Predicting the viability of archaic human hybrids using a mitochondrial proxy

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41 **Abstract**

42 Ancient DNA evidence has confirmed hybridization between humans and
 43 Neanderthals and revealed a complex pattern of admixture between hominin lineages.
 44 Many segments of the modern human genome are devoid of Neanderthal ancestry,
 45 however, and this non-random distribution has raised questions regarding the
 46 frequency and success of hybridisation between ancient human lineages. Here, we
 47 examine the hypothesis that hominin hybrid offspring suffered a reduction in fertility
 48 by comparing patterns of sequence divergence of mitochondrial and nuclear DNA
 49 from numerous hybridising pairs of mammals. Our results reveal a threshold
 50 separating species pairs whose divergence values fall within two categories: those
 51 whose hybrid offspring can successfully reproduce without backcrossing with their
 52 parent species, and those whose hybrid offspring cannot. Using this framework, we
 53 predict that the potential hybrid offspring of Neanderthals, Denisovans, the ancient
 54 individuals from the Sima de los Huesos and anatomically modern humans would not
 55 have suffered a reduction in fertility.

56

57 **Introduction**

58 Though numerous interspecies hybrids have been recorded, predicting their viability
 59 and relative fertility has proved difficult. For example, the absence of Neanderthal
 60 mitochondrial genomes in the modern human population led some to suggest that
 61 anatomically modern humans (AMH) and Neanderthals did not hybridise¹⁻³. Recent
 62 analyses of whole genome sequences derived from ancient individuals, however, have
 63 demonstrated that archaic hominins including Neanderthals and Denisovans did in
 64 fact hybridise with AMH⁴⁻⁶. For many anthropologists, this result corroborated
 65 previous conclusions (based on morphological grounds) that Neanderthals and AMH
 66 could have produced fertile offspring (*e.g.*⁷).

67

68 A recent paper, however, conflated the observation of genetic deserts devoid of
 69 Neanderthal ancestry on modern human autosomes, and especially the X
 70 chromosome, with reproductive incompatibilities between humans and Neanderthal⁸.
 71 Though this study also suggested that demographic factors likely played a role in the
 72 formation of these deserts, two studies^{9,10} concluded that most selection against
 73 Neanderthal DNA in genic regions was not due to reproductive incompatibilities, but
 74 instead was the result of the Neanderthals' high genetic load. In addition, the initially

75 high proportion of Neanderthal admixture¹¹ suggests that early male and female
76 hybrids of AMH and Neanderthals were normally fertile, and that any Neanderthal
77 variants that may have interfered with fertility within an AMH genetic background
78 were likely confined to a few genetic loci. Lastly, multiple factors including
79 ecological drivers can lead to genomic deficits of introgression within hybrid
80 offspring that are otherwise fertile¹², suggesting the existence of genetic deserts may
81 not imply reproductive incompatibility.
82
83 More generally, assessing whether any two species are capable of producing viable or
84 fertile offspring is difficult in the absence of nuclear genomic data, captive
85 experiments or field data. In addition, there has been no robust, quantitative biological
86 measure of the expected fitness of hybrid offspring, and thus no rigorous method to
87 ascertain the specific viability of archaic human hybrids, or the potential for
88 hybridisation between more ancient hominin lineages. Determining whether distinct
89 hominin lineages were capable of producing fertile offspring is important not only for
90 understanding the patterns of hybrid ancestry within modern humans, but also for
91 ascertaining the degree to which reproductive isolation (as a result of either cultural or
92 biological phenomena) existed between AMH and other hominin lineages.
93
94 Though numerous studies have speculated about the potential for hominin lineages to
95 produce viable hybrids, few have made use of the numerous pairs of mammalian
96 species known to produce hybrid offspring along a continuum of male (and to a lesser
97 extent, female) fertility. Based on the qualitative correlation that the fertility of
98 hybrids generally decreases with divergence time, two recent studies^{13,14} speculated
99 that, given their relatively recent temporal divergence, AMH and Neanderthals could
100 have retained the ability to produce fertile offspring. These studies could not rule out
101 the fact that the male hybrids may have possessed lower fertility as a result of
102 Haldane's Rule which states that if one sex is rare, absent or sterile among hybrid
103 offspring, it is the heterogametic sex.
104
105 In order to obtain a quantitative measure of whether two lineages can interbreed, we
106 developed a framework based on the correlation between mitochondrial genetic
107 distance between mammalian species pairs, and the manifestation of hybrid male
108 sterility. Some previous studies have questioned this approach and stated that

