GENETIC ANALYSIS OF FORMALIN FIXED TISSUES FROM NON-HUMAN PRIMATES

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INTRODUCTION

Therapeutic development is increasingly taking advantage of the expanding field of human genetics. The genetic similarity between humans and non-human primates has increased the importance of the non-human primate as a model to develop new therapeutic modalities. DNA analysis of targeted genetic loci has revealed genetic variation and similarities that underlie observed idiosyncratic and generalized trends of responses to xenobiotics.

The cynomolgus monkey is the most frequently used non-human primate model for efficacy and safety studies and may derive from several geographical sources, that include Vietnam, Cambodia, Thailand, Malaysia, Indonesia, Mauritius, Philippines, Japan, Burma, China, Borneo, and Java. They have been used for many years without a careful evaluation of their genetic diversity. Currently no information is provided with the animals regarding their genetic background or pedigree prior to entering into a study.

There is a growing body of knowledge suggesting that genetic background can directly influence study parameters and responses to therapeutic agents. Behavior, disease resistance and pathology have been shown to be influenced by genetic background (Bethea, et. al. 2005; Shibata et. Al. 1997: Stevison and Kohn, 2009, Vidal, et. al. 2010.)

Group sizes in a typical non-human primate study are relatively small and when combined with unknown genetic heterogeneity can produce results with a large variance around test parameters. Previous genetic studies of the cynomolgus macaque clearly indicate population differences depending upon their geographical source (Bonhomme et al. (2005, 2008)

Physical/morphological characteristics of individuals differ geographically providing additional evidence of their overall genetic diversity and possible hybridization with other macaque species.

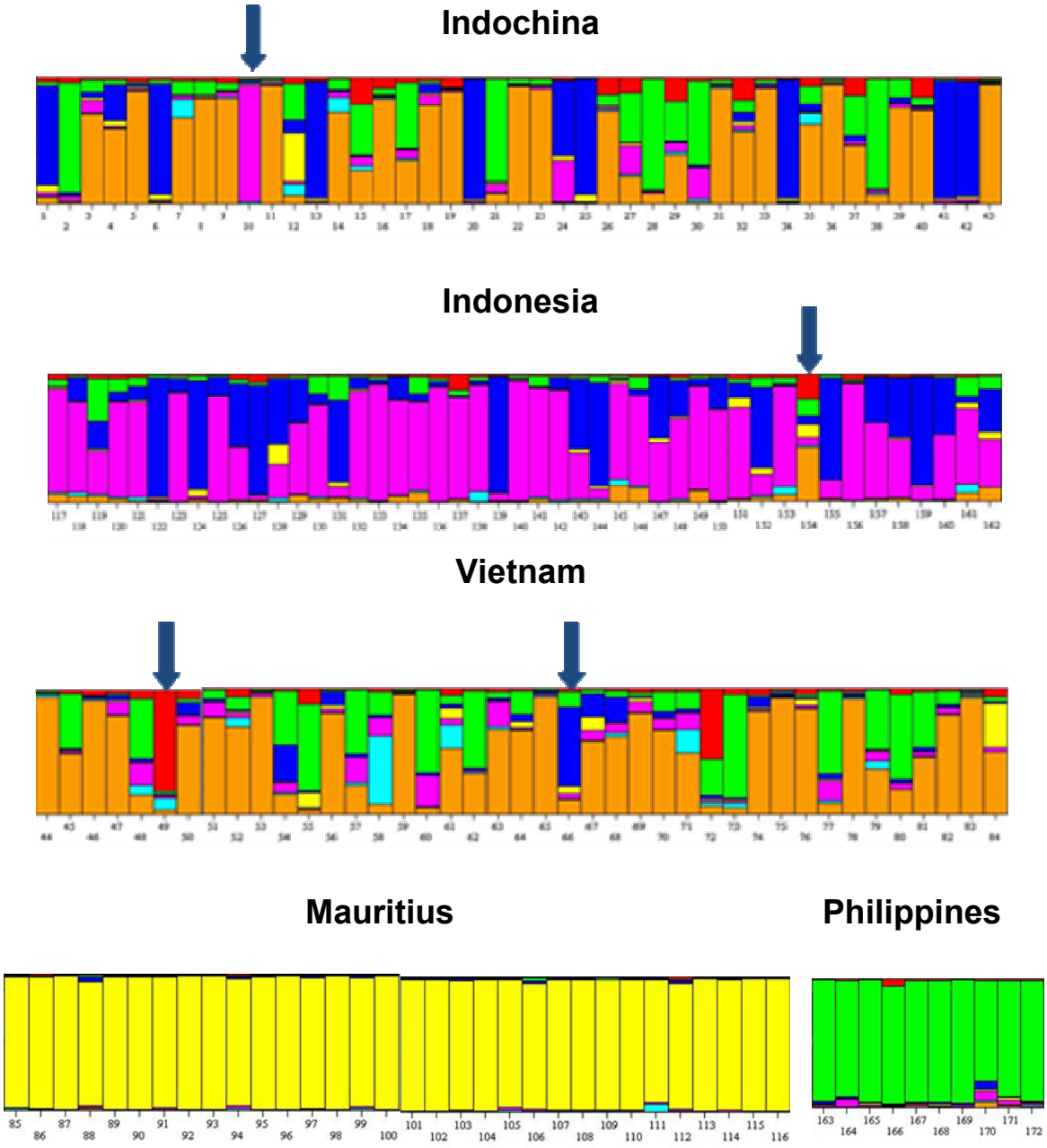
Examination of the genetic profiles using structure analysis of animals used in studies provides clear evidence that significant genetic background differences may occur in an animal within a study group (See Figure 1). This could lead to responses which are completely different from others in their test groups.

