DNA Methods of Assessing Dingo Purity

Alan N. Wilton

School of Biochemistry and Molecular Genetics,
University of New South Wales, Sydney 2052

ABSTRACT

The effective conservation of the dingo in Australia requires that a purebred animal can be reliably distinguished from a hybrid domestic dog. Even though the dingo and the dog are closely related differences in DNA between them can be used as the basis of a test to detect hybrids. Genetic markers that show high variability between individuals may show differences between related groups. Microsatellite loci show very high variability and many are available for the dog. From 72 microsatellites tested, 12 show distinct differences in the alleles found in dingoes and dogs. Microsatellites that are diagnostic, that is show no overlap between alleles in the dingo and the dog, are the most useful for testing. However, conditions are not ideal for detecting these since some dog breeds have dingo ancestry and the purity of dingoes currently in captivity is not guaranteed. Estimates of purity can still be made by comparing the relative likelihoods of an animal belonging to one group or another based on the frequency of the alleles found in that animal in the groups being compared.

Introduction

The dingo in the wild is endangered due to hybridisation with domestic dogs. Estimates of the proportion of hybrids in populations are as high as 78% in some areas near the populated centres on the east coast (Corbett 1995).

Skull measurements and other physical characteristics have been used to obtain these estimates (Newsome *et al.* 1980, Newsome and Corbett 1982, 1985). While they have been demonstrated to be reliable for differentiating dogs and from dingoes, they are difficult to obtain and their ability to detect hybrid-dingo backcrosses i.e. 3/4 dingo - 1/4 dog has not been determined. They are also not applicable to surveying populations on a regular basis as the animals must be killed to make the skull measurements.

DNA testing is a common method used to examine relationships between species. For the dog and dingo the chromosomes that carry the DNA are indistinguishable down the microscope. Both have 39 pairs of chromosomes. The genes of dingoes and dogs are also very similar at the DNA sequence level. However, only 3-5% of the 3 billion bases of DNA in canids are genes and the remainder is often referred to as junk DNA most of which has no effect on the physical characteristics of the animal.

Microsatellite loci on the chromosomes in the nucleus and DNA sequences of the D-loop in mitochondria are two types of DNA markers that have been used to examine evolutionary relationships between closely related species because they show high amounts of variation (Gottelli *et al.* 1994, Roy *et al.* 1994). What is required to detect hybrids is a marker for which the dog has types that do not exist in the dingo. Presence of this dog type in a wild canid is then an indication of dog ancestry.

Results for several microsatellites that are highly variable can be pooled to create a DNA fingerprint which is virtually unique for each animal. DNA fingerprinting is used in forensics for identification and in parentage testing in humans and animals.

Microsatellite loci are sites on the chromosomes where there are tandem repeats of short DNA sequence motifs of between 1 and 5 bases (Weber and May 1989). The most common repeat is the two nucleotides (AC)n. The number of copies (n) of the repeat at any site is variable. For each of the two copies of any microsatellite in each individual, one from each parent, the number of repeats can be measured simply and reliably by using the molecular techniques of polymerase chain reaction (PCR) to amplify the DNA from the sites and gel electrophoresis to separate the DNA products by size.

PCR is used to exponentially amplify the DNA specific to the microsatellite of interest using DNA primers designed to match unique DNA flanking the microsatellite. Million-fold amplification allows DNA testing on small samples of blood or other

Pages 49 - 56 in *A Symposium on the Dingo* edited by C. R. Dickman and Daniel Lunney, 2001. Royal Zoological Society of New South Wales, Mosman NSW.

tissue. This makes it possible to sample populations and test the level of crossbreeding in the population or in individual animals using less invasive methods than previously used. It may even be possible to apply the tests to samples such as scats or hair that could be collected without trapping the animals.

Materials and methods

Subjects

Dingoes: 77 animals held in captivity with the physical appearance of dingoes and no known dog ancestry. Animals are being held by dingo conservation groups, zoos and wildlife parks.

Dogs: 55 dogs of mixed breed from the Sydney area.

Wild canids: fifty animals captured and released in Kosiuszko National Park as part of a dingo tracking study by David Jenkins.

