppy being a nuclear clone of the donor Afghan.

On the basis of the results of both a microsatellite analysis of genomic DNA and a sequence comparison of mtDNA, it is highly unlikely that Snuppy came either from extreme inbreeding or from blastomere separation (also known as twinning). It is virtually certain that Snuppy was generated from somatic-cell nuclear transfer, as claimed by Lee *et al.*⁵.

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