Title: Genetic variation in *Pan* species is shaped by demographic history and harbors lineage-specific functions

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Abstract

Chimpanzees (*Pan troglodytes*) and bonobos (*Pan paniscus*) are the closest living relatives of humans, but the two species show distinct behavioral and physiological differences, particularly regarding female reproduction. Despite their recent rapid decline, the demographic histories of the two species have been different during the past one to two million years, likely having an impact on their genomic diversity. Here, we analyze the inferred functional consequences of genetic variation across 69 individuals, making use of the most complete dataset of genomes in the *Pan* clade to date. We test to which extent the demographic history influences the efficacy of purifying selection in these species. We find that small historical effective population sizes (N_e) correlate not only with low levels of genetic diversity, but also with a larger number of deleterious alleles in homozygosity and an increased proportion of deleterious changes at low frequencies. To investigate the putative genetic basis for phenotypic differences between chimpanzees and bonobos, we exploit the catalog of putatively deleterious protein-coding changes in each lineage. We show that bonobo-specific non-synonymous changes are enriched in genes related to age at menarche in humans, suggesting that the prominent physiological differences in the female reproductive system between chimpanzees and bonobos might be explained, in part, by putatively adaptive changes on the bonobo lineage.

Introduction

Chimpanzees (*Pan troglodytes*) and bonobos (*Pan paniscus*) are two closely related species with an estimated divergence time of 1 to 2 million years ago (Mya) and share a most recent common ancestor with humans 5 to 13 Mya (Langergraber & Prüfer 2012; Fischer et al. 2011; Prado-Martinez et al. 2013; de Manuel et al. 2016). They are interesting species to study genetic changes in comparison to humans for at least two reasons: First, they have experienced different demographic histories since their divergence. For example, the population history of bonobos is marked by population bottlenecks in the past and a smaller long-term small effective population size (N_e) (Prado-Martinez et al. 2013; de Manuel et al. 2016).

They are also known to have been geographically isolated in central Africa for a long time (nowadays Democratic Republic Congo) (Thompson 2003; Takemoto et al. 2017), which is expected to increase their genetic homogeneity. This is in stark contrast to the history of chimpanzees, which inhabited a much wider region across Africa (from Tanzania to Guinea, at the east and west edges of Africa). Most chimpanzee subspecies, except of the western population, are thought to have had a larger population size and to have maintained a greater level of genetic diversity compared to bonobos (de Manuel et al. 2016). The western chimpanzees are thought to have spread from a very small ancestral population (Shimada et al. 2004; Sá et al. 2013), while central chimpanzees appear to have been more continuously inhabiting central Africa (de Manuel et al. 2016).

A second reason why comparing the two sister species is interesting is that, despite many similarities, they show recognizable phenotypic differences likely due to genetic differences. One of their most distinct differences is that bonobos, but not chimpanzees, have a prolonged maximal sexual swelling, where females appear to be estrous even when they are not ovulating (*e.g.* during lactating period, Thompson-Handler et al. 1984; Furuichi 1987). Maximal or exaggerated sexual swellings in the perineal part around their ovulation period is a distinct feature of the *Pan* clade among the great apes (Nunn 1999), but bonobos are different from chimpanzees in that they present it even when they are not ovulating, and more often than female chimpanzees do. This phenotype is likely to have a physiological mechanism, and indeed at the physiological level, measuring urinary testosterone hormonal levels, it has been shown that female bonobos are on average three years younger than their chimpanzee counterparts when they experience the onset of puberty, which is an important clue for their differential sexual development (Behringer et al. 2014). It has been suggested that these differences are a source of different social dynamics between bonobos and chimpanzees. Chimpanzees show a clear hierarchy and frequent violence among males, while bonobos show more egalitarian interactions (Nishida 1983; Goodall 1986; Furuichi 1987; Idani 1990), frequent female-female sexual interactions (Kuroda 1980) and high female social status (Furuichi 1987), compared to female chimpanzees. Prolonged maximal sexual swellings in bonobos have been suggested as a mechanism to explain the egalitarian and peaceful social dynamics in bonobos, first