DNA extraction

DNA was extracted from 5 ml whole blood samples taken in EDTA tubes. Red cells were lysed by washing 3 times in TE. The remaining cells were digested with proteinase K in 5 ml TE in presence of 0.3% SDS overnight. The protein was precipitated by adding 2 ml 5 M NaCl and spinning. The solution was extracted with one volume of chloroform, the phases separated and aqueous phase combined with 2 volumes of ethanol. The precipitated DNA was spooled onto a glass rod and dissolved in TE. Tissue samples were processed in a similar way except they were ground into small pieces before digesting overnight as above.

Genetic Markers

Seventy-two canine microsatellite loci were tested on an initial sample of 16 dogs and 16 dingoes. Loci that appeared to have large differences in allele distributions in the two groups were tested on all of the samples. The markers tested were AHT101, AHT103, AHT107, AHT109, AHT125, AHT126 (Holmes *et al.* 1993), CXX30, CXX123, CXX127, CXX172, CXX204, CXX250, CXX255, CXX263, CXX314, CXX377, CXX402, CXX403, CXX406, CXX409, CXX410, CXX414, CXX423, CXX429, CXX434, CXX438, CXX441, CXX442, CXX443, CXX460, CXX502, (Ostrander *et al.* 1993), FH2001, FH2010, FH2054, FH2060, FH2079, FH2096, FH2138, FH2152, FH2159, FH2168, FH2175, FH2186, FH2199, FH2201, FH2226, FH2233, FH2247, FH2248, FH2250, FH2257, FH2261, FH2266, FH2278, FH2279, FH2289, FH2293, FH2295, FH2301, FH2304, FH2305, FH2312, FH2313, FH2318, FH2320, FH2321, FH2323, FH2324, FH2326, FH2346, FH2347, FH2361 (Mellersh *et al.* 1997) LEI008 (Mellersh *et al.* 1994)

PCR amplification and genotyping

Pairs of nucleotide primers specific for the loci above were purchased from Research Genetics. Amplification was performed in a reaction containing 0.2 mM of each dNTPs, 5 mM of each primer, ~ 2 mM $MgCl_2$, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.2 - 2 μM fluoro-labelled dUTP, 0.5 U AmpliTaq Gold DNA polymerase (PE Biosystems) and 50 ng target genomic DNA. Fluoro-labelled dUTPs were either [R110]dUTP or [R6G]dUTP (PE Biosystems). Reactions were put through a thermocycling program of 95°C for 10 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds and extension at 72°C for 1 minute, with one 5 minute extension step at 72°C at the finish.

GS500 size standards in formamide loading dyes (4 μl) were added to 1 μl PCR products and denatured at 95°C for 3 minutes. Samples were run on 36 cm denaturing polyacrylamide gels (4.25% 19:1 acrylamide: bisacrylamide) with 6 M urea in TBE (Tris-HCl 45 mM, borate 45 mM, EDTA 1 mM). Gels were run at 3000 V for 2 hours on ABI377 Automated DNA Sequencer. Genescan and Genotyper software (PE Biosystems) were used to size the PCR products relative to the GS500 size standard in each lane (see Fig. 1 for example).

Statistics

A relative likelihood score can be used to assign an individual to a population based on genotype information at several loci and information on the relative frequencies of each genotype in the alternative populations. The logarithm of the relative likelihood or Lod (log of odds) is commonly presented. Forty dogs and 50 dingoes were typed for 10 microsatellite loci to determine frequencies of alleles in each population. The probability of finding an animal in population 1, i.e. dingoes, with any particular combination of genotypes at the 10 loci is the product of the frequency of each genotype in the population. The probability of finding an animal with the same genotype in population 2, i.e. dogs, can be calculated using the genotype frequencies in that population. The Lod score based on these probabilities indicates how much more likely the animal is to come from population 1 than population 2.

If the question is whether the sample is from

a pure dingo or a hybrid then the appropriate score is based on the frequencies in these two populations. The frequencies of each genotype were averaged between the dingoes and dogs to create frequencies in a population with 50% dog genes. The dingo and dog frequencies can be weighted to give frequencies expected in populations with 75% dingo and 25% dog, etc. Comparisons of wild dogs with these hybrid populations are more appropriate than with the dog population because the question is whether the animal is more likely to be a pure dingo than a hybrid.