Since most studies are performed on animals without any genetic background information, it would be of great value to retrospectively evaluate the genetics of an animal or animal groups which respond differently from the expected outcomes when compared to others within a study group or testing in previous groups.

Each vertical bar is a single individual macaque. Colors indicate the mixture of genotypes that assign the individual to a group or location. Arrows indicate animals which differ significantly from the others from the same source.

Figure 1

Structure assignment of Cynomolgus macaques sourced from five geographic locations.



MATERIALS AND METHODS

Samples evaluated were from recently collected and previously collected specimens.

(Study 1) Frozen blood (w/EDTA), liver and heart tissues, and formalin fixed paraffin embedded liver and heart tissues from 4 cynomolgus macaques were obtained from a recent study.

(Study 2) Fixed paraffin embedded liver and heart from 6 cynomolgus macaques were obtained from a previous study.

DNA Extraction:
DNA was extracted from blood samples using the QIAamp Blood Kit and from frozen heart and liver tissues using the QIAamp Mini Kit (Qiagen, Courtaboeuf). DNA was extracted from one or two shaved sections of each formalin fixed, paraffin embedded tissue using the MagneSil Genomic, Fixed Tissue System (Promega, Corp, #MD1180) according to that protocol. Two separate extractions were carried out for each formalin fixed tissue and individual. Samples were started on the same day and one extracted the next day (1) following the protocol and the second set (2) was extracted 1 week later to test how much time could elapse between the steps in the protocol.

Microsatellite Analysis: All samples were genotyped for 12 human microsatellite loci following conditions in Bonhomme et al. (2005, 2008) and include D1S548, D2S1326, D3S1768, D4S2365, D5S820, D7S2204, D8S1106, D10S1432, D14S306, D16S402, D17S791, and D18S536. PCR reactions used 1 X PCR buffer; 1.5 mM MgCl2; 0.2 mM dNTPs; 4 pmol of each primer; 0.5 U GoTaq Flexi DNA polymerase (Promega, Corp.) and 40 to 90 ng genomic DNA in a total volume of 12.5 µl. PCR products were separated using the Beckman/Coulter CEQ8000 capillary electrophoresis system. The DNA Analysis System Software, version 4.3.9 (Beckman Coulter, Inc.) was used to visualize and size all fragments. Forward primers were fluorescently labeled with WellRed™ Beckman/Coulter dyes.

