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Original Research

Variation in *CCL3L1* Copy Number in Rhesus Macaques (*Macaca mulatta*)

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We used real-time quantitative PCR (qPCR) methodology to examine copy number variation (CNV) of the *CCL3L1* gene among pure Indian-origin, pure Chinese-origin, and hybrid Indian–Chinese rhesus macaques (*Macaca mulatta*). CNV among purebred macaques fell within expected ranges, with Indian macaques having lower copy numbers than those of Chinese macaques. Compared with the purebred macaques, Indian–Chinese hybrid rhesus macaques showed much greater variance in copy number and an intermediate average copy number. Copy numbers of *CCL3L1* in rhesus macaque trios (sire, dam, and offspring) were consistent with Mendelian inheritance.

Abbreviations: CCL3L1, chemokine (C-C motif) ligand 3-like 1; CCR5, chemokine (C-C motif) receptor 5; CNPRC, California National Primate Research Center; CNV, copy number variation; ONPRC, Oregon National Primate Research Center; qPCR, real-time quantitative PCR; STAT6, signal transducer and activator of transcription 6.

Copy number variation (CNV) is a form of genetic structural variation whereby specific segments of genomic DNA are duplicated or deleted, giving rise to greater or fewer gene copies in the duplicated region. These regions vary in size from 1 kb to greater than 1 Mb.[8,9] Extensive research on CNV has been done in both humans[29,32,38] and nonhuman primates, including chimpanzees, humans' closest primate relative.[30] Regions of CNV have been identified in various other mammalian species, including pigs,[6,31] cows,[7,19] and mice.[1,3,5,17] CNV appears to be a regular occurrence and accounts for more than 10% of the human genome. More than 90% of CNV regions are more than 1 kb long and can include intrachromosomal, interchromosomal, interspersed (genes or regions interspersed between duplications), and tandem segmental duplications.[32] If the genomic DNA in a CNV contains a coding region that is actively transcribed and translated, the resultant proteins may be produced at varying levels, affecting inter- and intracellular function as well as susceptibility to disease.[14,20,21]

Although CNV is widespread in several model species, such as rhesus macaques (*Macaca mulatta*) and mice (*Mus muculus*), studies of the effects of population interbreeding and hybridization on markers of this class are unavailable. We used rhesus macaques in the current study in light of their genetic similarities to humans and their wide usage in biologic and biomedical studies, especially in HIV and AIDS research.[11,15,25] Macaques are susceptible to SIV, which provides the best animal model for HIV. The rhesus macaque genome contains an ortholog of the human *CCL3L1* gene, located on chromosome 16, and studies of CNV have involved rhesus macaques[4,18] Rhesus macaque *CCL3L1* encodes the chemokine (C-C motif) ligand 3-like 1 protein, which is a direct competitor of SIV for the chemokine (C-C motif) receptor 5 (CCR5) membrane proteins that SIV uses as a coreceptor to infect $CD4^+$ T cells.

Recent studies in humans have postulated that having more *CCL3L1* copies results in more gene product and, therefore, more successfully precludes binding of HIV to CCR5, lowering infection rates.[22,26] Although a study in MamuA*01 Indian rhesus macaques found that *CCL3L* copy number had no influence on SIV susceptibility,[18] a similar study using statistical models and analysis found a correlation between longer survivorship after SIV infection and *CCL3L1*.[4] Specifically, Indian rhesus macaques have lower copy numbers of this gene and are more susceptible to SIV infection than are Chinese rhesus macaques, which have higher copy numbers and are less susceptible to SIV infection.[4]

In light of previous findings,[4] we sought to test whether copy number follows Mendelian segregation and, therefore, that hybrid rhesus macaques (50% Indian and 50% Chinese ancestry) would have *CCL3L1* copy numbers that are intermediate between those of Indian and Chinese rhesus macaques. Studies of transmission of CNV between parents and their Indian–Chinese hybrid offspring have not previously been conducted. Our data show that the copy number of *CCL3L1* in offspring is consistent with Mendelian inheritance and can be deduced from the number of copies in both parents.