Results

The majority of microsatellites tested showed similar distribution of alleles in the dingo and the dog. The variability in the dingoes was lower than that found in dogs for most loci. Six microsatellite loci were identified that appeared to be highly informative in that little or no alleles were shared between dingoes and dogs. For CXX30, a single basepair difference in sizes between dogs and dingoes that was previously found (Wilton *et al.* 1999) was confirmed (Fig. 2a). CXX402 was mostly homozygous for the 170 bp allele in the dingoes while sizes ranged from 140 bp to 178 bp in the dog (Fig. 2b). Markers such as these that show little or no overlap in allele distributions are the most

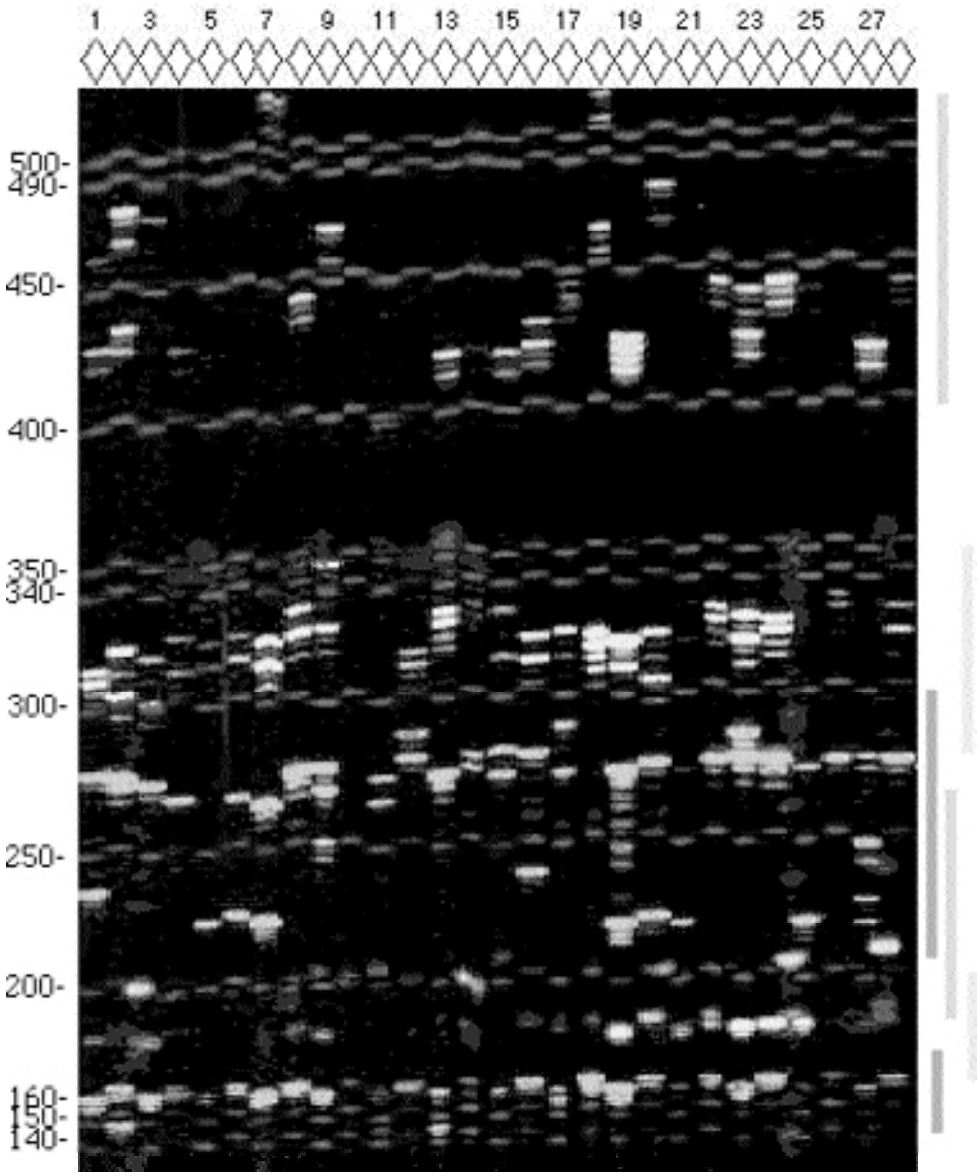

Figure 1. Genotyping gel containing six microsatellites for 28 animals. Each lane represents a different animal and different sizes of the microsatellites are estimated from the internal lane standards for which the sizes in basepairs are indicated on the left. Size range of each microsatellite is indicated by bars on right. (see back cover for colour).

useful in identifying hybrids, as it is obvious if a dog allele appears in a suspected dingo or a wild canid. For FH2257 the alleles in dingoes were smaller than those on dogs except for a few cases (Fig. 2c). For CXX410 there were 2 very common alleles in dingoes which were also found in dogs but the dogs had a number of other alleles sizes (Fig. 2d). For FH2138 the alleles in dingoes were generally larger than in dogs (Fig. 2e). FH2199 showed a patchy distribution of allele sizes but with little overlap between dogs and dingoes (Fig. 2f).

