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A mitochondrial genetic divergence proxy predicts the reproductive compatibility of mammalian hybrids

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Numerous pairs of evolutionarily divergent mammalian species have been shown to produce hybrid offspring. In some cases, F_1 hybrids are able to produce F_2s through matings with F_1s. In other instances, the hybrids are only able to produce offspring themselves through backcrosses with a parent species owing to unisexual sterility (Haldane's Rule). Here, we explicitly tested whether genetic distance, computed from mitochondrial and nuclear genes, can be used as a proxy to predict the relative fertility of the hybrid offspring resulting from matings between species of terrestrial mammals. We assessed the proxy's predictive power using a well-characterized felid hybrid system, and applied it to modern and ancient hominins. Our results revealed a small overlap in mitochondrial genetic distance values that distinguish species pairs whose calculated distances fall within two categories: those whose hybrid offspring follow Haldane's Rule, and those whose hybrid F_1 offspring can produce F_2s. The strong correlation between genetic distance and hybrid fertility demonstrated here suggests that this proxy can be employed to predict whether the hybrid offspring of two mammalian species will follow Haldane's Rule.

1. Introduction

Though hybrids between mammalian species have been catalogued for decades [1], the extent and frequency of gene flow between evolutionarily divergent taxa has only been recognized since the availability of high-coverage nuclear genomes. Recent studies have revealed rampant gene flow between multiple species of bears [2], canids [3], felids [4–6], cetaceans [7,8], birds [9–11], suids [12,13] and bovids [14]. Genome analyses of invertebrate lineages including butterflies [15,16] and mosquitoes [17] have also revealed similarly extensive patterns of ancient and contemporary introgression.

This demonstrated frequency of introgression is perhaps surprising given the significant barriers that maintain reproductive isolation in species pairs that diverged millions of years ago. In mammals, genomic barriers manifest in accordance with Haldane's Rule [18] as the unisexual sterility of the heterogametic sex (XY males) in F_1 hybrid offspring. In cases where matings between F_1s fail to produce F_2s, fertile offspring can often be produced through backcrosses between the fertile F_1 females and males from one of the parent species. Occasionally, however, F_1s produced from interbreeding between closely related mammalian species pairs can produce viable and fertile F_2 offspring.

If the calculated pairwise genetic distance between two species correlated with the ability of their hybrid offspring to produce F_2s, these values could serve as a proxy to predict this occurrence. Though at least one study [19] reported that genetic divergence values do generally correlate with species boundaries, others [20,21] have questioned whether this correlation exists, and have instead stated that measures of genetic distance between species are not reliable predictors of hybrid sterility. A recent empirical study of damselflies, however, demonstrated a strong correlation between the genetic distances between species pairs and their relative reproductive isolation [22].

Establishing whether genetic distance and reproductive isolation are correlated is also critical for our understanding of the genetic architecture of reproductive isolation. Doing so firstly requires knowing whether any two species are capable of producing viable or fertile offspring, but there is a general paucity of captive breeding experiments or field data that have unequivocally established this. An alternative approach is to develop a metric that can accurately predict the relative fertility of the F_1 hybrids of any two species that makes use of interspecific crosses whose offspring have been reproductively characterized. Here, we developed a robust, quantitative framework based on the correlation between mitochondrial genetic distance between mammalian species known to produce F_1 pairs to obtain a quantitative measure of whether F_1 hybrids of both sexes are likely to be capable of breeding, or if they instead manifest Haldane's Rule. We tested the accuracy of the proxy in a well characterized felid hybrid system, and then applied it to a hominin case study to assess the relative potential sterility of hybrids between humans and their closest extinct relatives.

2. Results and discussion

(a) Categorizing hybrid incompatibility

We first explicitly defined two dichotomous categories along the spectrum of hybrid incompatibility. Category 1 is defined by mammalian species pairs capable of producing fertile F_1 offspring of both sexes that can reproduce without backcrossing with a parent species (even if there are observed asymmetries in gene flow and variation in male fertility among the hybrids) (electronic supplementary material, table S1). category 2 is defined by pairs of species that can produce viable F_1 offspring, but follow Haldane's Rule, and thus only female F_1s can reproduce by backcrossing with a parent species. category 2 also includes species pairs whose hybrids are infertile (electronic supplementary material, table S1). We determined the categorical assignment of each species pair (electronic supplementary material, table S1 and figure S1) by following a decision tree (electronic supplementary material, figure S2) based upon empirical evidence derived from experimental studies of F_1 hybrid fertility. We confidently placed seven species pairs into category 1, and six others into category 2.