Materials and Methods

Source and composition of samples. Animals involved in this study are maintained in compliance with IACUC regulations that prescribe the humane care and use of laboratory animals. Blood draws for DNA extraction from the study animals were performed in accordance with protocols approved by the University of California Animal Care and Use Committee. The DNA of 151

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Table 1. Oligonucleotide sequences of primers and probes used in the study

Target gene	Purpose	Sequence	Melting point (°C)
CCL3L1	Forward primer	5′ CCA GTG CTT AAC CTT CCT CC 3′	55.3
	Reverse primer	5′ TCA GGC ACT CAG CTC CAG GT 3′	60.8
	Probe	5′ FAM–AGG CCG GCA GGT CTG TGC TGA CC–MGB–NFQ 3′	not applicable
STAT6	Forward primer	5′ CCA GAT GCC TAC CAT GGT GC 3′	58.4
	Reverse primer	5′ CCA TCT GCA CAG ACC ACT CC 3′	57.9
	Probe	5′ VIC–CTG ATT CCT CCA TGA GCA TGC AGC TT–MGB–NFQ 3′	not applicable

FAM, 6-carboxyfluorescein; MGB, minor groove binder; NFQ, nonfluroescent quencher; VIC, 4, 7, 2′-trichloro-7′-phenyl-6-carboxyfluorescein

individual rhesus macaques (*Macaca mulatta*) was used for this study. Of the samples collected, 92 samples were unrelated individuals, cousins, or half-siblings for which we had no parental samples; the remaining 59 samples comprise 23 mother–father–offspring trios. In addition, 3 sires and 2 dams were parents of multiple offspring belonging to different trios. This overlap allowed us to perform a limited half-sibling analysis.

Samples of whole blood from 121 macaques were provided by the California National Primate Research Center (CNPRC), and DNA samples of 30 additional Indian rhesus macaques were purchased from the Oregon National Primate Research Center (ONPRC). Of the 151 rhesus macaque sampled, 43 were descendants of pure Indian-origin rhesus macaques, and 100 were descendants of pure Chinese-origin rhesus macaques. The remaining 8 macaques were hybrid-origin (50% Indian and 50% Chinese) animals, as determined by colony records. The 10 trios from the ONPRC and 3 of the trios from the CNPRC were purebred Indian macaques, whereas those of the other 10 CNPRC trios were purebred Chinese animals.

Except for 5 pure Chinese-origin rhesus macaques of unknown pathogen status, all of the CNPRC rhesus macaques used in this study have been identified as standard (n = 43), SPF (n = 11), or 'super SPF' (n = 92) in terms of pathogen status. SPF macaques are free of B virus (herpesvirus 1), SIV, simian type-D retrovirus, and simian T-lymphotropic virus. Super SPF macaques are free of B virus, SIV, simian type-D retrovirus, and simian T-lymphotropic virus rhesus cytomegalovirus, rhesus rhadinovirus, and simian foamy virus.[13] The ONPRC samples all originated from standard pure Indian-origin macaques.

Sample preparation. DNA was extracted from whole blood by using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. All extracted DNA samples were quantified by using a Qubit 1.0 fluorometer (Invitrogen, Carlsbad, CA), and all DNA preparations were normalized to 4.69 ng/μL.

qPCR studies. Copy number of *CCL3L1* was measured by using qPCR on a 7300 Fast Real-Time PCR System with Sequence Detection Software (version 1.4.0.25, Applied Biosystems, Foster City, CA), and individual samples were analyzed in triplicate on 96-well qPCR plates. We used the gene for signal transducer and activator of transcription 6 (*STAT6*), which encodes a member of the STAT family of transcription factors,[12,16] as our internal positive control because only one copy is present per haploid genome in both rhesus macaques and humans.[2,4]

The primer and probe sequences that we used (Table 1) have been described previously.[4] For each reaction, 2 μL (9.38 ng) normalized DNA was added to a mixture containing 7.65 μL *Taq*Man Universal PCR Master Mix, 2.75 μL H_2O, 0.5 μL each of forward and reverse primers for *CCL3L1*, 0.5 μL each of forward and reverse primers for *STAT6*, and 0.3 μL each of probe for *CCL3L1* and *STAT6* (total volume per well, 15 μL). Cycling conditions included an initial denaturation cycle at 94 °C for 10 min, followed by 40 cycles of 20 s denaturation at 94 °C, 30 s annealing at 55 °C, and 40 s elongation at 72 °C. On each plate, an additional 3 wells were devoted to DNA from the A431 human cell line for use as a reference baseline, and one well contained our negative control. The A431 human cell line has 2 copies of *CCL3L1* and 2 copies of *CCL3* and therefore produces a consistent copy number of 4 for *CCL3*-related genes with the primer set we used.[34]