Other microsatellites that showed some alleles that were common in dogs, but absent or very rare in dingoes, were CXX406, CXX434, CXX460, FH2079, FH2175, FH2293, FH2313 and FH2346. Even though some of these loci have alleles that occur in both dogs and dingoes, they can still be used to detect hybridisation because the probability of finding several of these uncommon types in a dingo is very small and the frequencies can be used to calculate how likely.

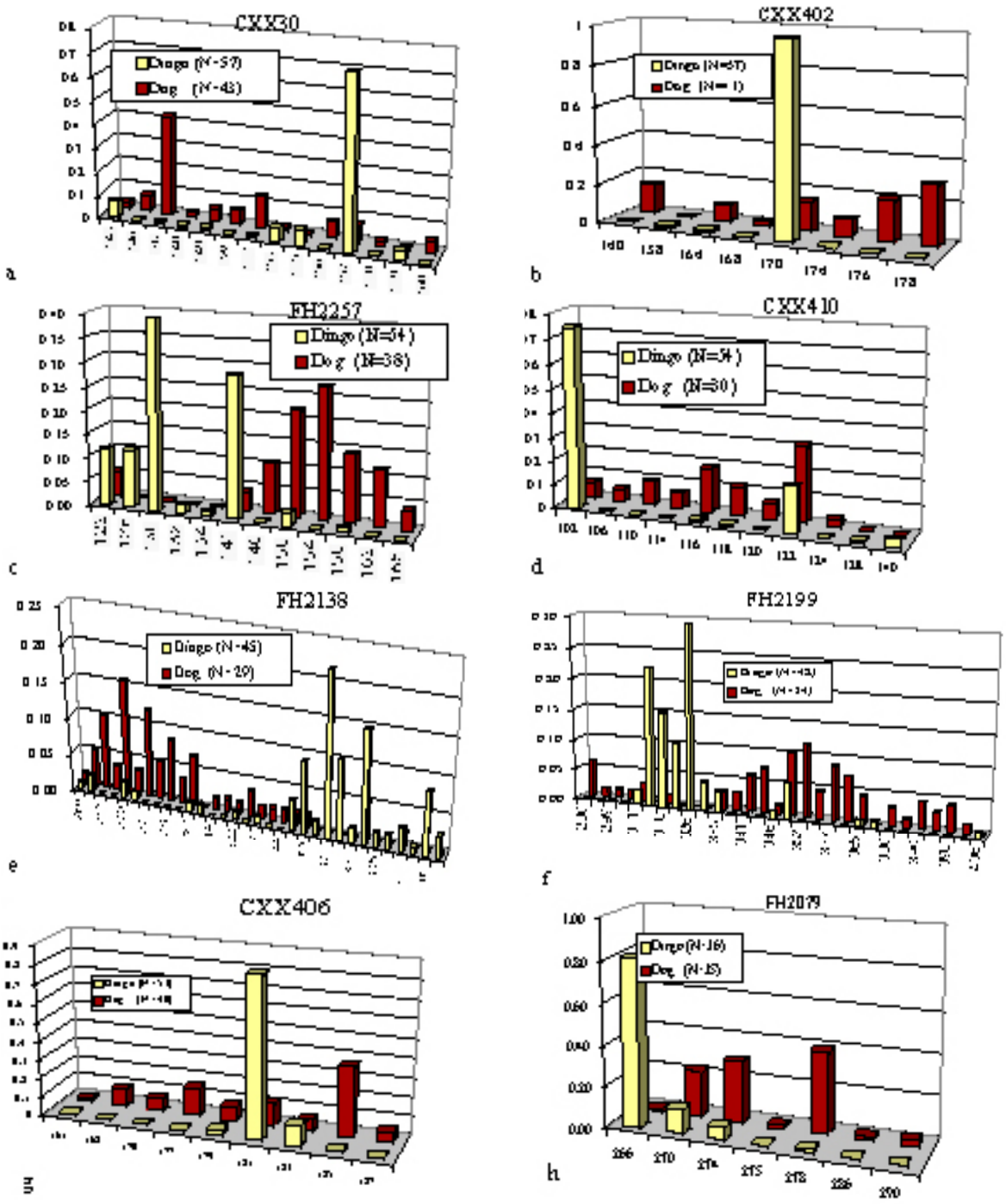

Figure 2. Distribution and frequency of alleles in dingoes and dogs for eight microsatellite loci. Allele sizes are shown in basepairs on the X-axis.

Diagnostic alleles

One method of assessing the purity of an animal is to look for alleles at these 12 loci that have not been previously found in dingoes but are found in dogs. Two or three such alleles among the 12 loci would be strong evidence of some dog ancestry. A single dog allele would not be enough to prove a dingo was not purebred because it may have occurred as a new mutation or it may exist in the pure dingo population but at a very low level or may only occur in some dingo populations which were not sampled.

It was also difficult to determine which alleles are unique to dogs and which to dingoes because the purity of the animals used to select the useful microsatellites was not guaranteed. Several breeds of dog, e.g. Australian cattle dog, had been established from crosses to dingoes and so carry some dingo alleles. While the dingoes used as controls look and behave like dingoes, it is possible that, in a few animals, there was some dog ancestry from a hybridisation event in the wild many generations ago.

Probability approach

One approach that can circumvent these problems is to use probability calculations to assign an animal to its most likely group - dingo, dog, or hybrid - and even estimate the extent of introgression of dog genes into a population. Using the frequencies of the alleles in the two groups, dogs and dingoes, as an estimate of the occurrence in the general purebred populations, and in populations of various mixtures, e.g. 50% dog or 25% dog (Table 1), relative probabilities in the form of Lod scores were calculated for each sample (Fig. 3). For alleles found in one population but not the other, a frequency of 0.5 rather than zero was used to calculate the probability to be conservative. This results in totals larger than 100%.

