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Germ-line chimerism and paternal care in marmosets (*Callithrix kuhlii*)

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The formation of viable genetic chimeras in mammals through the transfer of cells between siblings *in utero* is rare. Using microsatellite DNA markers, we show here that chimerism in marmoset (*Callithrix kuhlii*) twins is not limited to blood-derived hematopoietic tissues as was previously described. All somatic tissue types sampled were found to be chimeric. Notably, chimerism was demonstrated to be present in germ-line tissues, an event never before documented as naturally occurring in a primate. In fact, we found that chimeric marmosets often transmit sibling alleles acquired *in utero* to their own offspring. Thus, an individual that contributes gametes to an offspring is not necessarily the genetic parent of that offspring. The presence of somatic and germ-line chimerism may have influenced the evolution of the extensive paternal and alloparental care system of this taxon. Although the exact mechanisms of sociobiological change associated with chimerism have not been fully explored, we show here that chimerism alters relatedness between twins and may alter the perceived relatedness between family members, thus influencing the allocation of parental care. Consistent with this prediction, we found a significant correlation between paternal care effort and the presence of epithelial chimerism, with males carrying chimeric infants more often than nonchimeric infants. Therefore, we propose that the presence of placental chorionic fusion and the exchange of cell lines between embryos may represent a unique adaptation affecting the evolution of cooperative care in this group of primates.

callitrichid | genetic chimerism | genomic conflict | social behavior

Genetic chimerism, the mingling of two or more genomic lineages within an individual (1), is rare in mammals, but chimerism is prevalent in the hematopoietic tissues of marmosets and other callitrichid primates (2, 3). In these species, fraternal twins exchange cell lines through chorionic fusion during early development (2, 4, 5). On the basis of karyotypic evidence from *Callithrix jacchus* (2, 3), estimates are that 95% of pregnancies result in the birth of hematopoietic chimeric twins. Chorionic fusion of the twins' placentas begins on day 19 and is complete by day 29, forming a single chorion with anastomoses connecting the embryos, which are still at a presomite stage in development (4–7). The fusion of the chorions and a delay in embryonic development at this stage allows the exchange of embryonic stem cells via blood flow between the twins (2, 8). As a result, the infants are genetic chimeras with tissues derived from self and sibling embryonic cell lineages (2, 3, 8).

Although there is little doubt that tissues derived from hematopoietic origin are universally chimeric (9), the existence of chimeric cells in nonhematopoietic tissues, including germ-line cells, has not been established. Karyotypic analysis of *C. jacchus* revealed that testes cells express unusual orientation during meiosis, and this evidence suggested that the germ-line cells might include female cells present because of chimerism (8, 10). However, further karyotypic analysis refuted these findings, and an analysis of sex ratios in captive colonies of *C. jacchus* suggested that germ-line chimerism was not present (11). To investigate whether chimerism occurs in tissues other than those derived from the hematopoietic system, species-specific micro-

satellite markers were used to examine the extent and distribution of chimerism.

The existence of chimerism throughout somatic and germ-line tissues may have important implications for the evolution of paternal and alloparental care characteristics of this taxon, through genomic conflict or altered perceptions of relatedness between members of a family group (12, 13). Genomic conflict in individuals with genetic heterogeneity has been identified as a possible evolutionary mechanism, influencing behavioral and developmental traits (14–17). Conflict within an individual may influence the development of kin recognition mechanisms. Specifically, how do chimeric organisms identify an individual and determine relatedness to another chimeric individual (12)? Somatic chimerism may provide individuals with self-matching kin recognition cues, causing an overestimate of their relatedness to chimeric offspring. Although the exact mechanisms of kin recognition are unknown in primates, baboons appear to be capable of recognizing paternal offspring, which may involve phenotype matching (18). Phenotype matching has been conclusively demonstrated to occur in at least one mammal species (19). If chimerism in marmosets involves more than hematopoietic tissues, then we predict differential parental behavior toward chimeric and nonchimeric infants and altered estimates of relatedness from those expected for nonchimeric mammals.

Results

We examined the prevalence of chimerism in tissues derived from different embryonic origins by analyzing genotypes of microsatellite loci with a probability of detecting chimerism of 98% based on parental genotypes for these loci. A total of 92 intergenerational individuals that included 36 twin sets of *Callithrix kuhlii* (Wied's black tufted-ear marmosets) and their parents were assessed. The samples were genotyped in an appropriate blind fashion such that the identity of the individual and the tissue type were unknown. All alleles were noted for each locus, and samples were identified as potentially chimeric if they contained three or four allelic variants at a single locus. The samples were then matched to identity, and twins were noted to be chimeric at a tissue only if the alleles were found to match both the parents as well as their twin. Further, a majority rule approach was used to assign alleles as “self” (i.e., diploid and inherited vertically from the parents) and “sibling” (inherited horizontally from the twin *in utero*) [see example in supporting information (SI) Fig. 3]. Of the 36 twin sets surveyed, 26 (72.2%) were determined to carry chimeric tissues. Exchange of alleles