CNV analyses. The statistical analysis used for this study follows that used previously.[4] A normalized relative copy number was obtained by comparing each sample's *CCL3L1* levels with those of the *STAT6* internal positive control and the reference human cell line. In each well on the qPCR plates, the *CCL3L1* cycle threshold value was subtracted from that for *STAT6*, and the individual differences of each group of triplicate estimates were averaged to infer a copy number for each sample. This inferred relative copy number then was divided by the inferred relative copy number of the A431 reference human cell line to normalize the sample data. Finally, the normalized copy number of each sample was multiplied by 4 to reflect the 4 copies of *CCL3* genes in the A431 cell line. This product was rounded to the nearest integer to provide a final copy number (CNV) for each sample. All discussion and analyses were conducted by using the resultant rounded CNV values.

Once an integer copy number was obtained for both *CCL3L1* and *STAT6* from our rhesus macaque samples, rounded CNV values for the pureblood samples (n = 143) were compared with normal distributions by using statistical analysis. The hybrid samples (n = 8) then were compared with the purebred samples by using boxplots to determine whether the results were consistent with simple Mendelian inheritance. In addition, the rounded CNV values for the 23 sire–dam–infant trios (n = 59) were used in a parent–offspring, full-sibling, and half-sibling regression analysis to assess whether the phenotypes of relatives are consistent with complete (that is, 100%) heritability. Mendelian inheritance models were used to deduce all possible genotypes for the CNV phenotype of each member of all trios. For both the parents and offspring, every allele possible according to each copy number phenotype was taken into account, and combinations that were not possible under Mendelian inheritance were discounted.

Results

Controls. The *STAT6* CNV values had a consistent median of 4 copies in every run. Conversely, the human reference samples produced some inconsistencies, with several of the reference

