MOLECULAR GENETICS

DNA analysis of a putative dog clone

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

In August 2005, Lee *et al.* reported the first cloning of a domestic dog from adult somatic cells[1]. This putative dog clone was the result of somatic-cell nuclear transfer from a fibroblast cell of a three-year-old male Afghan hound into a donor oocyte provided by a dog of mixed breed. In light of recent concerns regarding the creation of cloned human cell lines from the same institution[2,3], we have undertaken an independent test to determine the validity of the claims made by Lee *et al.*[1].

Duplicate sets of blood samples were provided from the original fibroblast donor dog (Tai, an Afghan hound), the surrogate mother (a Labrador retriever) and Snuppy, the putative clone. Samples were drawn in heparinized tubes and delivered to us on ice overnight. Collection and mailing of samples was supervised by In Kwon Chung, a member of the investigative committee at Seoul National University in South Korea. Samples were not provided from the oocyte donor, which was unavailable for sampling. Samples were coded by a third party and laboratory personnel were blind to sample identifiers.

In addition to these six samples, the test panel included previously purified DNA samples from 11 Afghan hounds collected in the United States and registered with the American Kennel Club (AKC); eight of these shared no common parents or grandparents, and the other three were half-siblings that shared a common sire. Pedigree analysis revealed that one of the American-collected dogs was a first cousin of Tai, the fibroblast donor, and that five of the others had distant maternal and paternal relatives in common with him. Other samples on the test panel included a pure-bred female Labrador retriever, purportedly unrelated to the surrogate mother, and Tasha, the boxer dog used for the reference canine sequence[4].

We tested both nuclear and mitochondrial markers. Nuclear markers included 16 microsatellite markers routinely used for canine paternity testing by the AKC[5,6]. (A seventeenth marker was discarded because it failed to amplify from 25% of the DNA samples.) For all nuclear markers tested, Snuppy and Tai, the clone and donor, had identical genotypes (Table 1).

Table 1 | Microsatellite genotypes of 16 dogs, including the putative clone, the nuclear-DNA donor and the surrogate mother

	FH2010	FH2054	FH2079	Pez01	Pez05	Pez06	Pez08	Pez10	Pez11	Pez12	Pez13	Pez15	Pez16	Pez17	Pez20	Pez21
Boxer	242	178	292	134	120	199	244	306	161	293	235	224	319	226	191	109
	242	178	292	138	124	202	244	306	161	315	243	240	323	230	194	109
Labrador	246	166	288	130	128	187	256	310	153	285	239	228	311	222	194	109
	254	174	288	138	128	187	264	310	165	285	243	236	327	226	194	120
Afghan-1	246	170	288	138	120	198	252	283	145	300	243	228	315	226	194	109
	246	186	288	138	120	203	252	310	145	315	250	232	335	226	198	112
Afghan-2	246	174	288	130	120	194	252	283	145	289	nd	232	315	226	198	109
	254	186	288	138	120	198	252	298	145	320	nd	232	335	226	198	116
Afghan-3	246	174	292	113	120	199	252	283	145	289	243	232	315	226	198	112
	250	186	308	138	120	199	252	298	153	326	243	232	327	226	198	120
Afghan-4	246	186	288	113	124	199	252	302	150	293	250	228	335	226	194	112
	246	186	288	113	132	203	252	302	157	300	250	228	335	226	198	116
Afghan-5	246	174	288	130	120	194	252	298	145	315	235	228	315	226	198	109
	250	186	308	138	120	199	264	298	157	326	243	232	335	226	198	116
Afghan-6	246	174	292	138	120	195	252	283	145	275	235	232	315	226	194	112
	246	194	292	138	124	203	252	310	150	293	243	232	335	230	198	116
Afghan-7	246	174	288	130	132	203	252	298	150	289	219	228	315	226	194	112
	258	190	288	138	132	207	252	322	153	289	250	232	319	230	194	116
Afghan-8	246	174	288	113	120	194	252	294	157	289	219	232	311	226	198	116
	246	186	292	138	124	203	256	310	166	315	243	240	315	226	198	116
Afghan-9	246	186	282	138	124	199	252	294	166	315	219	228	315	230	198	109
	254	194	308	138	124	203	264	294	166	315	219	228	315	234	198	116
Afghan-10	246	186	288	113	132	195	241	283	157	312	219	232	323	230	198	112
	254	186	292	138	132	199	252	294	166	326	252	232	335	230	198	116
Afghan-11	246	174	nd	138	120	195	252	326	153	nd	243	228	315	226	194	109
	246	194	nd	138	132	198	252	nd	157	nd	250	228	330	226	198	116
Donor	246	186	288	130	124	199	252	294	153	300	235	232	307	226	194	112
	246	194	288	138	132	199	252	298	153	326	235	232	315	230	194	116
Snuppy	246	186	288	130	124	199	252	294	153	300	235	232	307	226	194	112
	246	194	288	138	132	199	252	298	153	326	235	232	315	230	194	116
Surrogate	250	170	292	138	120	198	256	298	145	285	215	228	311	230	194	116
	254	170	296	138	124	203	256	298	153	297	243	236	323	234	198	120

Alleles are named for the total length of the segment amplified; nd, allele not determined.

Methods. DNA was extracted and purified from all blood samples using the QIAamp DNA blood mini kit (Qiagen). The conditions and protocol used for the polymerase chain reaction are available at http://research.nhgri.nih.gov/dog_genome. Amplicons were genotyped using an ABI 3730xl DNA analyser. For all 19 samples, a 614-base segment of the mitochondrial DNA control region was sequenced using ABI BigDye terminator chemistry and standard protocols (see http://research.nhgri.nih.gov/dog_genome for details and raw data). Primers for both amplification and sequencing were designed using publicly available information (www.genome.ucsc.edu) and were: forward, 5′-TGAATCACCCCTACTGTGCTATGT-3′ and reverse, 5′-ACCTTGATTTTATGCGTGAGTTGA-3′. All markers and sequences were run in duplicate in independent assays, yielding more than 99% of potential genotypes (see also Table 3).

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 donor[7] (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 University[10]. Without this sample, we are unable to confirm the original experimental details[1], 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.

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.

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Verification that Snuppy is a clone

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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 low[4]. 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.