Many additional live hybrid offspring have been reported in the literature than are included in figure 1 or in the electronic supplementary material, figure S1. We identified 17 species pairs known to produce viable offspring, but for which there was insufficient evidence to confidently assign them into either category (electronic supplementary material, table S2 and figure S3). The framework and threshold values depicted in figure 1 allow us to predict the fertility of these offspring given the definitions described above, and their placement into categories 1 or 2. These pairs are listed in the electronic supplementary material, table S2 and their relative positions are depicted in the electronic supplementary material, figure S3.

We then obtained published sequence data across all species (electronic supplementary material, table S3) from both the cytochrome *b* gene (*CYTB*) ($n = 1795$) and complete mitochondrial genomes ($n = 30$) (excluding the control region) from multiple individuals per species. By matching the phylogenies derived from the alignments to available nuclear species trees, and by only including sequences that fell into reciprocally monophyletic clades, we ensured that the selected mitochondrial sequences for each species were neither mislabelled, nor nuclear copies of mitochondrial genes (NUMTs), nor derived from hybrid populations. Using the sequence alignments, we then calculated average pairwise genetic distances between each species pair using both the number of raw differences, and differences scaled by several different nucleotide substitution models. In order to test the correlation between the mitochondrial proxy and estimates obtained from nuclear loci, we calculated genetic distances for four genes (cholinergic receptor nicotinic alpha (*CHRNA1*), growth hormone receptor (*GHR*), zinc finger X-chromosomal protein (*ZFX*), and zinc finger Y-chromosomal protein (*ZFY*)) that were available for 10 of the primate species pairs [23] (electronic supplementary material, figure S4).

Plotting the calculated distance values using *CYTB* revealed a limited overlap associated with the two categories (figure 1; electronic supplementary material, figure S1), and a Student's *t*-test (two tailed) showed significantly lower genetic divergence values of species pairs in category 1 relative to those in category 2 ($p < 0.003$). The category 1 pair with the greatest divergence value was a pair of guinea pig species that were 8.0% divergent, and the category 2 pair with the lowest divergence was a pair of vole species that were 7.2% divergent. Several pairs of species fall within this 0.8% overlapping region suggesting that this level of *CYTB* distance is the zone where some F_1 mammalian offspring begin to require a backcross to generate an F_2. The existence of a genetic distance threshold separating the two categories also held true for the complete mitogenomes (electronic supplementary material, figure S4).

Towards the upper end of distance values between species pairs, both the male and female hybrid offspring of domestic pig (*Sus domesticus*) × babirusa (*Babyrousa celebensis*) (12.9%) were shown to be infertile [24]. In addition, controlled, exhaustive efforts failed to produce any viable hybrids between mountain hares and European rabbits (17.3%) [25] (figure 1). The large distance values between these species pairs corroborates previous studies showing that along the continuum of speciation, infertility in both sexes evolves prior to inviability [26–28].

Importantly, the two categories of fertility defined here are not strictly linked with gene flow. For instance, though