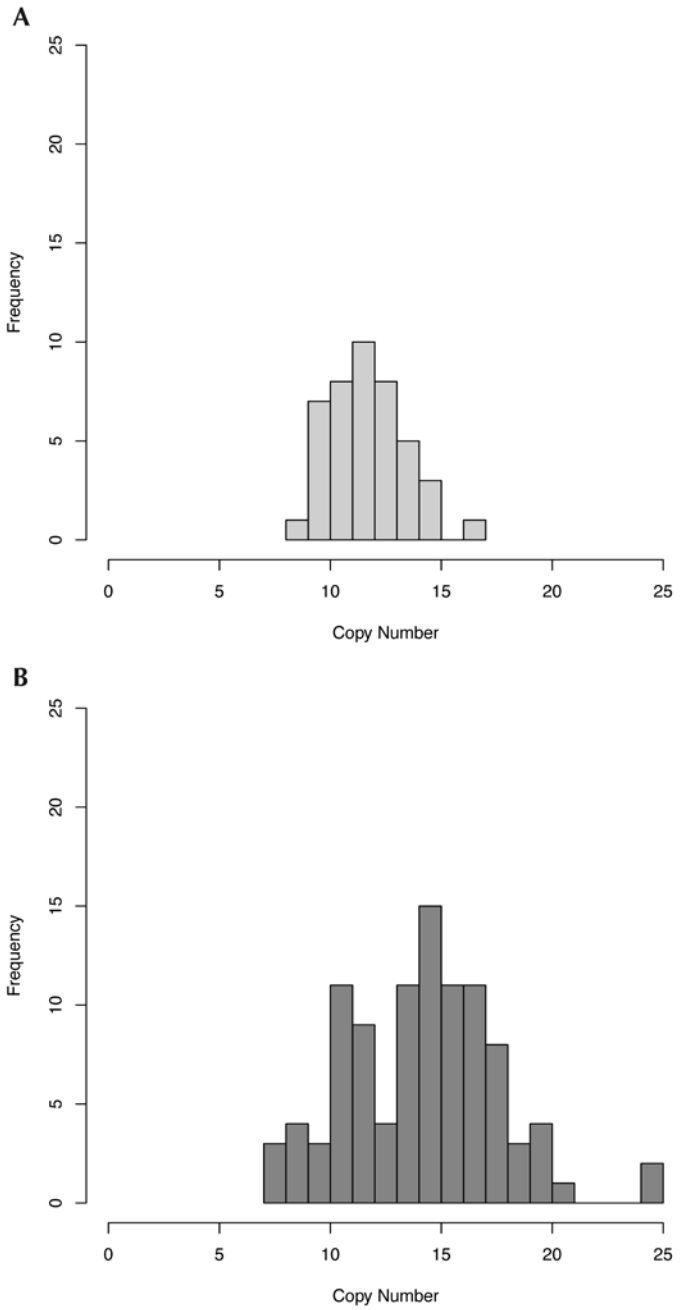

Figure 1. Histograms of the qPCR-estimated copy numbers of *CCL3L1* genes for both the (A) 43 Indian *M. mulatta* samples and (B) 100 Chinese *M. mulatta* samples.

A431 human cell lines not producing usable results when run in triplicate; therefore, these qPCR plates were excluded. In each of these instances, the qPCR reaction was repeated, and the second run yielded appropriate results every time.

CNV results by geography. The median copy number of *CCL3L1* for all 151 rhesus macaques was 13, with an arithmetic mean of 12.9± 3.3 copies. Measurements of copy number ranged from 8 to 17 in Indian-origin rhesus macaques and from 7 to 25 in Chinese-origin rhesus macaques (Figure 1). CNV data for *CCL3L1* and *STAT6* are summarized in Table 2. Considering the values for the first and third quartiles within each boxplot, the largest proportion of Indian-origin rhesus macaques had 10 to 12 copies. The more highly variable Chinese rhesus macaques had a range of 11 to 16 copies. Indian-origin animals had a median of 11 copies compared with 14 copies for their Chinese-origin counterparts. The arithmetic means of the 2 samples were very similar to their respective medians. The Indian rhesus macaques had a mean average of 11.2 copies, whereas the mean average of the Chinese rhesus macaques was 13.7 copies, as illustrated by the boxplot data.

We then verified whether the sample data statistically significantly deviated from a normal distribution by using 3 standard normality tests (Table 3). The sample data for 3 of the tests (Shapiro–Wilk, Anderson–Darling, and Cramér–von Mises) were analyzed both as a whole and separately by geographic origin. In every case, the null hypothesis that the distribution is normal ($P < 0.05$) was rejected. For this reason, further statistical analysis of the Indian-origin and Chinese-origin samples was conducted by using 2 nonparametric tests. A one-sample test against a normal distribution and a 2-sample test of the Indian-origin samples compared with the Chinese-origin samples were conducted by using the Kolmogorov–Smirnov test. A one-sample signed rank and 2-sample ranked-sum Mann–Whitney–Wilcoxon tests also were performed. The null hypothesis for the nonparametric tests was that the 2 groupings belonged to a common, continuous distribution at the 0.05 level of probability. The results of these tests (Table 4) rejected the null hypothesis for at least the 0.05-level of probability, suggesting that the *CCL3L1* CNV values obtained for the Indian and Chinese rhesus macaque samples were parts of 2 separate distributions. Figure 2 compares CNV density distribution

Table 2. CNV boxplot data for all rhesus macaques (by ancestry)

		% Chinese ancestry			
		0%	50%	100%	Human
CCL3L1					
	First quartile	10	10.5	11	4
	Minimum	8	8	7	4
	Median	11	11.5	14	4
	Maximum	17	18	25	4
	Third quartile	12	14.3	16	4
STAT6					
	First quartile	4	3.8	3	4
	Minimum	3	3	3	4
	Median	4	4	4	4
	Maximum	5	4	5	4
	Third quartile	4	4	4	4

Table 3. Normality tests of *CCL3L1* CNV data

	Shapiro–Wilk		Anderson–Darling		Cramér–von Mises	
	W	*P*	A	*P*	W	*P*
Indian	0.937	0.0204	0.817	0.032	0.133	0.039
Chinese	0.964	8.44×10^{-3}	0.790	0.039	0.121	0.058
All data	0.956	6.86×10^{-5}	1.56	4.97×10^{-4}	0.259	1.00×10^{-3}