by lowering the operational sex ratio (the temporal ratio of adult males to estrous females in the group, Randall Parish 1994; Furuichi 2011) and second by strengthening female-female sexual interactions (females are more attracted to other females that show sexual swellings, which builds female affiliative relationships and a strong social stance in a male-philopatric society, Ryu et al. 2015). Considering that the high social status of bonobo females is also viewed as a driving force of selection on non-aggressive males (Hare et al. 2012), the prolonged sexual swelling can be considered a key aspect of bonobo-specific evolutionary features. Here, we investigate the genetic differences between these species, as they are likely to they underlie their physiological differences. We use derived alleles in each lineage, which are the obvious candidate changes for such lineage-specific traits, particularly non-synonymous changes and other genetic changes with potential functional impact.

Bonobos have a smaller N_e (11,900–23,800) than central chimpanzees (24,400–48,700). However, chimpanzee subspecies differ substantially, with the N_e of central chimpanzees being about four times larger than that of western chimpanzees (9,800–19,500) (Prado-Martinez et al. 2013) (summarized in Table S2). Whether different demographic histories influence the efficacy of purifying selection in populations has been a debated topic in the field of population genomics. Many studies have shown that in human populations with small N_e and with population bottlenecks, genetic diversity is lower (the number of heterozygous genotypes per individual; Lohmueller et al. 2008; Kidd et al. 2012; Torkamani et al. 2012; Hodgkinson et al. 2013), while the rate of random fixation of derived alleles is higher (the proportion of fixed substitutions and number of homozygous derived alleles per individual; Lohmueller et al. 2008; Kidd et al. 2012; Torkamani et al. 2012) than in populations that have maintained a larger N_e. These patterns are generally explained by the effects of random genetic drift, in the framework of the neutral theory (Kimura 1968), which assumes most genetic variation to be neutral or nearly neutral (Ohta 1972; Ohta 1973). In order to assess the efficacy of purifying selection, it is crucial to consider the proportion of deleterious changes, particularly at high frequencies within the population. It has been inferred that almost half of all non-synonymous single nucleotide changes (SNCs) in a single genome are

deleterious (Subramanian & Lambert 2012). Such deleterious allele changes are, for example, predicted to alter protein function, which is typically strongly conserved across species, or are associated with an increased risk of disease; hence, they are proposed to be under purifying selection that reduces their probability of fixation in the population (Jukes & Kimura 1984). Slightly deleterious changes are particularly interesting because they are relatively well tolerated, appear as polymorphisms (Henn et al. 2016), and thus could be informative about the effects of purifying selection. We expect a larger proportion of slightly deleterious SNCs at high frequencies in a population with relaxed purifying selection pressure than in one with more efficient purifying selection, in agreement with the nearly neutral theory (Ohta 1972; Ohta 1973). This hypothesis has been tested in different species, including archaic and modern humans (Lohmueller et al. 2008; Castellano et al. 2014), dogs (Marsden et al. 2016), and rice populations (Liu et al. 2017). Several studies have also analyzed the efficacy of purifying selection in the *Pan* clade: Using the exomes of central, eastern and western chimpanzees, (Bataillon et al. 2015) showed that the efficacy of natural selection correlates with past N_e, in agreement with (Cagan et al. 2016), where lineage-specific selection signatures from all the great ape genomes led to the same conclusion. On the other hand, (de Valles-Ibáñez et al. 2016) interpreted data of various great ape species, including the very similar eastern and Nigerian-Cameroon chimpanzees (Prado-Martinez et al. 2013), that the load of loss-of-function (LoF) mutations, which probably have severe consequences, is not influenced by the demographic history. However, given the differences in demographic history across chimpanzee subspecies, it seems important to include all four recognized subspecies, particularly the central and western chimpanzee populations, which had the largest and the smallest N_e, respectively (Prado-Martinez et al. 2013). Bonobos in turn experienced population bottlenecks and are believed to have maintained a small N_e since their split from chimpanzees (Prado-Martinez et al. 2013). Comparing the mutational load in all the chimpanzee lineages and bonobos together based on their well-known demographic history, which has not yet been studied, would provide us with a comprehensive picture on the interplay between purifying selection and the demographic history.