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Table 1. The number of *Callithrix kuhlii* individuals chimeric for each tissue type

| Tissue | Tissue type | Genotyped, no. | Chimeric, no. | Chimeric, % |
|-------------------------------|-------------|----------------|---------------|-------------|
| Samples from deceased animals | | | | |
| Placenta | H | 7 | 7 | 100.0 |
| Blood | H | 2 | 2 | 100.0 |
| Spleen | H | 28 | 14 | 50.0 |
| Liver | H | 39 | 15 | 38.5 |
| Heart | S | 30 | 7 | 23.3 |
| Hair | S | 35 | 6 | 17.1 |
| Lung | S | 30 | 4 | 13.3 |
| Kidney | S | 33 | 4 | 12.1 |
| Gonad | G | 21 | 2 | 9.5 |
| Skin | S | 36 | 2 | 5.6 |
| Brain | S | 31 | 1 | 3.2 |
| Muscle | S | 34 | 1 | 2.9 |
| Samples from living animals | | | | |
| Sperm | G | 7 | 4 | 57.1 |
| Saliva | S | 31 | 16 | 51.6 |
| Blood | H | 45 | 22 | 48.9 |
| Hair | S | 50 | 13 | 26.0 |
| Fecal | S | 22 | 2 | 9.09 |

H, hematopoietic; S, other somatic; G, germ line.

between twins was not always bidirectional. In 14 twin sets, only one twin was found to have chimeric tissue types, whereas the other 12 chimeric twin sets revealed chimerism in both twin's tissues.

Chimerism was found to be present in every tissue that was analyzed, and the occurrence of chimerism in tissues harvested from marmoset cadavers differed significantly across liver, spleen, kidney, heart, lung, brain, muscle, skin, and hair ("Samples from deceased animals" in Table 1; $n = 25$, Cochran's $Q = 51.6$, $df = 8$, $P < 0.001$). To determine which tissue type accounted for the variance among the tissues, the tissues were ranked according to percentage of occurrence of chimerism and then grouped. A comparison of liver and spleen revealed a nonsignificant difference between the tissue types ($n = 25$, $Q = 4$, $df = 1$, $P = 0.15$). The addition of hair samples revealed a significant difference between the tissue types ($n = 25$, $Q = 14.3$, $df = 2$, $P < 0.001$). The grouping of all other tissues (heart, hair, lung, kidney, skin, brain, and muscle) resulted in no significant difference between the tissue types for the presence of chimerism ($n = 25$, $Q = 10.3$, $df = 6$, $P > 0.1$). Hematopoietic tissues were significantly more likely to be chimeric than all other tissue types ($\chi^2 = 4.88$, $df = 1$, $P < 0.05$). The assessment of chimerism in tissues collected from living marmosets revealed nonsignificant differences between the tissue types ("Samples from living animals" in Table 1).

The presence of sibling-derived alleles in multiple tissues suggested that all embryonic cell lineages in *C. kuhlii* might be affected by chimerism, including gametic tissue. In fact, gonadal tissue was found to be chimeric (2/21), and sperm samples were also chimeric (4/7). Additionally, the 36 twin sets analyzed for chimerism comprised multiple generations within 15 family lines. We determined that individuals in 5 of the 15 families passed on alleles to their offspring that represented gene lineages inherited horizontally from the sibling (see examples in Fig. 1 and SI Fig. 4). One breeding female, whose uterine twin was a male, produced offspring that inherited her sibling's alleles. This documents the possibility that an XY primordial germ cell is capable of maturing and producing viable eggs in a female, a phenomenon that has not been documented for primates. Although we are not currently able to document the fate of the Y chromosome during development of the female's oocytes, our

data suggest the intriguing possibility that a female may pass on a Y chromosome to her offspring.

The presence of cells derived from different lineages within an individual may impact behavioral decisions. Genetic chimerism may give rise to genomic conflict such that an individual's decision to cooperate within a group and care for members of the group may depend on the true, or perceived, genetic relatedness between the individuals (12, 16). To illustrate this with a simple example, we consider the increased proportion of shared alleles, because of genetic chimerism, between male twins produced by nonchimeric parents. A chimeric individual's coefficient of relatedness to his twin could increase from the expected fraternal twin value of $r = 0.5$ to as much as $r = 1$ in certain tissues. Based on the prevalence of chimerism, the proportion of cells within a tissue that carry sibling alleles, and the probability of the direction of exchange obtained from our data, we estimate that male twins are on average related by $r = 0.574$ (see SI Text for calculations). More specifically, in a case of unidirectional exchange in which the soma of the donating twin is nonchimeric, he is related to the sperm of the recipient twin by an average r of 0.625 (see SI Text). The relatedness calculations suggest that chimeric callitrichid siblings are more closely related to each other than typical nonchimeric mammalian siblings. Calculations of relatedness under more complex scenarios and involving parental chimerism are beyond the scope of this report; thus, at this stage, it is not known how parental-offspring relatedness may be affected by chimerism.