For those individuals included in the Structure diagram (Figure 1), genotype scoring errors were monitored by re-amplification of approximately 10 individuals at all loci and allele binning by plotting fragment size distribution per locus for each genotype by hand (Amos et al. 2007). Possible scoring errors and null alleles were also checked using Microchecker V.2.2.3 (van Oosterhout et al. 2004).

Statistical analysis: MStools: Microsatellite Toolkit v 3.1 for PC Microsoft Excel (Park 2001) was used to calculate allele frequencies observed and expected heterozygosities. Structure analysis: The Bayesian clustering method, Structure, (Pritchard et al. 2000) was used to infer the number of populations based on genetic variation, without information regarding origin, and assign individuals to a population based on their composite genotype. Genotype data from all individuals was analyzed with a burn-in of 100,000 iterations, followed by 200,000 iterations.

RESULTS

Study 1 (Short term fixation)

Blood and Frozen Tissue:

PCR reactions were performed for each individual DNA sample. Additional reactions, verified the accuracy of allele calls and genotype scores. All samples were genotyped.

Formalin Fixed Tissues:

Fixed paraffin embedded liver and heart tissue from four animals were processed. The two separate extractions, carried out for each tissue and individual one week apart showed that the samples extracted after one day were successfully genotyped (4% failure rate) when compared with samples that sat for one week (16% failure rate).

A total of 96 genotypes were evaluated (2 tissues from 4 animals at 12 loci).

The success rates for the samples are below:

Tissue	**Failure Rate (%)**		
	Day 1 Extraction	1 Week Extraction	Combined
Liver (n=4)	32.0	16.0	13.6
Heart (n=4)	4.5	16.0	0

Genetic variability indices (individual heterozygosity) were also evaluated.

Animal	**Heterozygosity**	
1	10 of 12 loci	83%
2	7 of 12 loci	58%
3	4 of 12 loci	33%
4	8 of 12 loci	67%

Populations of macaques range from 70% to 85% variability on average per individual.

Study 2 (Longer duration fixation)

Formalin Fixed Tissues:

Fixed paraffin embedded liver and heart tissue from six animals were processed. One extraction was carried out for each tissue and individual.

A total of 144 genotypes were evaluated (2 tissues from 6 animals at 12 loci).
120 of the possible 144 (83%) were scored.

One liver sample failed in the analysis.
One locus also failed to amplify in most individuals.
Five genotypes (3%) failed randomly across the matrix.

The summary of failure rates are below:

Tissue	Failure Rate (%)
Liver (n=6)	28.0
Heart (n=6)	7.0

Genetic variability indices (individual heterozygosity) were also evaluated.

Animal	Heterozygosity	
1	6 of 11 loci	69
2	5 of 10 loci	50
3	9 of 12 loci	75
4	9 of 12 loci	75
5	11 of 12 loci	92
6	8 of 11 loci	73

Compiled data from the two tissues provided an overall success rate of 94.4% for all genotypes.

The amount of fixed tissue which enters into the extraction step directly affects the success as tissue slices can vary in thickness and the amount of tissue within the section. When required, two separate extractions can provide complete genotype data for most samples. Some loci may not amplify reliably in formalin fixed tissues and must be carefully selected. Time from tissue set up to extraction does not seem to affect the amplification success rate, although paraffin can create a problem if allowed to cool during the DNA extraction process.

CONCLUSIONS

Genotyping of the formalin fixed embedded tissues was successful for both the new and the historical samples; however, an additional extraction was required to obtain adequate DNA for processing in some cases. The heart consistently provided better results for both the freshly prepared formalin fixed embedded samples and the historical samples from a previous study when compared to the liver specimens.

The ability to retrospectively evaluate the genetic profile of animals may provide an important tool to follow-up on studies with divergent responses leading to data which are difficult to interpret.

Further investigations are required to better identify alternative tissues and the conditions which may result in complete degradation of samples over longer periods of time.

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