Logic

To determine whether any sample is likely to be from a hybrid we ask the following questions. How likely are we to find a dingo that looks exactly like this? How likely are we to find an animal in a hybrid population (e.g. 25% dog) that looks exactly like this? How much more likely are we to find this genotype in a purebred dingo than in a hybrid (i.e. the ratio of the likelihoods)? Taking logs of the ratio gives easier numbers to work with. A score of 2 is considered significant, that is 100 times more likely to be from a purebred dingo than a hybrid. A score of 6 indicates that pure dingo is 1,000,000 times more likely. A negative number indicates it is less likely to be purebred dingo.

Making the appropriate comparison

All of the dingoes from captive populations that were used to create the dingo allele frequencies used in the test gave very high positive Lod scores when comparing dingoes to dogs as expected (Fig. 3). All of the dogs gave very low Lod scores. However, a more appropriate comparison is to a hybrid rather than to a dog. Two comparisons of hybrid to dog (i.e. does it have any dingo genes) and dingo to hybrid (i.e. is it more dingo than a hybrid) show the comparisons are useful as they will differentiate between pure dingoes, known crossbred animals and dogs (Fig. 4). Pure dingoes show positive Lods for all tests, showing significantly more dingo content (Lod>2) than even an animal

Table 1. Distribution of alleles in dingoes and dogs for microsatellite CXX402 with the probabilities of finding each allele in a dingo, dog, hybrid (1/2 dingo) and 3/4 dingo hybrid populations.

Alleles	Numbers		Probabilities of allele in population			
Size (bp)	Dingo	Dog	dingo	dog	hybrid	3/4 dingo
140	0	12	0.44%	14.63%	7.32%	3.66%
158	1	0	0.88%	0.61%	0.44%	0.66%
164	1	7	0.88%	8.54%	4.71%	2.79%
168	0	2	0.44%	2.44%	1.22%	0.61%
170	112	12	98.25%	14.63%	56.44%	77.34%
174	0	7	0.44%	8.54%	4.27%	2.13%
176	0	17	0.44%	20.73%	10.37%	5.18%
178	0	25	0.44%	30.49%	15.24%	7.62%
Total	114	82	102.19%	100.61%	100.00%	100.00%