A different issue, but also with potential behavioral implications, is perceived relatedness through kin recognition. To investigate whether the presence of chimeric epithelial tissues, those most likely to mediate kin recognition, are associated with changes in parental care, maternal and paternal infant carrying effort for the first 2 weeks of life was compared between infants determined to be epithelial chimeras ($n = 10$) and those determined to be nonchimeric ($n = 20$). Alloparents do not typically provide care during this early period (20) and thus were not included in the analysis. Females carried chimeric infants significantly less than nonchimeric infants ($F_{1,28} = 7.61$; $P = 0.01$), but fathers carried chimeric infants significantly more than nonchimeric infants ($F_{1,28} = 12.08$; $P = 0.002$) (Fig. 2). No significant differences in carrying time by mothers or fathers

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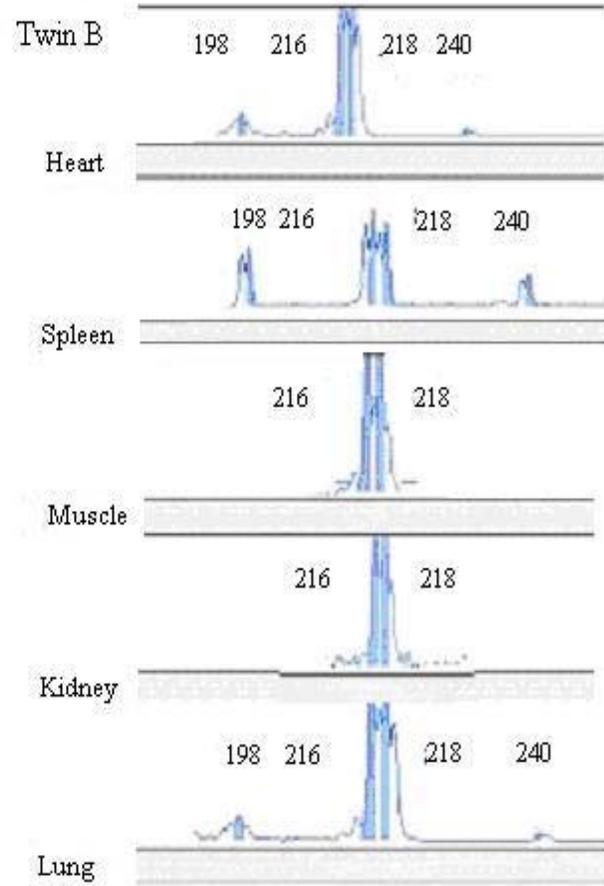
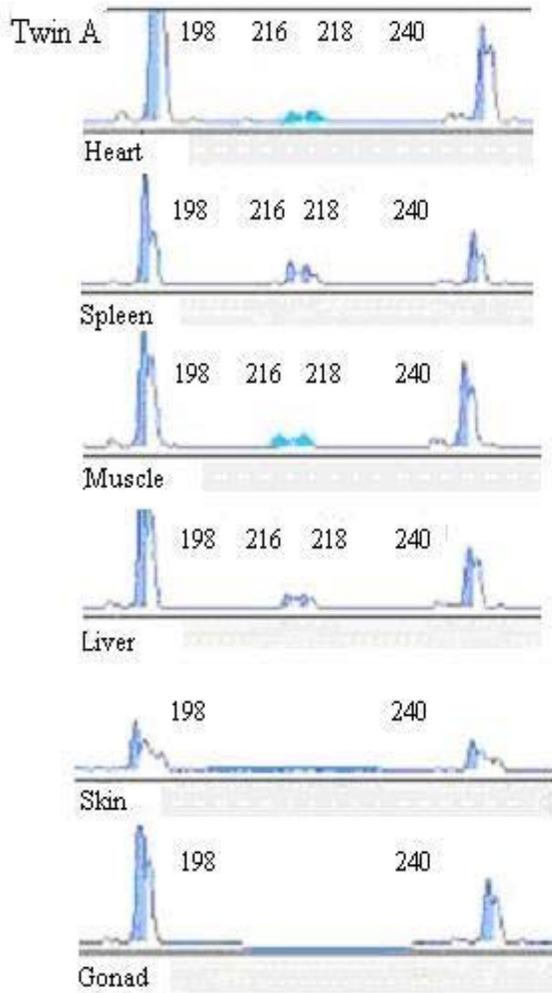
Supporting Information

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Fig. 3. An example of genotype data for two twins, twin A has a designated self genotype of 198/240 for this locus as determined by majority rule, and twin B has a designated self genotype of 216/218. This figure also demonstrates that twin A is chimeric for heart, spleen, muscle, and liver tissue and nonchimeric for skin and gonad tissue, whereas twin B is chimeric for heart, spleen, and lung tissue and nonchimeric for muscle and kidney tissue.