In this study, we examine 59 chimpanzee and 10 bonobo genomes (de Manuel et al. 2016) to investigate the accumulation of putatively deleterious derived alleles, with the goal of testing how their demographic histories have shaped the distribution of such alleles. We analyze changes in the bonobo and chimpanzee lineages, assessing the enrichment of genes associated to different phenotypic traits using human a GWAS database. We interpret an enrichment in genes related to these traits as indication of a genetic basis for lineage-specific differences between the two *Pan* species.

Materials and Methods

Data Preparation

Chimpanzee and bonobo genomes used in this study were generated in previous studies (Prado-Martinez et al. 2013; de Manuel et al. 2016). The data consists of 59 chimpanzee and 10 bonobo genomes sequenced to high coverage, including four chimpanzee populations: 18 central, 20 eastern, 10 Nigerian-Cameroon and 11 western chimpanzees (Fig. S1 and Table S1). All genomes were mapped to the human genome assembly *hg19* (ENSEMBL GRCh37.75), and previously published genotype calls for single nucleotide changes (SNCs) – variable sites across all the genomes of the *Pan* populations – in mappable regions on the autosomes were used (de Manuel et al. 2016). We applied the ENCODE 20mer uniqueness filter, and required all the heterozygous loci to be allele balanced, with ratios of the raw reads between 0.25 and 0.75, in order to exclude sites with potential biases or contamination. Across 69 individuals, the initial VCF file contained 36,299,697 loci in total. After applying all filters, we used 33,946,246 loci where at least one individual shows a reliable genotype, while only 5,795,261 loci have information for all 69 individuals. For the analysis with eight individuals per population at highest quality, we exclude samples which could have had a small percentage of human contamination. To do so, we counted positions where all *Pan* individuals carried an allele different from the human reference allele in homozygous state, with the exception of one single sequencing read in one individual. For the same individual, we required at least five sequencing reads to match the derived allele in other *Pan* individuals to assign the single human-like sequencing read as contamination. We then selected individuals with

fewer than 10,000 such observations across the entire genome, choosing those individuals with the highest coverage (Table S1). For these analyses, we only considered sites where high-quality genotypes were available for all individuals. Furthermore, where stated, a subset of ten central chimpanzees for a fair comparison with bonobos was used (Table S1). In the analysis of GWAS-associated genes described below, a more permissive population-wise filtering was applied, using sites at which at least 50% of individuals in each species (chimpanzee and bonobo) pass the above filters, and one of the lineages carries the derived allele at more than 90% frequency, while the other population carries less than 10% of the derived allele. Population-wise frequencies were calculated as proportions of the observed counts.

The functional effects for each SNC were inferred using the ENSEMBL Variant Effect Predictor (VEP) v83 (McLaren et al. 2016) on all segregating sites across the 69 individuals. Non-synonymous and synonymous SNCs were used as defined by VEP, and the following functional categories were retrieved from the ENSEMBL annotation: 5' UTR, 3' UTR, regulatory elements, splice sites, transcription factor binding sites, upstream variants, downstream variants. Each variant can carry multiple annotations, and all annotation categories are treated separately in the subsequent analyses. In rare cases where a variant overlaps with multiple genes, both genes would be associated to that variant. Genomic regions and positions were analyzed using *R/Bioconductor* (Huber et al. 2015) and the packages *biomaRt* (Durinck et al. 2005) and *GenomicRanges* (Lawrence et al. 2013). The catalog of variation and functional inference in *Pan* species is available under (will be available before publication).

Terminology

"Derived change" refers to the allele state different from the reference allele state. In this study, the human genome was used as reference, and since it is an outgroup to the *Pan* clade it represents closely the ancestral state at *Pan*-specific mutations. "Ancestral state" refers to the allele state same as the human reference allele state. "Lineage-specific derived changes" refers to the derived changes fixed or almost fixed (>=90%) in one lineage (either chimpanzee or bonobo), where the other lineage appears fixed or almost fixed for the ancestral state (<10% derived). Doing so, we avoid neglecting actual fixed sites which

appear not fixed due to sequencing errors, and include sites which are influenced by gene flow between populations.