measures of species divergence are not necessarily reliable predictors of hybrid sterility^{15,16}. Other studies have reported that genetic divergence values do correlate with species boundaries¹⁷ and time to speciation. A more specific recent study of damselflies demonstrated a strong correlation between the genetic distances separating two species and their relative reproductive isolation¹⁸.

Hybrid fertility exists along a continuum. For the sake of simplicity and ease of distinction, we first explicitly defined two dichotomous categories amongst hybrid offspring. Category 1 contains eight terrestrial mammalian species pairs that are capable of producing F1 offspring that can reproduce without backcrossing with a parent species (even if there are observed asymmetries in gene flow and variation in male fertility amongst the hybrids) (Table S1). Category 2 consists of nine pairs of species that can produce viable F1 offspring, but these hybrids either cannot reproduce without backcrossing with a parent species, or are completely infertile (Table S1). In order to determine the categorical assignment of each species pair, we relied upon empirical evidence derived from experimental studies of F1 hybrid fertility (Table S1). We also compiled 22 additional species pairs that are known to produce viable offspring, but for which there was insufficient evidence to confidently assign them into either category (Table S2, Fig. S2).

We then aligned sequences of both the cytochrome b gene (*CYTB*) and mitochondrial genomes (excluding the control region) from multiple individuals per species and confirmed (by matching the phylogenies to nuclear species trees) that the selected mitochondrial sequences for each taxon pair were neither mislabelled, nor nuclear copies of mitochondrial genes, nor derived from hybrid populations. From the sequence alignments we calculated average pairwise genetic distances between each species pair using both Hamming (raw) distances and evolutionary models. In addition, we calculated genetic distances between ten primate species pairs using four nuclear loci (*CHRNA1*, *GHR*, *ZFX*, *ZFY*)¹⁹ (Fig. S3).

Results and Discussion

The calculated divergence estimates using *CYTB* revealed clear thresholds distinguishing the two categories (Fig 1, Fig. S1). More specifically, none of the eight

Category 1 pairs that were capable of producing fully fertile offspring possessed *CYTB* sequences with raw average pairwise divergence values greater than 7.60%. Other than two vole species that possessed a divergence value of 7.54%, all other Category 2 pairs possessed divergence values greater than 7.73%. The existence of a genetic distance threshold also held true for the mitogenomes (Fig. S3). In addition, the male and female hybrid offspring of the two most divergent pairs were shown to be completely infertile corroborating previous studies²⁰⁻²² showing that along the continuum of speciation, infertility evolves prior to inviability. Lastly, a Mann-Whitney U test showed significantly lower genetic divergence values of species pairs in Category 1 relative to those in Category 2 ($p < 0.0001$).

Importantly, the two categories of fertility defined here are not strictly linked with gene flow. For instance, though both male and female hybrid offspring of all species pairs in Category 1 can reproduce without requiring a backcross with a parent species gene flow asymmetries have been demonstrated in virtually all Category 1 pairs including house mice (*Mus musculus musculus* x *Mus musculus domesticus*)²³, and brown (*Ursus arctos*) and polar bears (*Ursus maritimus*)¹² (Table S1). In addition, gene flow has also been demonstrated between Category 2 species (including *Mus musculus* and *M. spretus*²⁴) whose F1 hybrid offspring cannot produce viable F2s. Since both fertility and the potential for gene flow vary along a continuum, it is interesting to note that the divergence values associated with the two fertility categories defined here do not overlap more substantially. As a result, our results suggest that pairwise mitochondrial genetic distance values can be used to make predictions of male hybrid sterility, at least in terrestrial mammals.