SI Figure 4

Fig. 4. Vertical transmission of sibling alleles through *Callithrix kuhlii*. The grandfather and grandmother (P) are individuals that had self alleles (218/220) and (206/216), respectively. They gave birth to twins (F1) with self genotypes of (206/218) and (206/220). The male (206/220) was found to have sibling alleles (206/218) present in his blood samples, which represented $\approx 50\%$ of cells in those tissues and were not present in hair samples. This male was paired with a female (218/224), who was chimeric, having the allele (240) from her sister, which represented 30% of the cells present. The pair's twin infants (F2) were both heterozygous; sibling 1 inherited one allele from the father (206) and one from the mother (218). Sibling 2 inherited 224 from the mother, and because only the mother possessed the 224 allele, the allele 218 was inherited from the father, which is in fact the sibling allele that the father had acquired through horizontal exchange with his twin. Sibling 1 is also chimeric with his blood having sibling cells (218/224) present at $\approx 40\%$.

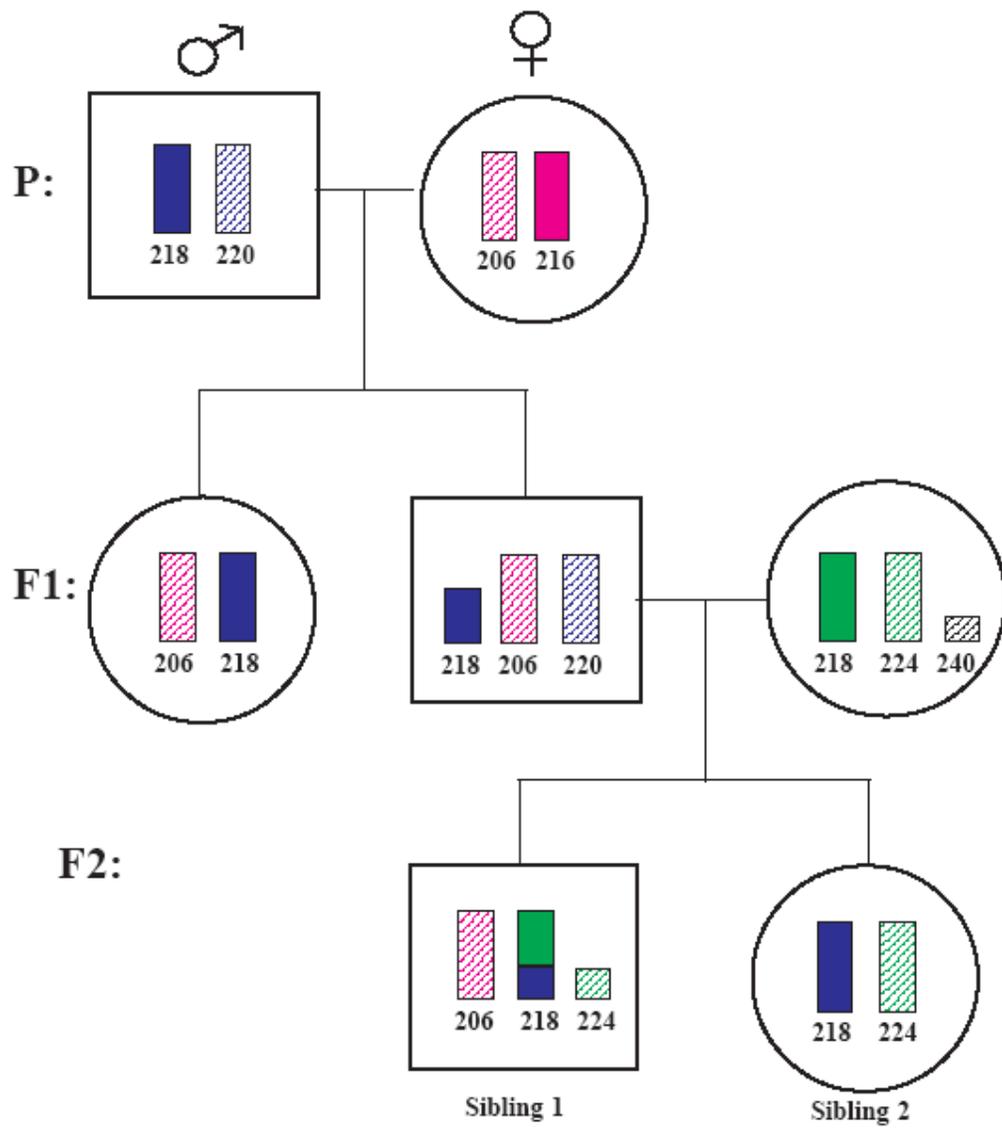
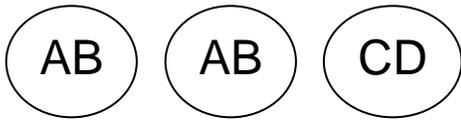
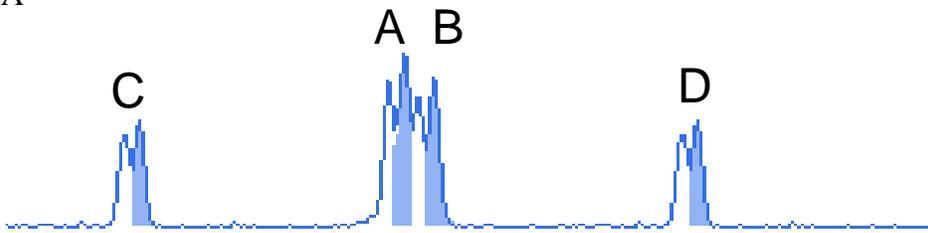