Simulations

We used the forward-simulator SFS_CODE (Hernandez 2008), in order generate three populations diverging from one source population, following the demographic parameters of bonobos, central and western chimpanzees, which we find most relevant in interpreting our observation in relationship of the demographic history and the mutational load. We have generated 8 individuals per population of bonobos, central and western chimpanzees, for one locus of 10,000 base pair length in 200 iterations. We used the simplified N_e in (Prado-Martinez et al. 2013), with a divergence of 40,000 generations between chimpanzees and bonobos, and 28,000 generations between central and western chimpanzees. We simulated neutral fragments (sfs_code 3 1 -TS 0 0 1 -Td 0 P 0 10 -Td 0 P 1 22 -TS 28 1 2 -Td 28 P 1 2.7 -Td 28 P 2 0.45 -TE 40 -W 0 -L 1 10000 -n 8 -a N --theta 0.001 --rho 0.001) and fragments under purifying selection with a gamma of 0.17 for 70% of the sites (Bataillon et al. 2015) (sfs_code 3 1 -TS 0 0 1 -Td 0 P 0 10 -Td 0 P 1 22 -TS 28 1 2 -Td 28 P 1 2.7 -Td 28 P 2 0.45 -TE 40 -W 1 0.17 0 0.7 -L 1 10000 -n 8 -a C --theta 0.001 --rho 0.001).

Deleteriousness assessment

We used the following methods to assess deleteriousness: Grantham score, C-score, GWAVA, SIFT, PolyPhen-2 and GERP score. We did so in order to avoid a bias coming from a particular method and ensure that our results are robust to the method, some based on conservation across different taxa, others on physicochemical property or known consequences of the variants. More specifically: The Grantham Score (Grantham 1974; Li et al. 1984) represents only the physical/chemical properties of amino acid changes. A custom cutoff 150 (>150) was used to determine deleterious/radical changes. The C-score (Kircher et al. 2014) measures deleteriousness on a genome-wide scale for both coding and non-coding variants, integrating a variety of known functional information. A cutoff of 10 was used to select the top

10% most deleterious changes. GWAVA (Ritchie et al. 2014) predicts the functional impact of genetic variants, based on genome-wide properties. A custom cutoff at 0.5 (>0.5) was used to select potentially functional changes. SIFT (Kumar et al. 2009) predicts the deleteriousness of amino acid changes, based on the degree of conservation inferred from sequence alignments. All changes diagnosed as 'deleterious' was analyzed. PolyPhen-2 (Polymorphism Phenotyping v2) (Adzhubei et al. 2010) predicts the possible impact of amino acid changes on the structure and function using both physical property and multiple sequence alignments. All changes diagnosed as 'probably_damaging' were analyzed. Finally, GERP (Genomic Evolutionary Rate Profiling) scores (Davydov et al. 2010) compare, based on multiple alignments, the number of observed substitutions to the number of hypothetical substitutions given that they are neutral changes. A cutoff of 4 (>4) was used to select the changes with 'large' functional consequences. They assume a deficit of observed substitutions as "Rejected Substitutions", a natural measure of constraint on the element. For our analyses, we used precomputed base-wise scores for *hg19* (http://mendel.stanford.edu/SidowLab/downloads/gerp/). Neutral loci were defined as described in (Gronau et al. 2011).

Analysis of non-synonymous lineage-specific SNCs

We used the software *FUNC* (Prüfer et al. 2007) to determine an enrichment of Gene Ontology terms. We ranked the genes by the number of all lineage-specific non-synonymous changes, divided by the number of lineage-specific deleterious SNCs. In order to assess whether particular gene categories are enriched for lineage-specific deleterious SNCs in each lineage, we used the Wilcoxon rank test.