Though the lack of available sequences for nuclear loci (relative to mitochondrial loci) limited our ability to test whether nuclear genes produced the same general pattern as the mitochondrial loci across all our species pairs, we were able to identify four nuclear genes: Zinc finger Y-chromosomal protein (ZFY), Zinc finger X-chromosomal protein (ZFX), Growth Hormone Receptor (GHR), Cholinergic Receptor Nicotinic Alpha (CHRNA1), that had been sequenced in 10 of the primate pairs (Fig. S3) that were known to produce viable hybrid offspring¹⁹. We generated pairwise distances for each of these genes using the same methodology employed in the mitochondrial analyses. We then assigned each species pair to Category 1 or

177 Category 2 based upon their *CYTB* divergence values within the original framework.
 178 In each case, though the order of the taxa based upon pairwise divergence values
 179 varied relative to the pattern generated using *CYTB* (owing to the significantly smaller
 180 divergence values in nuclear loci), for each locus the limited overlap in divergence
 181 values associated with the two categories was consistent with the mitochondrial
 182 assessment (Fig. S3).

183
 184 In order to further substantiate both this correlation and the robustness of *CYTB* as a
 185 proxy for hybrid fertility, we tested the utility of this system for predicting fertility in
 186 a well-known hybrid system. To create hybrid pets, cat breeders have crossed
 187 domestic cats (*Felis catus*) with three species of wild felid: Jungle cats (*F. chaus*),
 188 Leopard cats (*Prionailurus bengalensis*), and Servals (*Leptailurus serval*)²⁵. In all
 189 cases, the first generation male hybrids are sterile. To regain fertility while
 190 maintaining some wild cat characteristics, breeders must backcross the F1 female
 191 offspring with male domestic cats to establish a breeding population of pets²⁵. Given
 192 that backcrossing is required for all three crosses, our framework would firstly predict
 193 that the *CYTB* distances between all three pairs should be close to or greater than
 194 ~7.5% and that they should all fall into the Category 2 range. Secondly, the pairs with
 195 greater genetic distances should require more numerous backcrosses with domestic
 196 cats (halving the wild cat ancestry with each subsequent generation) before full
 197 fertility is restored and a breeding pet population is established.

198
 199 Both of these predictions are borne out by the data (Fig 1, Table S3). All three pairs
 200 possess *CYTB* distances greater than or equal to 7.54% and the increasing molecular
 201 distances between the pairs correlate with an increase in the number of required
 202 backcross generations to regain fertility. Specifically, distances between Jungle cats,
 203 Leopard cats, Servals and domestic cats (7.54%, 10.94%, and 11.28% respectively)
 204 are consistent with both the observed minimum (2, 3, and 4 respectively) and average
 205 (3, 4, and 5, respectively) number of backcrosses with domestic cats required for
 206 hybrid males to acquire fertility²⁵. These results are also consistent with an early
 207 hybrid experiment using guinea pigs in which hybrids between *Cavia fulgida* and *C.*
 208 *porcellus* (8.02% *CYTB* divergence) were able to regain male fertility after the third
 209 generation of backcrosses²⁶ (Table S1).

210

Recent accidental hybrids in zoos also confirm the predictive power of this proxy. In 2006, the Copenhagen Zoo placed a domestic sow (*Sus scrofa domesticus*) in a pen with a male babirusa (*Babirusa celebensis*) with the expectation that the two species were sufficiently evolutionarily divergent that they would be incapable of producing offspring. The *CYTB* divergence between the two species (12.89%), however, falls within the Category 2 range. Months later, five infertile piglets were born and though two died from maternally induced trauma, the other three (two males, and one female), all survived²⁷ (Table S1, Fig. S1). Historically, zoos have often accidentally produced hybrids offspring between distantly related species (Table S2), though the relative fertility of the F1s was rarely established.

Having approximated the *CYTB* threshold values within the framework, we then predicted the relative fertility of hybrids between pairs of other hominin lineages. To do so, we calculated the average pairwise divergence in *CYTB* sequences between AMH and three extinct hominin lineages: Neanderthals, Denisovans, and the ancient population from the Sima de los Huesos cave in Spain²⁸. To avoid overestimating the genetic divergence that results from comparing modern and extinct populations, we also generated distance values using the *CYTB* sequences derived solely from ancient AMH found in archaeological contexts.