Figure 4

SI Figure 5

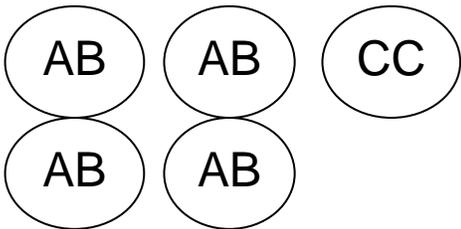
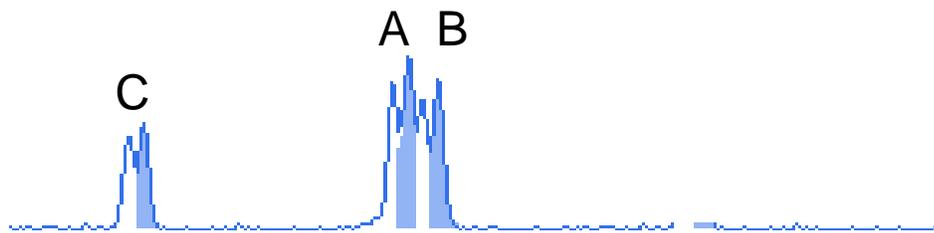
Fig. 5. Estimation of the proportion of sibling cells within a tissue (PSC). (A) The average peak strength (height) of the sibling alleles (C and D) are 50% of the height of the self alleles (A and B), resulting in a ratio of 2A:2B:1C:1D, meaning that there are twice as many AB cells in the tissue as there are CD cells. Therefore, CD cells make up 33% of the tissue sample. (B) The peak strength of the sibling allele (C) is 50% of the height of the self alleles (A and B), resulting in the ratio of 2A:2B:1C:1C, meaning that there are four times as many AB cells in the tissue as there are CC cells. Therefore, CC cells make up 20% of the tissue sample.

