Refinement of Non-human Primate Use in Toxicology: Evaluation of Genetic Diversity in Three Geographical Sources of Cynomolgus Monkey (Macaca fascicularis)

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INTRODUCTION

Animal models for research are constantly reviewed and improved. In particular, small animal models have been refined over many years to insure a consistent response when experiments span several shipments of animals and in some cases from multiple sources.

Pharmacologic and toxicologic responses to new agents can be greatly influenced by a number of factors, including background genetic variability. The refinement of rodents through breeding of known strains has provided a supply of genetically homogeneous animals that yield consistent responses to the challenges of new agents. Some rodent animal models are selected based upon their well-understood characteristics for a particular use. For example, the Fisher 344 rat is preferred over the Sprague Dawley rat for metabolism studies due to their similar metabolic pattern for some classes of structures. Conversely, the Sprague Dawley rat is a better model for reproduction studies, as they have a greater fertility rate and an increased number of offspring.

It is interesting to note that the species which are well defined often have large numbers of animals per study group whereas less defined larger animal species usually have smaller number of animals per group in both pharmacologic and toxicologic research.

The cynomolgus macaque (*Macaca fascicularis*) is the most commonly used non-human primate in biomedical research. They have been used for many years without a careful evaluation of their genetic diversity. Cynomolgus monkeys used in research may derive from several geographical sources. Typical sources include Vietnam, Cambodia, Thailand, Malaysia, Indonesia, Mauritius, Philippines, Japan, Burma, China, Borneo, and Java. China does not have an indigenous population of cynomolgus monkeys. They originate from breeding programs of animals derived from other geographic areas and some are possible hybrids with rhesus macaques.

Although end users usually request research animals sourced from a single geographical area, this selection may be based upon past experience, price and availability with minimal regard for genetic background. Currently no information is provided with the animals regarding their genetic background or pedigree.

There is a growing body of knowledge suggesting that genetic background can directly influence parameters which may be important when selecting a population of animals for a particular research project. *Macaca mulatta* alleles have been introgressing into the northern *M. fascicularis* stocks over time. This may be correlated with the higher level of aggression exhibited by Indochinese *M. fascularis* relative to their insular conspecifics (Stevison and Kohn, 2009). Behavior and disease resistance have both been shown to be influenced by the genetic background (Bethea, et. al. 2005; Shibata et. Al. 1997)

Genetic uniformity can insure a more consistent response to a test agent and reduce the occurrence of outlier responses. Several considerations direct us to better understand the genetic homogeneity of the animals used in a study. The group sizes in a typical non-human study are relatively small and when combined with unknown genetic heterogeneity, analyses can result in a large variance around test parameters.

Previous genetic studies of the cynomolgus macaque clearly indicate population differences depending upon their geographical source. Physical characteristics of individuals differ geographically providing additional evidence of their overall genetic diversity. The geographic ranges of the rhesus and cynomolgus monkeys adjoin in Indochina and have lead to a degree of hybridization in zones of contact (Stevison and Kohn, 2009). These studies have measured significant divergence in the populations from the mainland and island populations. Therefore, cynomolgus monkeys from isolated colonies should have less genetic variance between individual animals whereas animals from geographically less isolated areas could be more genetically diverse. Non-human primates for research are sourced from both types of genetic backgrounds. It is clear that the genetic profiles of the cynomolgus monkey can vary greatly depending on location of the source population.

We present here results from our genetic analysis to further characterize the genetic diversity among three population sources and assess the levels of genetic variation within populations currently available and used for research purposes. These data were generated to determine if the animals currently in the supply chain follow the genetic patterns of animals as defined by previous studies.

METHODS AND MATERIALS

***DNA Extraction**:* Blood samples, preserved with EDTA, were obtained from Primate Products Inc. originating from breeding facilities in three countries: China, Vietnam, and Mauritius. DNA was extracted using the QIAamp Blood Kit (Qiagen, Courtaboeuf) and protocol.

Microsatellite analysis: All individuals were genotyped for eight human microsatellite loci (D1S548, D3S1768, D5S820, D7S2204, D8S1106, D10S1432, D14S306, D18S536)(Bonhomme et al. 2005, 2008). All loci were amplified following conditions in Bonhomme et al. (2005) using 1 X PCR buffer; 1.5 mM $MgCl_2$; 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.

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. 2003).

Statistical analysis: MStools: Microsatellite Toolkit v 3.1 for PC Microsoft Excel (Park 2001) was used to calculate allele frequencies observed and expected heterozygosities and to convert data files for other applications. Observed and expected heterozygosities over all loci per population were also calculated using GENEPOP v 3.3 (Raymond and Rousset 1995). GENEPOP was also used to test for linkage disequilibrium between all pairs of loci and significance was determined using Fisher's Exact test with 1000 permutations. FSTAT v 2.9.3 (Goudet 1995) was used to calculate the average number of alleles per locus and F_{IS} per population (Weir and Cockerham 1984). Genetic distance among populations, measured as pairwise F_{ST}, was also calculated in FSTAT and GENEPOP.

Structure analysis: The Bayesian clustering method, Structure, version 2.1 (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. For each value of K, 5 repetitions were conducted. The method of Evanno et al. (2005) was used to determine the true value of K, the number where K plateaus and the value of delta K.

RESULTS

A total of 98 individuals were analyzed from three geographic regions: China (n=33), Vietnam (n=32), and Mauritius (n=33). The following results were observed:

1. Heterozygosity

Average variability measures the proportion of individuals in a population that have two alleles for the markers examined.

Average variability (observed heterozygosity)

Source	
Chinese	77%
Vietnamese	75%
Mauritius	55%

Chinese and Vietnamese macaques are more variable than the population from Mauritius.