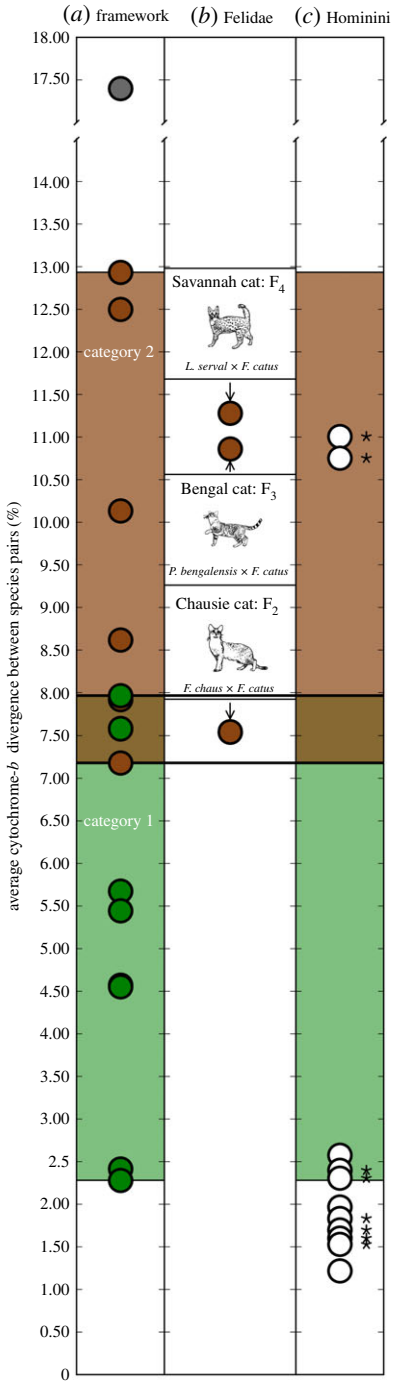

Figure 1. A depiction of the correlation between CYTB divergence between mammalian species pairs and the relative fertility of their hybrid offspring. In column (*a*), the green circles represent species capable of producing fully fertile F_1 offspring which can reproduce independently of their parent species (category 1). Brown circles represent species pairs that follow Haldane's Rule and require backcrossing of a female F_1 with a parent species, or both sexes are sterile (category 2). The single grey dot represents the distance between mountain hares and European rabbits that, despite numerous attempts, failed to produce any offspring. The green and brown shaded regions represent the range of divergence values of the two categories. Column (*b*) depicts the divergence between three wild felid species and domestic cats, as well as the minimum number of generations of backcrosses with domestic cats before full fertility of the hybrid is restored. The white circles in column (*c*) depict the divergence between three ancient hominins and anatomically modern humans (AMH), as well as the distances between AMH and chimpanzees and bonobos (in category 2). The asterisks represent those pairs that include modern samples of AMH. The lack of an asterisk signifies that only sequences derived from archaeological AMH were used to compute the divergence values. Details regarding the specific species pairs are listed in the electronic supplementary material, figure S1 and table S1. (Online version in colour.)

both male and female category 1 hybrid offspring can reproduce without requiring a backcross with a parent species, gene flow asymmetries have been demonstrated in virtually all of these species pairs including house mice (*Mus musculus musculus* × *Mus musculus domesticus*) (2.3%) [29], and between brown bears (*Ursus arctos*) and polar bears (*Ursus maritimus*) (2.4%) [2] (electronic supplementary material, table S1). Gene flow has also been demonstrated between category 2 species (including *M. musculus* and *Mus spretus* [30]) (8.6%). Because both fertility and the potential for gene flow vary along a continuum, it is striking that the divergence values associated with the two fertility categories defined here do not overlap more substantially.

The lack of available nuclear sequences (relative to mitochondria) reduced our ability to test whether nuclear genes generally produced the same pattern as the mitochondria across all our species pairs. Despite this limitation, we were able to identify four nuclear loci: *ZFY*, *ZFX*, *GHR*, and *CHRNA1*, that have been sequenced in 10 primate pairs (electronic supplementary material, figure S4) known to produce viable hybrid offspring [23]. We generated pairwise distances for each of these genes using the same method employed in the mitochondrial distance calculation. We then assigned each species pair to category 1 or category 2 based upon their *CYTB* divergence values within the original framework. In each case, though the order of the taxa based upon pairwise divergence values varied relative to the pattern generated using *CYTB* (owing to significantly fewer variable sites and thus smaller divergence values in nuclear loci), for the two most variable nuclear loci, *ZFX* and *GHR*, there was no overlap in divergence values between the two categories, consistent with the mitochondrial assessment (electronic supplementary material, figure S4). The other two loci, *ZFY* and *CHRNA1*, possessed very limited interspecific nucleotide variability, but generally followed the same overall pattern.