The divergence values for each pairing between three *Homo* groups (Sima de los Huesos, Neanderthals, and AMH) all possess divergence values at the bottom of the Category 1 range (Fig 1, Fig. S1, Table S1). Interestingly, the divergence values of Denisovan-Neanderthal and Denisovan-AMH are the largest of the *Homo* pairings, and are consistent with the suggestion that Denisovans possess a mitochondrial lineage that may have introgressed from another source population²⁹.

In order to assess hybrid fertility within the hominin lineage more deeply, we calculated the pairwise *CYTB* divergence between humans and our closest living relatives: Chimpanzees (*Pan troglodytes*) and bonobos (*P. 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 stage³⁰. Recent molecular clock assessments have suggested that AMH and chimps diverged ~5-6 Mya³¹, well beyond both the two million year threshold

purported by other studies as the upper limit to hybrid fertility¹⁴, and the average time to speciation³².

Interestingly, our analysis places the divergence values between AMH and chimps, and AMH and bonobos within Category 2 (Fig 1, Fig S1). Six other species pairs (including two of the wild cat and domestic cat pairs) possess divergence values equivalent to or greater than AMH, chimps, and bonobos (Table S1, Fig. S1). In all six cases, however, each of the pairs possesses an identical number of chromosomes. Though karyotype is not a strict barrier to the production of hybrids (Table S1), AMH possess two fewer chromosomes than chimps and bonobos (Table S1).

Conclusions

When placed within the context of other mammalian species, ancient hominin lineages were likely not sufficiently divergent from each other to expect a biological impediment to the generation of fertile offspring. Although some studies have inferred a low level of introgression³³, the relative ease of hybridisation between hominin lineages is demonstrated by the fact that archaic populations interbred with AMH on at least four occasions³⁴. The absence of a biological barrier shifts the focus toward cultural considerations and demographic factors to explain the lack of Neanderthal and Denisovan genomic contribution on the AMH X chromosome, and the lack of mitochondrial DNA from either species within AMH. Palaeoanthropologists disagree as to what differences in culture, behaviour and cognition existed between AMH and Neanderthals (e.g.^{35,36}). Though these factors may have altered the sexual symmetry of the hybrids, they appear not to have precluded introgression in either direction³⁷.

This study demonstrates the power of genetic distances as a proxy to predict the relative sterility of hybrids resulting from matings between mammalian species. Hybrid sterility is both fluid and governed by numerous mechanisms at multiple levels of biological organization. In addition, gene flow between species whose hybrids require backcrossing is possible (Table S1), as are asymmetries in gene flow in species pairs whose F1 offspring can produce fertile F2s. We do not claim that *CYTB* plays a causative role in hybrid fertility (though a recent study proposed that speciation may be mediated by mitonuclear interactions³⁸), and the use of genetic divergence values as a proxy cannot be perfectly predictive. For example, under the

Dobzhansky-Müller model, incompatibility can arise from as few as two mutations in each of the admixing populations irrespective of time since divergence, meaning that it would be possible for closely related populations to be incapable of generating fertile hybrids³⁹, though no such examples have yet been described.

The value of any proxy, however, is determined by both its predictive power and the ease of generating the proxy data. Publicly available mtDNA sequences from thousands of mammalian taxa already exist and calculating pairwise divergence values is inexpensive, simple and fast. As a result, *CYTB* specifically (and genomic distances more generally) have substantial value as a means to predict the potential for any two mammalian species to produce viable offspring, and the relative degree of the hybrid's sterility. The discovery of additional extinct hominin populations that survived into the last 250,000 years, including *H. floresiensis* and *H. naledi*, has raised interest in understanding the limits to fertility and hybridization between extinct and extant *Homo* spp. 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 lead to a better understanding of the process of speciation and the biological and sociocultural mechanisms responsible for hybrid sterility. Our framework can also be applied more generally in contexts such as conservation biology, where predicting the consequences of biological hybridization is key to policy considerations.

Materials and Methods

Assessment of Hybrid Fertility and Rationale of Assignment into Categories

In order to ascertain if there was a relationship 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. Into Category 1 we placed eight species pairs that are capable of producing fertile F1 offspring of both sexes that can reproduce without backcrossing with a parent species. Though within these examples there have been observed asymmetries in gene flow and variation in fertility amongst the hybrids,

none of these examples requires (one or more generations of) backcrossing with a parent species for their offspring to regain fertility. More specifically, for an example to be listed in this category, we required evidence of fertility in both male and female F1 hybrids. The evidence and rationale for placing each of these pairs into Category 1 is listed in Table S1.