A



2A:2B:1C:1D

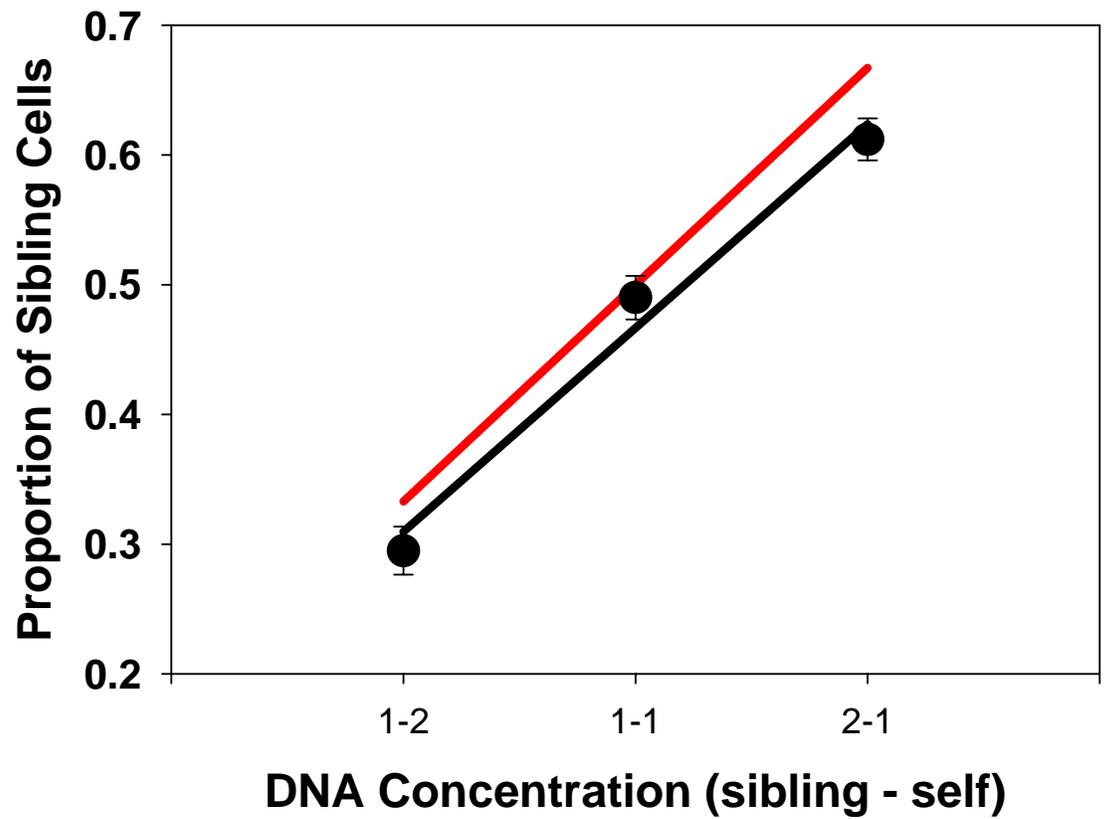
B



2A:2B:1C:1C

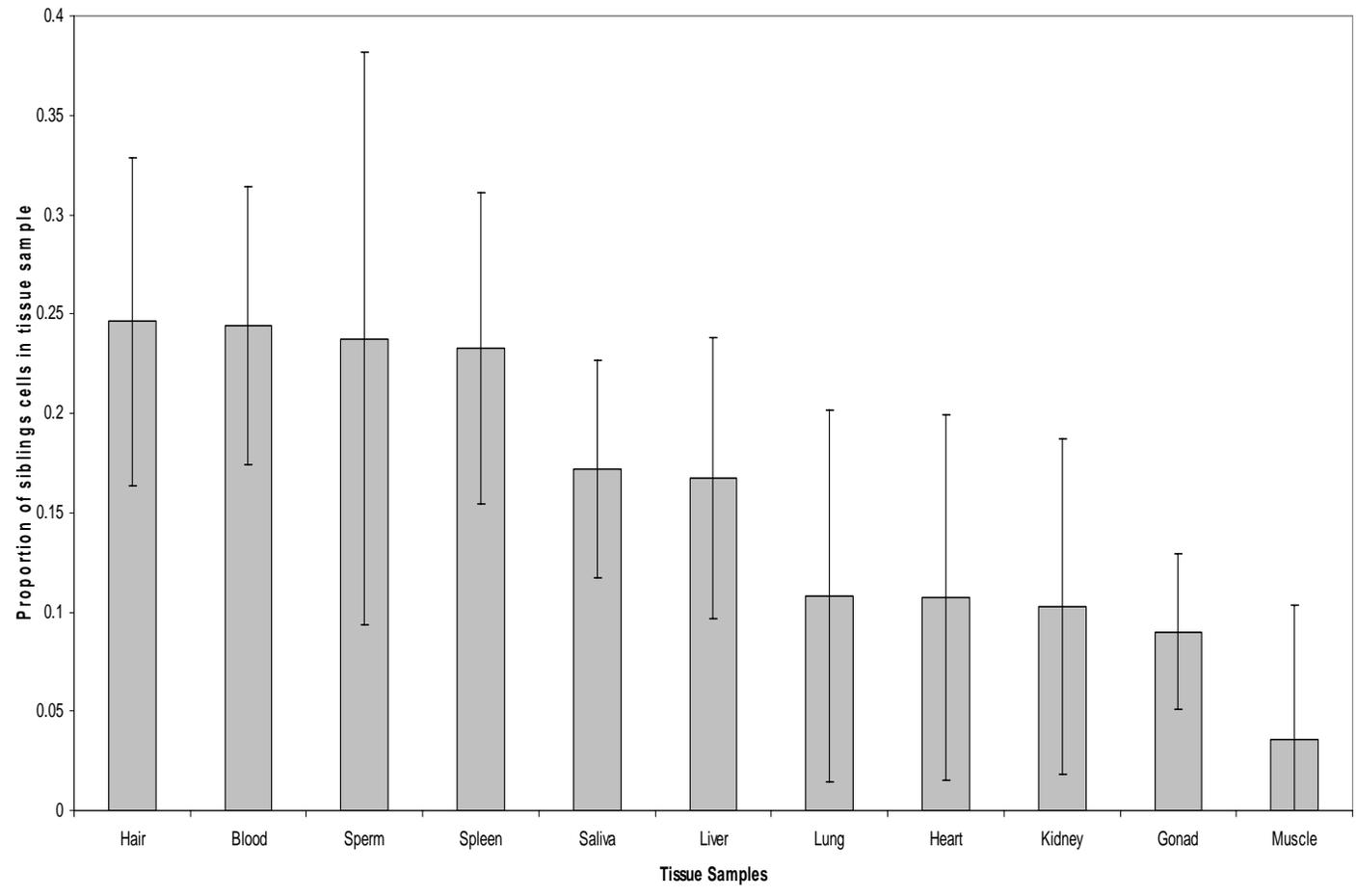
SI Figure 6

Fig. 6. The validation of the estimation of the proportion of sibling cells from peak strength of genotyped alleles. DNA of known quantity was mixed to form engineered chimeric samples in known mixed ratios, for example 1 unit sibling:1 unit self. Peak signal strength from 25 replications was used to determine the average proportion of sibling cells within each sample (black line, mean \pm SEM). The expected values are also plotted (red line).



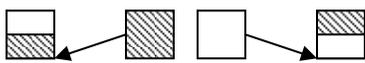
SI Figure 7

Fig. 7. A comparison of the average proportion of sibling cells (PSC) within a tissue sample (95% confidence intervals).



SI Figure 8

Fig. 8. The calculation of the average coefficient of relatedness of male twins derived from nonchimeric parents. The direction of exchange refers to the exchange of stem cells between twins such that in some cases there is no exchange (A), in some cases there is unidirectional exchange (B), and in some cases there is bidirectional exchange (C). The open square represents twin 1's self genotype, and the filled square represents Twin 2's self genotype.

| Direction of Exchange | Likelihood of pathway involving testes tissue | r | Weighted r |
|--|---|--------|------------|
|  | $0.275 + 0.119 + 0.083 = 0.477$ | 0.5 | 0.2385 |
|  | $0.156 + 0.2206 = 0.3766$ | 0.625 | 0.2354 |
|  | 0.1462 | 0.6875 | 0.1005 |

Total average r = 0.5744

Table 2. Microsatellite markers used for genotype analysis of *Callithrix kuhlii*

| Locus | Repeat Motif | N _A | H _o |
|-------|--|----------------|----------------|
| CJ1* | (CA) ₂₁ | 8 | 0.89 |
| CJ6* | (CA) ₁₇ | 6 | 0.76 |
| CJ13* | (CA) ₁₃ (TC) ₁₄ | 6 | 0.79 |
| CJ14* | (TC) ₂₁ (CA) ₅ | 10 | 0.76 |
| CK2 | (TCTA) ₂₃ (TC) ₈ | 13 | 0.96 |

*, Nievergelt et al. 1998. N_A, number of alleles. H_o, observed heterozygosity.

SI Text