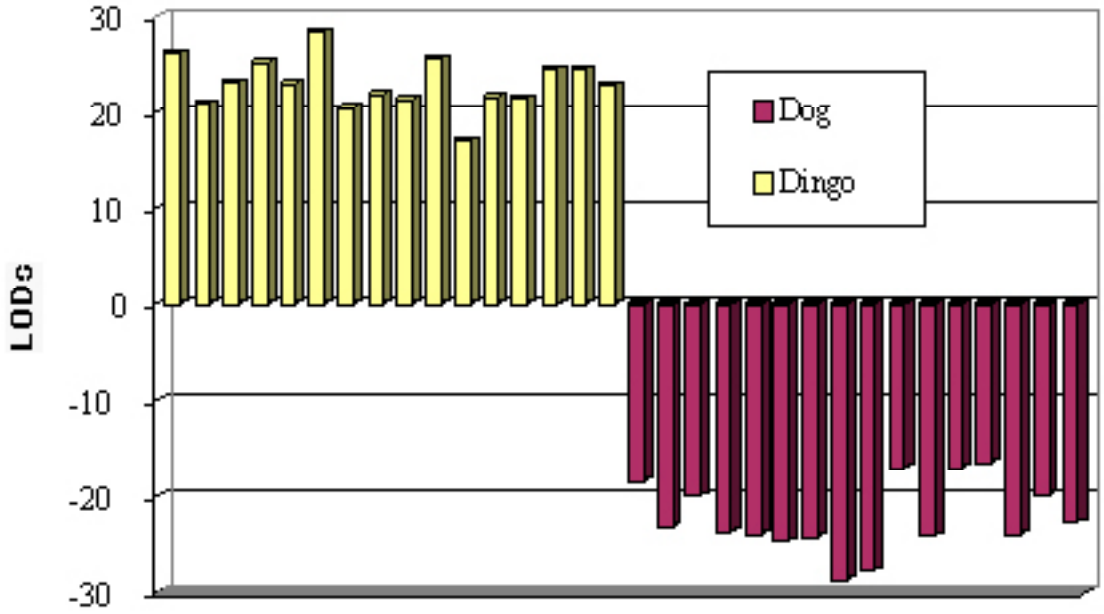

Figure 3. *Lod scores showing the relative likelihood that an animal is from a dingo population compared to a dog population for 16 dingoes (light bars) and 16 dogs (dark bars). Scores based on results from 12 to 14 microsatellites.*

that was three quarters dingo. Pure dogs show negative Lod scores for all tests indicating that they have significantly less dingo content than even hybrids. The five known or suspected hybrids (H1-H5) that were tested show a mixture of negative and positive Lods. They are more likely to be hybrids than pure dogs but less likely to be pure dingoes than hybrids with 75% dingo or 50% dingo (Fig. 4).

Preliminary wild animal results

Preliminary results from wild dingo samples taken by David Jenkins during trapping and release studies in the Kosiuszko National Park area suggest that the level of hybridisation may not be as extensive as has been suggested. Only a small number of microsatellites was used (<5) for these tests and a more accurate estimate of the hybridisation levels requires a more detailed analysis.