Table 4. Nonparametric independence tests of *CCL3L1* CNV data

	Kolmogorov–Smirnov		Wilcoxon or Mann–Whitney[a]	
	W	*P*	W or V[b]	*P*
Two-sample test (Indian compared with Chinese)	0.457	7.03×10^{-6}	3159.5	8.02×10^{-6}
One-sample test (all samples compared with standard)	1.000	$<2.2 \times 10^{-16}$	12246	$<2.2 \times 10^{-16}$

[a]This test has a varying nomenclature: for one-sample testing, it is called the Wilcoxon signed-rank test; for 2-sample testing, it is called the Wilcoxon rank-sum test or Mann–Whitney *U* test.
[b]The Wilcoxon signed-rank test produces a V value, whereas the Wilcoxon and Mann–Whitney rank-sum tests produces a W value.

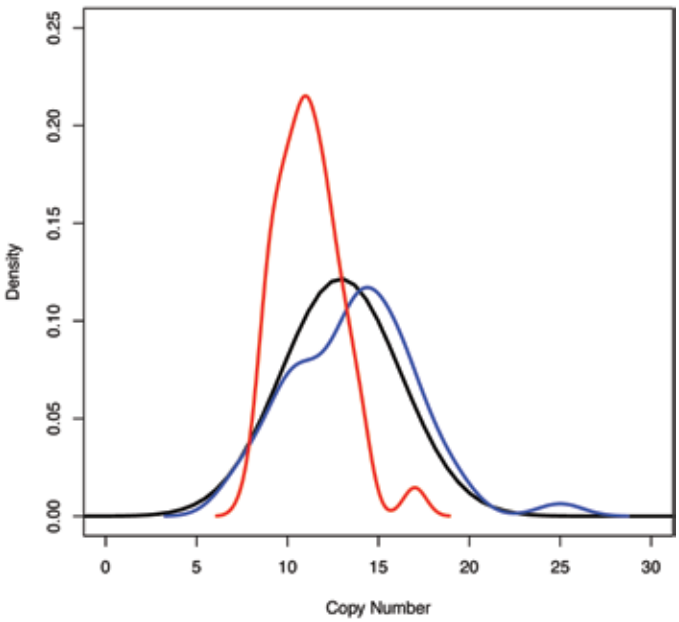

Figure 2. Density distribution for *CCL3L1* copy-number variation (separated). Indian-origin *M. mulatta* samples (red) show a narrow sample range with a lower mean than the normal distribution (black), whereas Chinese-origin *M. mulatta* samples (blue) show a uneven range with a higher mean than the normal distribution.

between Indian and Chinese rhesus macaques and includes a normal distribution reference line.

Indian–Chinese hybrid study results. The average *CCL3L1* copy number of the 8 macaques with 50% Indian, 50% Chinese ancestry generally fell between those of purebred Indian and Chinese rhesus macaques, with mean (12.5) and median (11.5) CNV values closer to those of purebred Indian-origin animals. In addition, the overall range (8 to 18 copies) of CNV for the hybrid macaques fell between the ranges for Indian-origin and Chinese-origin macaques. Furthermore, the qPCR runs for the 50%–50% hybrids produced a range (10.5 to 14.3 copies) that fell directly between those for the 2 purebred populations, as shown in boxplot analyses (Figure 3).

Mendelian inheritance study results. The *CCL3L1* copy number of the parents of the 23 offspring included in the trios ranged from 8 to 17 copies, compared with 9 to 18 in their offspring. By using Mendelian inheritance models for each trio set, it was possible to combine one or more hypothetical allele(s) from each of the 2 parental possible genotypes to produce a genotype that correctly matched the phenotype of 22 of the 23 offspring (Figure 4). In cases of full- or half-siblings, the only possible alleles chosen for each common parent were those that could have produced both offspring. Inherited allele possibilities ranged from 0 to 16 copies, and the largest number of allelic possibilities fell between 2 and 9 copies.