(b) Testing the proxy using known mammalian hybrids

In order to further substantiate both this correlation and the robustness of *CYTB* divergence as a proxy for hybrid sterility, we tested the use of this system for predicting fertility in a well-known hybrid system. Cat breeders have crossed domestic cats (*Felis catus*) with several wild felids, including the jungle cat (*Felis chaus*), leopard cat (*Prionailurus bengalensis*) and serval (*Leptailurus serval*) [31], to create three exotic cat breeds: Chausies, Bengals and Savannahs, respectively. In all cases, the F_1 male hybrids are sterile. To regain fertility while maintaining some wild felid characteristics, breeders must backcross the F_1 female offspring with male domestic cats to establish a breeding population of pets [31]. Given that multiple generations of unidirectional backcrossing was required for all three crosses to generate a fertile population, our proxy would firstly predict that the *CYTB* distances between all three pairs should be close to or greater than approximately 7.2%, and that they should all fall into the range encompassed by category 2. Secondly, the pairs with larger genetic distance values should require a greater number of backcrosses with domestic cats (halving the wild cat ancestry with each subsequent generation) before fertility is restored in hybrid males and a breeding pet population is established.

Both of these predictions are borne out by the data (figure 1; electronic supplementary material, table S3). All three pairs

show *CYTB* distances greater than or equal to 7.5% and the increasing molecular distances between the pairs correlate with an increase in the number of required backcross generations to regain fertility. Specifically, distances between domestic cats and jungle cats, leopard cats and servals (7.5%, 10.9% and 11.3%, respectively) are consistent with both the observed minimum (2, 3 and 4, respectively) and average (3, 4 and 5, respectively) number of backcrosses with domestic cats required for hybrid males to acquire fertility [31]. These results are also consistent with an early hybrid experiment using guinea pigs in which hybrids between *Cavia fulgida* and *Cavia porcellus* (8.0% *CYTB* distance) were able to regain male fertility after three generations of backcrossing [32] (electronic supplementary material, table S1).

Accidental hybrids in zoos also confirm the predictive power of this proxy. In 2006, the Copenhagen Zoo placed a domestic sow (*S. domesticus*) in a pen with a male babirusa (*B. celebensis*) with the expectation that the two species were sufficiently evolutionarily divergent that they would be incapable of producing offspring. Months later, however, five piglets were born and though two died from maternally induced trauma, the other three (two males and one female), all survived and were shown to be infertile [24] (electronic supplementary material, table S1 and figure S1). Historically, hybrid offspring between distantly related species have accidentally been produced in zoos (electronic supplementary material, table S2), though the relative fertility of the F_1s was rarely established. In this case, the *CYTB* distance between the two species (12.9%) is not much greater than the value between rhesus macaques and hamadryas baboons (12.5%) which were able to produce a live (infertile) offspring [33], thus suggesting that live offspring between these suids was possible.

(c) Assessing hominin hybrid incompatibility

The initial discovery of Neanderthals led some anthropologists, as early as the turn of the twentieth century, to speculate that anatomically modern humans (AMH) and their closest extinct relatives were capable of producing hybrid offspring [34]. The absence of Neanderthal mitochondrial genomes in the extant human population, however, led some to suggest that AMH and Neanderthals did not hybridize [35–37]. More recent analyses of whole ancient genome sequences have demonstrated that, in fact, archaic hominins, including Neanderthals and Denisovans, did produce hybrid offspring, not only with AMH [38–40] but also with each other [41]. The generation of these ancient genomes has also allowed for an assessment of the role that incompatibility may have played in the selection for and against hybrid introgression in modern humans [42]. The genomic confirmation of the existence of hominin hybrids supported the conclusions of two studies [43,44] that used a qualitative correlation between the divergence times between species pairs and the fertility of their hybrid offspring to suggest that, given their relatively recent temporal divergence, AMH and Neanderthals could have retained the ability to produce fertile offspring of both sexes.

We quantitatively assessed the relative fertility of hybrids between pairs of modern and ancient hominin lineages using the proxy established in this study. To do so, we calculated the average pairwise distance in *CYTB* sequences between AMH and three extinct hominin lineages: Neanderthals, Denisovans and the ancient population from the Sima de los Huesos cave in Spain [24,45]. To avoid overestimating the genetic distances resulting from the comparison of modern and extinct populations, we generated values using the *CYTB* sequences derived solely from ancient AMH found in archaeological contexts (electronic supplementary material, table S3).