In order to formally assess an enrichment of lineage-specific non-synonymous changes in gene clusters associated with known phenotypic traits in humans, we retrieved genome-wide association data from the NHGRI-EBI GWAS Catalog (MacArthur et al. 2017), containing 2,385 associated traits. We analyzed all the associated genes in the data, which have protein-coding SNCs either on the chimpanzee or bonobo lineage, respectively. We used the permissive set of non-synonymous SNCs (*Data Preparation*). For each trait from each study ("Disease trait"), we counted the number of non-synonymous SNCs on each lineage,

and performed a contingency table significance test (G-test) against the total number of non-synonymous SNCs, compared to the total number of genes associated to the trait and the total number of protein-coding genes. This test determines an enrichment of this trait considering the number of protein-coding changes. We also performed a G-test between the total numbers of all non-synonymous SNCs on each lineage compared to the numbers of non-synonymous SNCs on each lineage falling in genes associated to the trait, in order to determine whether or not the difference between the two species is significant. In both cases, a *P*-value cutoff of 0.1 was applied. These cutoffs are permissive, considering that the data relies on independent studies on different cohorts, with high significance cutoffs within each study. This also results in a large number of tests with 0 observation counts. Finally, we performed an empirical enrichment test, by creating 500 random sets of genes with similar length as the genes associated to each trait (± 10% of the length of each gene), and counting the number of lineage-specific non-synonymous SNCs in each random set. Here, we require 90% of random sets to contain fewer non-synonymous SNCs in a given lineage than the real set of associated genes, to determine a trait significant. We note that this test is analogous to a false discovery rate, since it empirically determines how often we would expect GWAS loci to fall in the genes of interest. Performing the same analysis on a randomly drawn set of genes did not show an enrichment of any GWAS trait, suggesting that no significant enrichment is expected after applying these three tests. Finally, we filter for traits with at least 10 associated loci in order to restrict the analysis to multigenic traits, and we report only significant traits where at least three genes carry non-synonymous SNCs.

We screened the genes which have lineage-specific non-synonymous SNCs at high frequency, and confirmed their expression in chimpanzees and bonobos, using the dataset from (Brawand et al. 2011). The RNA sequencing data was mapped to the human genome assembly *hg19* using *tophat2* (Kim et al. 2013), and gene expression was estimated with *samtools* (Li et al. 2009) and *htseq-count (Anders et al. 2015)*, which measures the total count of fragments falling in each gene. Genes were defined as expressed when either lineage showed a log2-normalized expression value larger than 3 (more than 2 fragments mapped to the whole gene).

Results

Neutrality index in populations

Previous studies have shown that the historical N_e has been lowest in western chimpanzees and highest in central chimpanzees (Won & Hey 2005; Prado-Martinez et al. 2013; de Manuel et al. 2016; Kuhlwilm et al. 2016) (Table S2). Accordingly, we observe differences in measures like average heterozygosity per base pair across each population, which is indeed highest in central chimpanzees (0.0014) and lowest in western chimpanzees (0.0006) (Table S2). To study the overall impact of these N_e differences on the efficacy of selection, we used inferred fixed derived (D) and polymorphic (P) non-synonymous (n) and synonymous (s) alleles in samples of equal size of eight individuals from each population, to calculate a population-wise version of the Neutrality Index (NI) (Rand & Kann 1996), which is the ratio of P_n/P_s and D_n/D_s. The NI quantifies the direction and degree of natural selection, based on the assumption that synonymous substitutions are neutral. In the classical NI, a value greater than one indicates an excess of non-synonymous polymorphism over fixed derived sites, implying negative selection, while a value lower than one indicates an excess of fixed non-synonymous alleles and may imply positive selection. We find that the NI across autosomes in western chimpanzees (1.51) is higher than in the other chimpanzee subspecies (1.19-1.28), caused by an excess of non-synonymous over synonymous polymorphisms (Fig. 1A, Table S3). This could be explained by reduced efficacy of purifying selection in this population as the result of a low N_e (Eyre-Walker 2006) that allows slightly deleterious alleles to accumulate as polymorphisms in the population. The direction of selection (DoS) is another statistic that uses *Pn*, *Ps*, *Dn* and *Ds* to quantify the accumulation of slightly deleterious mutations as a measure of reduced efficacy of purifying selection (Stoletzki & Eyre-Walker 2011). Here, when calculating the DoS, we observe the lowest value for western chimpanzees (-0.1) and the highest for central chimpanzees (-0.04) (Fig. 1B, Table S2), which suggests that positive selection is not abundant in these populations (no positive values) and that a larger proportion of deleterious sites is segregating in western chimpanzees compared to central chimpanzees (more negative value). In bonobos, the NI is higher (and DoS smaller) than in all non-