A Mendelian trend in *CCL3L1* copy number transmission was observed for all but one of the 23 trio sets (Figure 5). In this incongruent set, dam 36019 displayed a *CCL3L1* phenotype of 9 copies that, when paired with the sire's (35854) copy number phenotype of 14, could not produce a viable genotype to concomitantly account for the phenotypes of both her offspring, macaques 39405 and 40379. Whereas macaque 39405 had a copy number of 7, macaque 40379, its alleged full sibling, produced a copy number of 14. Because 35854, the alleged father of these offspring, also sired several of the offspring in the trio study, he was inferred to have conferred allele possibilities of 5, 6, or 7 copy numbers to each offspring. No unique allele for 36019 could be paired with the possible paternal alleles that would serve to explain both the copy number of 7 for sample 40379 and the copy number of 14 for sample 39405.

Discussion

Previous studies suggest because Chinese-origin rhesus macaques have more copies of CCL3-like genes, they also have a greater resistance to SIV than do Indian-origin rhesus macaques.[4] Although the difference in average copy number between the Indian and Chinese rhesus macaques in the present study was less than that reported previously,[4] our analysis is consistent with the previous findings[4] that suggest a link between variations in copy number in the 2 geographically defined rhesus macaque populations.

In addition to CCL3L1 copy number, Chinese and Indian origin macaques have other significant differences that may contribute to their different levels of SIV resistance. Several other contemporary theories of SIV resistance macaques that might differ between Indian and Chinese rhesus macaques include restriction by specific class I major histocompatibility complex alleles,[23,24,27] polymorphism in CCR5[40] (a key cell-surface coreceptor for HIV1), postentry inhibition of viral replication in $CD4^+$ T cells,[28] and amino acid substitution in the *TRIM5*α gene.[37] In addition, Indian and Chinese rhesus macaques show significant linkage disequilibrium at the proximal end of chromosome 1, in the vicinity of the *HIVEP3* gene;[35] this gene codes for a zinc finger protein whose only known function is to bind to HIV1[33] and has undergone positive selection in humans.[36]

The mean CNV levels for the Chinese–Indian hybrid samples were widely variable but fell in a linear fashion between those of the pure Indian-origin and pure Chinese-origin macaques (Figure 3). The higher CNV in the admixed animals is expected because these hybrids have been produced by different types of matings (that is, copy numbers in the 50% Chinese animals produced from