2. Genetic diversity

Diversity was measured by the average number of alleles (the forms a gene can take, eg alleles for blood type are A,B, O).

Source	# Alleles
Vietnamese	11.0
Chinese	9.8
Mauritius	4.6

The Mauritius population was lowest in genetic diversity.

3. Genetic differentiation

Differentiation of groups was assessed by the number of private alleles in each population. There were a total of 36 alleles unique to one of the three populations.

Source	# Private Alleles
Vietnamese	24
Chinese	11
Mauritius	1

Genetic differentiation was lowest in the Mauritius population

4. Genetic divergence

The term F_{st} indicates how different populations are from each other.

	Chinese	**Vietnamese**
Vietnamese	**0.0182**	
Mauritius	**0.1208**	**0.1575**

Mauritius has diverged from the other populations.

5. Genetic uniformity

The term F_{is} measures genetic uniformity of a population. Higher values are found in the least variable group.

Source	F_{is}
Chinese	0.031
Vietnamese	0.103
Mauritius	0.169

The highest F_{is} was found in the Mauritius population

FIGURE 1

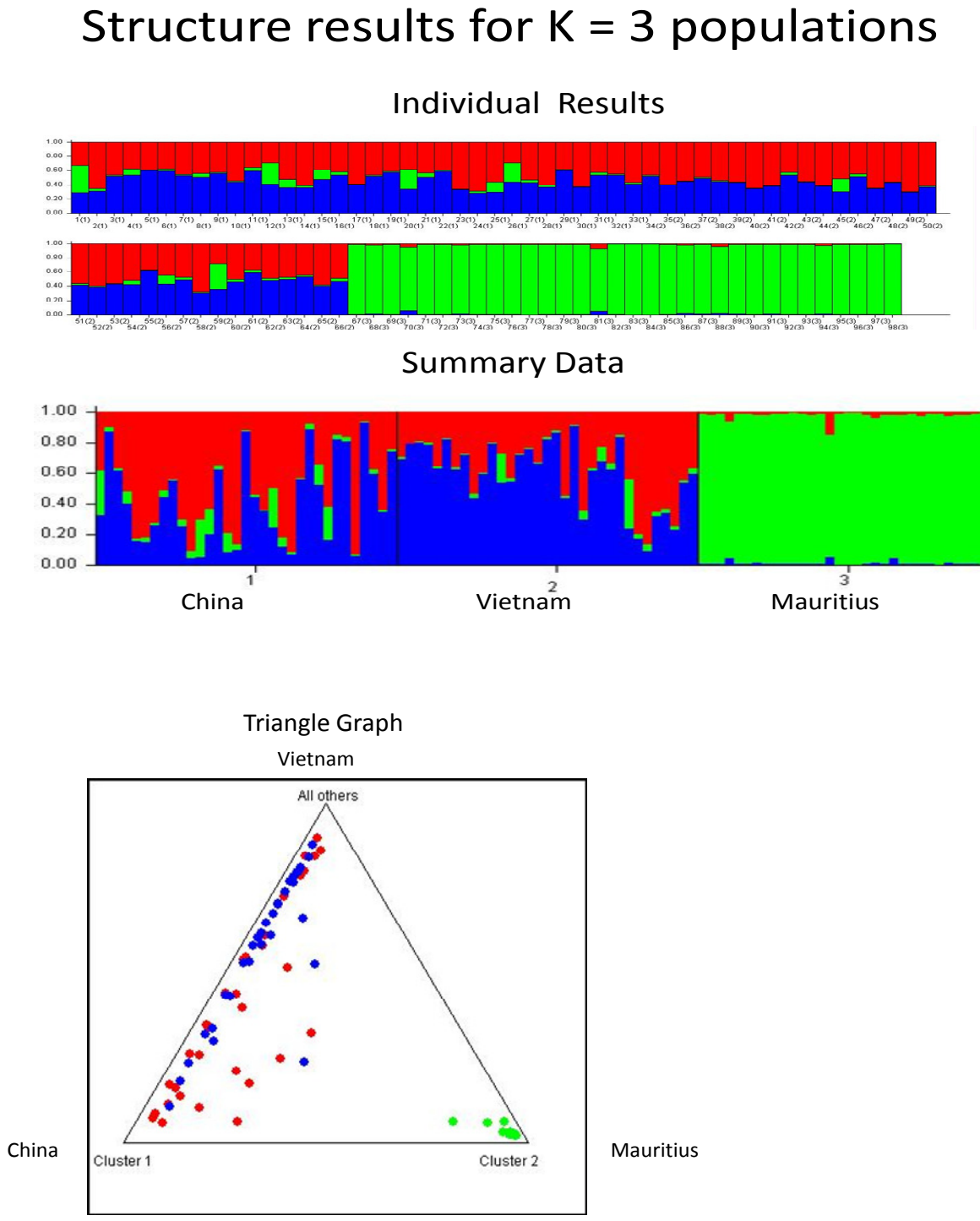

Figure 1. Bayesian Structure, version 2.1, analysis of individual cynomologus macaques using the admixture model with K set at 1 to 4. Results shown are for K = 3 populations. Histogram of assignments shows each individual as a vertical bar and clusters are identified with different colors. Triangular plot represents admixture between each of the three populations. China and Vietnam are represented with red and blue, Mauritius is green. The number of populations measured by Structure based on Delta K is 2

CONCLUSIONS

Higher levels of genetic variation and admixture were observed in the Chinese and Vietnamese populations. However, a surprising number of private unique alleles were present in each group indicating limited gene flow among geographic locations. These variants distinguish these two populations from each other suggesting there are other unique polymorphisms in the individual genome. The Mauritius macaques appear to be the most genetically uniform population. The adaptive value of the uniformity is yet to be determined.

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