The distance values for all of the pairings of three *Homo* groups (Sima de los Huesos, Neanderthals and AMH) occupy the bottom of the category 1 range. The distance values for Neanderthals and modern and ancient AMH specifically (1.6%) fall below all the mammalian pairs in this study including polar bears and brown bears (2.4%), and between subspecific crosses of *M. musculus* (2.3%) (figure 1; electronic supplementary material, figure S1 and table S1). When placed within this context, our data predict that ancient hominin lineages were probably not sufficiently divergent from each other to expect a significant biological impediment to the generation of fertile offspring. This is consistent with the ancient genomic evidence, which has shown not only that archaic populations interbred with AMH on at least four occasions [46], but also that introgression took place in both directions [47]. In addition, the distance values of Denisovan-Neanderthal and Denisovan-AMH are the largest of the *Homo* pairings, and are consistent with the suggestion that Denisovans possessed a mitochondrial lineage that may have been acquired through introgression with another, as yet unknown source population [48].

We also assessed hybrid sterility between more distantly diverged hominin lineages. Specifically, we calculated divergence values between humans and our two closest living relatives: chimpanzees (*Pan troglodytes*) and bonobos (*Pan paniscus*). Female chimpanzees inseminated with human sperm during a Soviet experiment in the 1920s failed to produce any offspring, and the reverse experiment did not progress beyond the planning stage [49]. Recent molecular clock assessments have suggested that AMH and chimpanzees diverged approximately 5–6 Ma [50], well beyond both the 2 Myr threshold suggested by other studies as the upper limit to hybrid fertility [43,44], and the average time to speciation [51]. Our analysis places the distance values between AMH and chimpanzees (11.0%), and AMH and bonobos (10.8%) within category 2, suggesting that even if hybrids could be produced, they would probably follow Haldane's Rule (figure 1; electronic supplementary material, figure S1).

3. Conclusion

The correlation demonstrated here between *CYTB* divergence (as well as genetic divergence in general) and relative hybrid sterility suggests that distance values can be used as a proxy to accurately and rapidly predict the relative sterility of hybrids that result from matings between pairs of mammalian species. More specifically, our results show that the F_1 offspring of some mammalian species pairs with greater than 7.2% *CYTB* distance have lost the ability to produce F_2s, and beyond 8.0%, all pairs of species in our dataset require a backcross to a parent species to produce fertile offspring. In addition, our results demonstrate that once a single backcross with a parent species is required to restore fertility in hybrid males, the number of additional necessary backcrosses increases with greater *CYTB* distances between the parent species.

Our emphasis here is on mitochondrial DNA, and though recent studies have proposed that speciation may be mediated

by mitonuclear interactions [52,53], our results should not be misinterpreted as a claim that *CYTB* plays a causative role in hybrid sterility. Nor can the use of genetic distance values as a proxy be perfectly predictive. For example, under the Dobzhansky–Müller model, incompatibility can arise from as few as two mutations in isolated populations irrespective of time since divergence. This means that it would be possible for closely related populations to be incapable of generating fertile hybrids [52,54], though no such examples have been described.

The value of any proxy is determined by both its predictive power, and the ease of generating the proxy data. Publicly available mitochondrial DNA sequences from thousands of mammalian taxa already exist and calculating pairwise distance values is inexpensive, simple and fast. As a result, mitogenomic distances have substantial value as a means to predict the potential for any two mammalian species to produce fertile offspring, and the relative degree of sterility in one or both sexes. As whole genomes become available from the same set of species, this analysis can be extended to determine which regions of the nuclear genome may also be more or less predictive.

The discovery of additional extinct hominin populations that survived into the last 250 000 years, including *Homo floresiensis* [55] and *Homo naledi* [56], has raised interest in understanding the limits to fertility and hybridization between extinct and extant *Homo* spp. [57]. If and when mitochondrial genomes from these samples can be obtained, the approach described here may provide an answer, even if nuclear genomic data are not obtainable. Lastly, establishing which species pairs violate the predictions of the framework will identify unique systems that may lead to a better understanding of the process of reproductive isolation, and the biological mechanisms responsible for hybrid sterility.