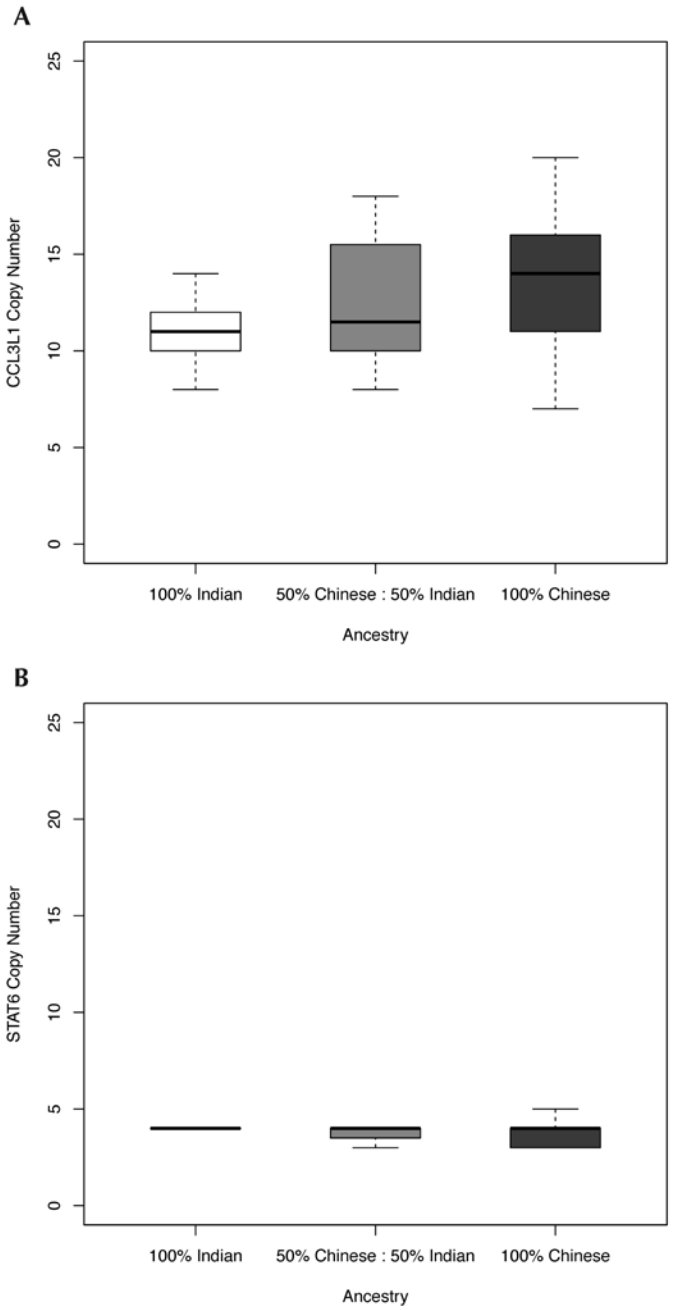

Figure 3. Detailed boxplots of *CCL3L1* (a) and *STAT6* (b) CNV for all *M. mulatta* individuals. Data is sorted by ancestry, with labels based on percentages of Indian and Chinese ancestry.

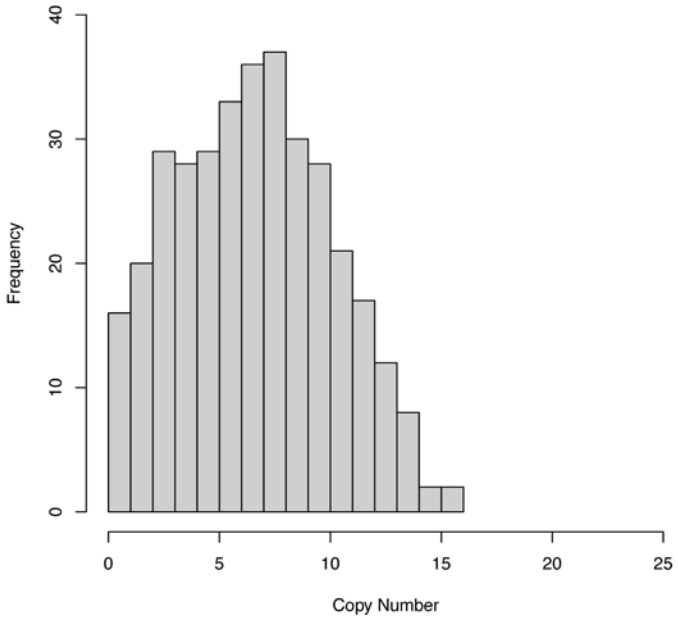

Figure 4. Possible *M. mulatta CCL3L1* alleles passed on through Mendelian inheritance.

mating fullbred Chinese with fullbred Indian macaques will be different from those from animals whose parents were hybrids themselves). Despite the variance, the mode of CNV inheritance among the hybrids appears to mendelize.

In addition, the segregation of nearly all potential diploid allele sizes in the trios is consistent with Mendelian inheritance. With total copy number phenotypes for all 151 rhesus macaques in the study ranging from 7 to 20, with two 25-copy-number outliers, we expect gamete alleles in a Mendelian model to produce results of CNV/2, or 3 to 10. Allele transmission predictions for trios in the present study were strongest between the copy numbers of 2 and 9, thus also supporting Mendelian inheritance. The copy number levels for either of the offspring of 36019, whose *CCL3L1* copy number phenotype was inconsistent with the copy numbers predicted for her offspring (39405), may be accurate if a randomly mutated gamete was created though errors in the recombination cycle. The copy number levels could have been overamplified or inhibited if 39405 displayed mosaicism or other genetic abnormalities. Finally, the probes or primers might have been unable to fully anneal due to basepair mutations in established copies of *CCL3L1*. Regardless, a Mendelian trend in *CCL3L1* CNV transmission was still observable in 22 of the 23 trios, including with 36019's other offspring (40379).

A qPCR-based method of estimating CNV is not perfect, but it provides a rapid and close approximation of CNV values.[39] We modeled our qPCR process to be extremely robust by using 2 validated references and basing it on a relativistic, qualitative approach rather than on static or absolute quantification. We chose this model because our main focus was qualitative comparison of *CCL3L1* CNV within the primate genetic context. Because we focused more on intersample precision during each qPCR run and less on the absolute accuracy of the copy number, the sensitivity and quality of the reference samples, as well as any noise produced by the qPCR amplification, carried less weight in the determination of our final CNV results.