The hybrid offspring of all of nine pairs of species in Category 2 either require one or more generations of female hybrids backcrossing with the male of a parent species to produce fertile offspring, or are completely infertile. For these pairs, we obtained evidence demonstrating no successful F2s from F1 hybrid couplings, an inability to produce offspring other than by backcrossing to a parent species, or other biological assessments (including a histological assessment of the testis from the hybrid males) that demonstrated complete infertility (Table S1).

For instance, in the case of hybrids between lions (*Panthera leo*) and tigers (*P. tigris*), there are numerous anecdotal reports regarding hybrid male sterility in ligers (male lion x female tiger) and the backcross hybrid offspring derived from females (e.g.⁴⁰. Here, we provide histological evidence of sterility based on H&E stained testes from an adult male liger (Fig. S4 panels a and b), and an adult male tiliger (male tiger x female liger; panels c and d). Testes show clear seminiferous tubule degeneration, lined only with Sertoli cells in the liger, and tubule degeneration with germ cell arrest in the tiliger (Fig. S4 panels c, d).

Many additional live hybrid offspring have been reported in the literature than are included in Fig. 1 or in Fig. S1. The relative fertility of the hybrid offspring, however, has not been adequately assessed and we were therefore unable to confidently place them into either category. The framework and threshold values depicted in Fig. 1 allow us to predict the fertility of these offspring given the definitions described above and their placement into Category 1 or 2. These pairs are listed in Table S2 and their relative positions are depicted in Fig. S2.

Genetic Distance Calculation

Both *CYTB* sequences and full mitogenomes (excluding the control region) of multiple individuals of each species were collected from Genbank (Table S4) and

aligned using Clustal Omega version 1.2.4⁴¹. In order to ensure that none of the sequences was either mislabelled or were nuclear copies of mitochondrial genes, we constructed Neighbour-Joining trees using Geneious version 6.1.8⁴² and removed all individuals that did not fall into monophyletic clades of each species. We first used *pModelTest* version 1.04⁴³ 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 version 8⁴⁴ and FASTTree version 2.1⁴⁵. We also generated raw distance values (i.e. the proportion of sites that differ between each pair of sequences) using the Hamming distance method which sums the number of basepair differences, regardless of whether they are transitions or transversions, 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 wrapper to wrap around the programs RAXML, FastTree, pModelTest, and a custom python script written to test Hamming distance of sequences making use of the distance version 0.1.3⁴⁶ and Biopython version 1.68⁴⁷ modules. The script was written for use in version 2.7 of the Terminal application in an OSX environment, and the source code is included at the end of this Supplementary Information. To generate the Hamming distance calculations, the aligned sequences had to be the identical length (meaning all partial sequences were rejected). In addition, gaps in the published sequences were ignored across those loci and across each alignment even if a nucleotide was present at those loci in other sequences to minimise the artificial inflation of the divergence value differences.

Mann-Whitney test of statistical difference between *CYTB* distance in hybrid categories

The statistical significance of observed differences in mitochondrial genetic distance between groups with reduced hybrid compatibility and those with full compatibility, was tested using the Mann-Whitney U test ($p = 5.439\text{e-}06$) implemented in the R-software package(R Core Team 2013⁴⁸).

Figure Legends

Figure 1

A depiction of the correlation between *CYTB* divergence between terrestrial mammalian species pairs and the relative fertility of their hybrid offspring. In column A, the green circles represent species capable of producing fully fertile F1 offspring which can reproduce independently of their parent species (Category 1). Brown circles represent species pairs whose F1 offspring either require backcrossing with a parent species or are completely infertile (Category 2). The lighter green and brown shaded regions represent the range of divergence values of the two categories and, given their general lack of overlap, can be used to determine the relative fertility of hybrid offspring. 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 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. All hominin pairs are positioned at the lowest (most fertile) end of Category 1, suggesting that their hybrid offspring are likely to have been fully fertile. Additional detail regarding the specific species pairs are listed in Fig. S1 and Table S1.

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