To calculate relatedness for members of the social group in callitrichids in light of chimerism it is necessary to estimate the prevalence of chimerism (reported in the main article) and the proportion of cells in each tissue type that represent sibling cells. To estimate the proportion of cells that contain self alleles and those carrying sibling alleles, peak heights of the GeneScan analysis (i.e., the strength of the signal for each allele) were compared for the chimeric tissues. The amount of PCR amplification product is proportionate to the amount of template DNA in the reaction; therefore, alleles that are present in a smaller fraction of the cells will not produce peaks as strong as the most frequently represented alleles. In cases of chimerism, a rough estimate of the proportion of sibling cells within a tissue (PSC) can be determined by comparing the peak strength of the sibling alleles to the individual's self alleles. To calculate the PSC, the GeneScan software, Version 3.1 (Applied Biosystems, Foster City, CA) was used to obtain a measure of the strength of the signal (peak height); then, the average height of the sibling alleles was divided by the average height of the self alleles. For example, if the peak height of heterozygous sibling alleles is 50% of the height of heterozygous self alleles then the ratio of self: sibling is 1:0.5, or 2:1, meaning that the sibling alleles are present in $\approx 33\%$ of the cells sampled. Whereas, if the peak height of homozygous sibling alleles are 50% of heterozygous self alleles, then the ratio is 4:1, or 20% of the cells are sibling cells (see SI Fig. 5 for examples). This assumption was tested experimentally by quantifying peak height from samples with known DNA quantity mixed in specific ratios. Each sample of known mixture was amplified by using PCR a minimum of 25 times and genotyped, and the resulting peak strengths were used to estimate the ratio of cells present. The measurement of peak strength was found to be an accurate estimate of cell representation within the sample (SI Fig. 6). Although this technique may only

give a crude estimate of cells that are chimeric, this estimate can be used to preliminarily evaluate coefficients of relatedness based on genetic exchange (SI Fig. 7).