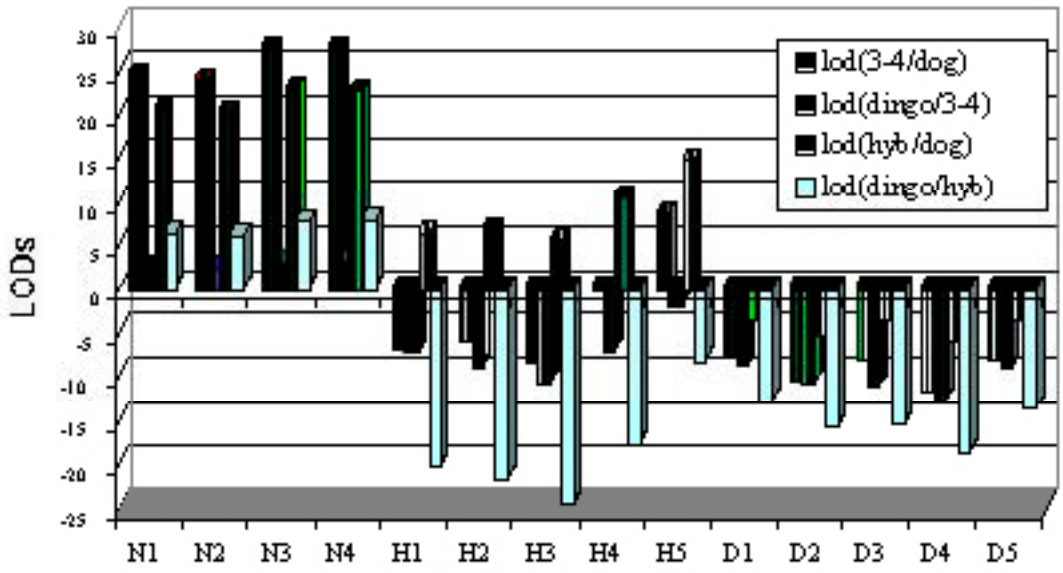

Figure 4. Relative likelihood tests for 4 dingoes (N), 5 suspected hybrids (H) and 5 dogs (D). Tests comparing purebreds to three quarters dingo as well as purebreds to 50% dingoes (hyb), are shown are in two pairs; the likelihood the animal is three quarters dingo rather than pure dog (dots) with likelihood it is pure dingo rather than 3/4 dingo (bricks); and the likelihood the animal is half dingo rather than pure dog (crosshatched) with likelihood pure dingo rather than half dingo.

DISCUSSION

Detection levels

One unanswered question is "what is the lowest level of hybridisation than can be detected by this method"? The answer will depend on the number of markers that are tested. The more tests that are applied, the more chance of detecting a past hybridisation event with dogs. For example, if only one marker was tested a crossbred would have one dog allele and one dingo allele but only half of the progeny of a backcross (hybrid x dingo) would have the dog allele. Further generations of backcrossing dilute the dog genes even further, by one half every generation. Therefore, by 3 generations of backcrossing only 1 in 8 tests would be expected to show evidence of the hybridisation event by the presence of dog genes. For this reason we need to do multiple tests and, even after using 12 microsatellites, a definitive answer as to whether an animal has any dog ancestry and a guarantee of purity cannot be given.

Need for pure baseline

A criticism of the study has been that there is no guarantee that the dingoes used as a control dingo population have absolutely no dog ancestry. The large number of microsatellites that show differences between the dingoes and the dogs suggest that the two group are genetically distinct and there is not a large amount of dog contamination of the dingoes. DNA samples extracted from dingo pelts collected by early explorers would create a good baseline for what would be expected from a pure dingo population.

CONCLUSION

DNA techniques can be used to detect past hybridisation events with dogs. They are best at detecting recent hybridisation events. They can be applied to assessing the purity of populations as well as individual animals. The current methods of obtaining samples are not practical for screening whole populations. The development of methods that can be applied to DNA extracted from scats and hair that can be collected by non-invasive means would allow population screening and assessment of the populations conservation value.

Acknowledgements

Thanks to all those that supplied blood and tissue samples for this study. Special thanks to David Jenkins, NSW Agriculture and members of the Rural Lands Protection Boards for their assistance with sample collection. The results presented were produced with technical assistance of Elisa Mokany, David Steward, Juliet Ward and Katina Zafiris. This research is supported by an ARC SPIRT grant in collaboration with Australian Dingo Conservation Association and Australian Native Dog Conservation Society and has received past support from ARC Small Grants.

References

Corbett, L.K., 1995 *The dingo in Australia and Asia.* University of New South Wales Press Ltd, Sydney

Gottelli, D., Sillero-Zubiri, C., Applebaum, G. D., Roy, M. S., Girman, D. J., Garcia-Moreno, J., Ostrander, E. A. and Wayne, R. K. 1994. Molecular genetics of the most endangered canid: the Ethiopian wolf *Canis simensis*. *Molecular Ecology* **3**: 301-12

Holmes, N. G., Mellersh, C. S., Humphreys, S. J., Binns, M. M., Holliman, A., Curtis, R. and Sampson, J. 1993. Isolation and characterization of microsatellites from canine genome. *Animal Genetics* **24**: 289-292

Mellersh, C., Holmes, N., Binns, M. and Sampson, J. 1994. Dinucleotide repeat polymorphisms at four canine loci (LEI003, LEI007, LEI008 and LEI015). *Animal Genetics* **25**: 125