4. Material and methods

(a) Assessment of hybrid fertility and rationale of assignment into categories

In order to ascertain if there was a correlation between genetic divergence and the fertility of hybrid offspring between species, we first collected published examples of species pairs that were capable of producing live offspring. We then split the hybrid pairings into two categories. category 1 consisted of seven species pairs that are capable of producing fertile F_1 offspring of both sexes, and for which we were able to obtain evidence of captive breeding experiments showing that the F_1s could mate to produce F_2s. The evidence and rationale for placing each of these pairs into category 1 is listed in the electronic supplementary material, table S1 and the decision tree we used to determine the categorization is shown in the electronic supplementary material, figure S2.

The hybrid offspring of all of six pairs of species in category 2 are either completely infertile, or require one or more generations of female hybrid backcrosses with the male of a parent species to produce fertile offspring. For these pairs, we obtained evidence demonstrating no successful F_2s from F_1 hybrid pairings, an inability to produce offspring other than by backcrossing to a parent species, or other biological measurements (including histological assessments of the testes from the hybrid males) that demonstrated complete infertility (electronic supplementary material, table S1 and figure S5).

(b) Genetic distance calculation

Both *CYTB* sequences and full mitogenomes (excluding the control region) of multiple individuals of each species were collected from Genbank (electronic supplementary material, table S4) and aligned using Clustal Omega v. 1.2.4 [58]. In order to ensure that none of the sequences were either mislabelled, or were NUMTs, we constructed neighbour-joining trees using Geneious v. 6.1.8 [59] and removed all individuals that did not fall into monophyletic clades consisting of individuals from each species. We first used pModelTest v. 1.04 [60] to determine the best model for the alignment of each set of sequences for both species. We then calculated pairwise distances between each species pair using RAXML v. 8 [61] and FastTree v. 2.1 [62]. We also generated raw distance values using the Hamming distance method which sums the number of base pair differences (ignoring transition or transversion status) and divides that number by the sequence length.

The distances were generated from the *CYTB* and nuclear gene alignments for each set of species pairings in fasta file format using a Python v. 2.7 wrapper to automate the terminal based programs RAXML, FastTree and pModelTest. A custom Python 3.6 program was written to calculate Hamming distances of sequences making use of the distance v. 0.1.3 [63] and Biopython v. 1.66 [64] modules. Gaps in the aligned sequences were treated as missing data.

The mean distance and standard errors for each pairwise comparison were calculated using the bootstrapping method on the assumption that the sets of pairwise distances between related species would not be normally distributed. Each pairwise comparison group containing Hamming distances was randomly resampled into sets of equal sample size and processed using a helper function in the custom software which made use of the bootstrapped v. 0.0.2, NumPy v. 1.10.1 [65] and SciPy v. 0.16.0 [66] Python modules. The source code of this script is available at https://github.com/BeebBenjamin/MrHamming.

The *CYTB* distances were compared with those generated in MEGA X for GNU/Linux [67] (which uses a slightly different method for treating missing bases) using the 'compute between group mean distance' method with the following settings:

(a) variance estimation method: bootstrap method;
(b) no of bootstrap replications: 500;
(c) substitutions type: nucleotide;
(d) model/method: p-distance;
(e) substitutions to include: d: transitions + transversions;
(f) rates among sites: uniform rates;
(g) gaps/missing/data treatment: complete deletion; and
(h) select codon positions: 1st, 2nd, 3rd, non-coding site.

Using a Student's *t*-test (two tailed), the differences between the results were found to be statistically non-significant ($t = -0.11222$, $p = 0.912504$). The distance values reported in the tables and figures were those generated using the Python 3.6 script described above.

(c) Student's *t*-test of statistical difference between cytochrome *b* gene distance in hybrid categories

The statistical significance of observed differences in *CYTB* divergence between categories 1 and 2 was tested using the Student's *t*-test ($p = 0.002942$) implemented in the R software package [68]. The suitability of a parametric test was determined using the Shapiro–Wilk normality test ($p = 0.6238$).

Data accessibility. Data has been uploaded as part of the electronic supplementary material.

Authors' contributions. R.A. and H.R. compiled and analysed data and wrote the paper. B.W.D., C.K., R.B., A.L., L.L., J.H., O.L., M.W., A.C.K. and L.F. wrote the paper. E.I.-P. analysed data and wrote the paper. W.J.M. generated data and wrote the paper. G.L. conceived of the study and wrote the paper.

Competing interests. We declare we have no competing interests.

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