Although the coefficients of relatedness between twins differ depending on the tissue type examined, for this example the calculations focus on the frequency of exchange in sperm tissue because these are the cells that directly contribute to the next generation. To calculate an average coefficient of relatedness in a simple case of male twins conceived by nonchimeric parents, there are three factors that contribute to the calculation. First, it is necessary to determine the proportion of male twins that are chimeric in the population. We found that in our population, 45% of male twins exhibit bidirectional exchange of stem cells, 27.5% exhibit unidirectional exchange, and 27.5% exhibit no evidence of stem cell exchange. Of those that are chimeric, there are only a portion for which the testes may be chimeric, as determined by the prevalence data shown in Table 1 of the Text. Finally, of those individuals that have chimeric testes, only a portion of sperm cells will be chimeric, as determined by the PSC data described above. Hence, there are three pathways which need to be calculated to account for these factors. Pathway A occurs when there is no stem cell exchange between twin 1 and 2 which involves the testes or sperm cells. Pathway B accounts for the probability that twin 1 has chimeric cells within his testes because of unidirectional exchange with his twin, or *vice versa*. Finally, pathway C accounts for the probability that both twin 1 and twin 2 have chimeric cells within their testes because of bidirectional exchange (SI Fig. 8).

To calculate pathway A, it is first the proportion of twins which exhibit no exchange in the population (0.275) plus the frequency for which exchange fails to involve the testes in cases of unidirectional and bidirectional exchange in the population. In cases of unidirectional exchange, the proportion of the population (0.275) is multiplied by the proportion of the population found to not have chimeric gonads ($1 - 0.56 = 0.43$; Table 1). Thus, $0.275 \times 0.43 = 0.119$, and this is added to pathway A. In cases of bidirectional exchange, the prevalence of nonchimeric testes likely in twin 1 is multiplied by the prevalence of nonchimeric testes likely in twin 2, which is then multiplied by the proportion of the population found to exhibit bidirectional exchange ($0.43 \times 0.43 \times 0.45 = 0.083$, which is added to pathway A. Thus, pathway A becomes $0.275 + 0.119 + 0.083 = 0.477$.

To calculate pathway B (cases of unidirectional exchange), the frequency of unidirectional exchange (0.275) is multiplied by 0.57 (the prevalence of chimeric testes in the population) (Table 1), which equals 0.156. Then, the likelihood of unidirectional exchange in testes within twins that exhibited unidirectional exchange is the prevalence of nonchimeric testes in one twin (0.43) multiplied by the prevalence of chimeric testes in the other twin (0.57) multiplied by the proportion of the population that exhibits bidirectional exchange (0.45) multiplied by two (this process can occur in either direction, twin 1 to twin 2 or twin 2 to twin 1), which equals 0.2206. Thus, pathway B is a result of $0.156 + 0.2206 = 0.3766$.

To calculate pathway C (cases of chimeric testes due to bidirectional exchange), it is the prevalence of chimerism in the testes of twin 1 (0.57) multiplied by the prevalence of chimerism in the testes of twin 2 (0.57) multiplied by the proportion of male twins that exhibit bidirectional exchange (0.45), which equals $(0.57 \times 0.57 \times 0.45) = 0.1462$.

Next, it is necessary to calculate the coefficient of relatedness for each pathway as a result of the proportion of chimeric cells present within the testes. For example, the r for pathway A is simply 0.5; no chimeric cells are present, and thus the average r is the same as in nonchimeric mammals. To calculate r for pathways B and C, which contain chimeric cells, it is necessary to account for the average proportion of sibling cells found within the sperm samples, an average of 25% as estimated from PSC described above. The alleles that are shared through horizontal exchange to the sperm have a relatedness of 1, thus 1×0.25 ; the alleles shared through parental descent have a relatedness of 0.5, thus 0.5×0.75 . Therefore, the relatedness for pathway B is $(1 \times 0.25) + (0.5 \times 0.75) = 0.625$. For pathway C, 75% of the sperm cells in an individual are self and are thus related to the twin such that $(0.75 \times 0.5 \text{ parental}) + (0.25 \times 1 \text{ horizontal})$. This is added to the 25% in an individual that are from the twin and are thus related to themselves in a manner of $(0.75 \times 1 \text{ horizontal}) + (0.25 \times 0.5 \text{ parental})$. Thus, for C, $[0.75 ((0.75 \times 0.5) + (0.25 \times 1)) + 0.25 ((0.75 \times 1) + (0.25 \times 0.5))] = 0.6875$.

Finally, each relatedness value needs to be weighted based on the probability of that pathway as determined above. Thus, for pathway A, the weighted r is $0.477 \times 0.5 = 0.2385$; for pathway B, the weighted r is $0.3766 \times 0.625 = 0.2354$, and for pathway C, the weighted r is $0.1462 \times 0.6875 = 0.1005$.

Thus, the overall average coefficient of relatedness between male twins from nonchimeric parents in our population is $0.2385 + 0.2354 + 0.1105 = 0.5744$.