Mellersh, C. S., Langston, A. A., Acland, G. M., Fleming, M.A., Ray, K., Weigand, N. A., Francisco, L. V., Gibbs, M., Aguirre, G. D. and Ostrander, E. A. 1997. A linkage map of the canine genome. *Genomics* **46**: 326-336

Newsome, A. E., Corbett, L. K. and Carpenter, S. M. 1980. The identity of the dingo. I. Morphological discrimination of the dingo and dog skulls. *Australian Journal of Zoology* **28**: 615-625

Newsome, A. E. and Corbett, L. K. 1982. The identity of the dingo. II. Hybridisation with domestic dogs in captivity and in the wild. *Australian Journal of Zoology* **30**: 365-374

Newsome, A. E. and Corbett, L. K. 1985. The identity of the dingo. III. The incidence of hybrids and their coat colours in remote and settled regions of Australia. *Australian Journal of Zoology* **33**: 363-375

Ostrander, E. A., Sprague, G. F. and Rine, J. 1993. Identification and characterization of dinucleotide repeats (CA)n markers for genetic mapping in dog. *Genomics* **16**: 207-213

Roy, M. S., Geffen, E., Smith, D., Ostrander, E. A. and Wayne, R. K. 1994. Patterns of differentiation and hybridization in North American wolflike canids, revealed by analysis of microsatellite loci. *Molecular Biology and Evolution* **11**: 553-570

Weber, J. and May, 1989. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *American Journal of Human Genetics* **44**: 388-96

Wilton, A. N., Steward, D. J. and Zafiris, K. 1999. Microsatellite variation in the Australian dingoes. *Journal of Heredity* **90**: 108-11

QUESTIONS & ANSWERS

DAVID JENKINS: David Jenkins from the ACT-New South Wales Wild Dog Management Project. Alan, with respect to extracting DNA from scats, how will you do that, because they are going to be full of all sorts of other proteins from what the dog has been eating, to say nothing of parasites it might be harbouring and things like that?

ALAN WILTON: The technique that we use is PCR for amplifying the DNA. There are three billion bases of DNA, and we only want a couple of hundred. We have to be able to target the pieces we want, using small DNA primers of about 20 base pairs. They will be specific to that particular site in the dog. It doesn't matter what sort of tapeworm DNA we get, or what sort E. coli DNA we get, the amplification will not work on those. What we need is at least a couple of molecules of dog DNA from the bit that we are looking for. That won't be a problem for microsatellites. If we are looking at mitochondrial DNA, where everything has mitochondrial DNA, and it is all very similar, that might be a problem, but we are not doing that at the moment.

ERIC DAVIS: Do you have any estimate of likely cost?

ALAN WILTON: For people that have been sending us animals for testing, we have been asking for something like $50 for a one-off sample. If we are doing it on a large scale, it would be much reduced. We are trying to get as many samples as we can for research, so, at the moment, we are certainly not going to charge anybody that wants to send us any samples to test.

ERIC DAVIS: I am just trying to anticipate if the public were required to undergo that sort of testing.

ALAN WILTON: They are trying to do this sort of thing for sheep farming, and things like that. They are working around the cost of $20 for doing it on a large-scale where that is what they do all the time. I don't think you would be doing it at that scale, so if you upgrade it a bit, somewhere between $20 and $50.

ERIC DAVIS: Thanks.

KEN ENGLAND: Ken England, National Parks and Wildlife Service. So you would be encouraging land-holders to send in skin samples from dogs, dingoes or whatever, that they have trapped, shot, poisoned or whatever. Will those results be made available back to the people that send the samples in?

ALAN WILTON: Yes. We would be happy to supply the results to people that send the samples in. It sometimes takes a little while. Unless it is an urgent matter for a sample to be processed because somebody is holding the animal and they want to know whether to destroy it or not, it would usually take a month or so before we got around to extracting the DNA.

Skin is a bit harder than blood samples. Blood is very easy to extract DNA from, so we have been storing a lot of skins to process all in one go. The turnaround on skins is not particularly fast, but we are happy to accept skins from anyone because we can get the DNA from them. We would be happy to supply anybody with the information, and if people have dingoes as pets, I guess we would have to keep the information confidential if people wanted it kept confidential. We are interested in getting samples from everywhere. I have copies of a paper that has come out recently on the work, if anyone is particularly interested in the scientific details.

DAN LUNNEY: Thanks Alan.