Any individual sample is subject to error, as might be the case with the trios that included dam 36019. A well that failed to generate a cycle threshold value for either *CCL3L1* or *STAT6* would not have been counted in the run, and data from the 2 remaining wells would have been averaged to produce a rounded CNV count for the macaque. Polymorphisms due to rare mutations at primer annealing or probe target sites might lead to erroneous measurements of copy number.[10] Our reference human cell line failed to yield usable cycle threshold readings during several plate runs. Because the results for all of the samples on the plate were analyzed based on the reference human cell line, all of the

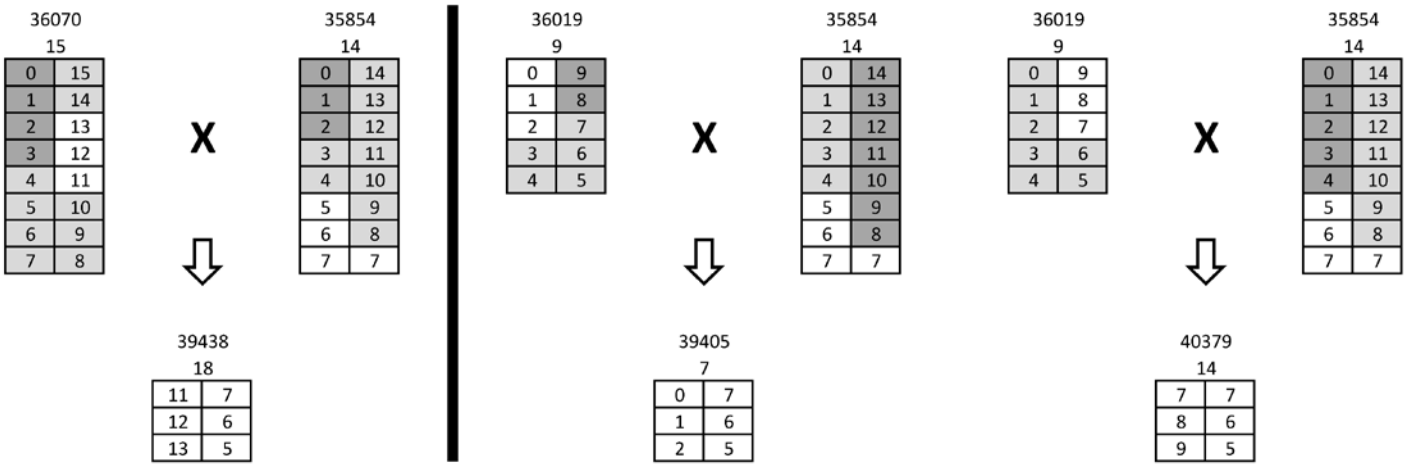

Figure 5. Inferred *CCL3L1* CNV allele transmission from parents to offspring. Sire 35854's allele possibilities were determined by graying out all alleles that are unable to produce any one of sire 35854's offspring. In a normal allele analysis (left panel), the possible sire alleles are transferred to the infant, and the dam's possible alleles are determined by subtracting the sire's allele possibilities from the phenotype. Only 2 trios of 23 (right panel) resulted in conflicting dam allele possibilities.

samples on the plate with the faulty cycle threshold readings for the human cell line might have been unreliable and unusable. These erroneous results might have occurred due to human error; however but the human cell line may have reacted to the *CCL3L1* and *STAT6* primers or probes in an unexpected manner. Because we were able to study only a limited number (n = 8) of hybridized Indian–Chinese rhesus macaques, a larger study should be undertaken to confirm their intermediate *CCL3L1* copy number.

Indian and Chinese rhesus macaques exhibit 2 distinct distributions of *CCL3L1* copy numbers, suggesting a link between CNV of *CCL3L1* and geographic origin that accounts for the differences in SIV resistance. In agreement with previous findings,[4] our Chinese-origin rhesus macaques carried more copies of *CCL3L1*; however, the difference in average copy between Indian and Chinese rhesus macaques we observed was lower than that reported previously.[4] The CNV of *CCL3L1* in the rhesus macaque trios (sire, dam, and offspring) exhibited values consistent with Mendelian inheritance, and allele transmission prediction was strongest in the 2- to 9-copy range.

Acknowledgments

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