western chimpanzees, suggesting a stronger accumulation of slightly deleterious alleles. When calculating the NI per gene, as in previous studies (Rand & Kann 1996), this pattern is confirmed (Table S2). However, we caution that only 131 genes carry a sufficient number of informative sites in these populations. The excess of potentially deleterious polymorphism in western chimpanzees is not exclusive for protein-coding changes, and it is present also in different categories of non-coding sites in functional elements, such as 5'UTRs and the upstream and downstream regions of genes (Fig. 1B, Table S2). This suggests that, also in non-coding loci, the efficacy of purifying selection was lowest in populations with smaller historical N_e.

Distributions of deleterious changes

We analyzed the population-wise ratio of deleterious to neutral derived alleles across the site frequency spectrum (SFS) (Fig. 2), using eight individuals from each population. Deleterious alleles in Fig. 2 were defined each by GERP (Davydov et al. 2010) and PolyPhen-2 (Adzhubei et al. 2010), which represents genome-wide and protein-coding predictions, respectively. We have used four other methods for diagnosis of deleteriousness of allele changes, in order to assess robustness of the methods. We note that phylogeny-based approaches, such as C-score and GERP scores, may have a bias in western chimpanzees, since this subspecies was used for the reference chimpanzee genome. Methods based on protein sequence and structure, such as SIFT and PolyPhen-2, could avoid such a bias. The Grantham score, on the other hand, measures only the chemico-physical changes of amino acids and might be the most conservative non phylogeny-based approach. We generally observe a much higher proportion of deleterious derived alleles at high frequencies in bonobos than chimpanzees (Fig. 2 and S2). When stratifying alleles into singletons vs. non-singletons, the deleterious-to-neutral ratio in non-singletons is highest or second-highest in bonobos, except for Grantham scores, compared to chimpanzee populations (Table S5). This suggests that bonobos, which experienced long-term small N_e and bottlenecks since the split from chimpanzees, have accumulated proportionately more deleterious alleles at high frequencies than chimpanzee populations. At low frequencies, we observe all non-central chimpanzee populations having higher proportions of

deleterious derived alleles, with western chimpanzee being particularly high. These patterns are generally similar using other deleteriousness estimates (Fig. S2). When simulating data based on the demographic history of bonobos, central and western chimpanzees, we also find that proportionately more deleterious changes than neutral changes accumulate in singletons and at low frequencies in the western-chimpanzee-like population in comparison to the central-chimpanzee-like population, which is in agreement with our data (Fig. S16).

Another way to assess the efficacy of purifying selection is to investigate the effects of population size on the individual mutational load. We estimated the mutational load, defined as the number of sites with putatively deleterious derived alleles per individual. We stratified them into two different classes, by counting either only heterozygous sites or only homozygous sites. When only heterozygous sites are considered, the western chimpanzee population shows the lowest level of mutational load among chimpanzee populations (Fig. 3A). This is significantly lower than in the other chimpanzee populations ($P < 0.001$; Wilcoxon rank test), and is largely due to their low genetic diversity. However, when only homozygous sites are considered, the mutational load of western chimpanzees increases drastically (Fig. 3B), and becomes significantly higher than that of central chimpanzees ($P < 0.001$; Wilcoxon rank test). This is probably because slightly deleterious alleles more often reach high frequencies, and are observed in homozygosity in populations with small N_e, since purifying selection is less efficient. Regarding the other chimpanzee populations, the mutational load shows a gradient with a non-significant correlation trend with their long-term N_e (de Manuel et al. 2016) and heterozygosity (Table S2), negative in homozygous sites ($R=-0.87$, $p=0.05$, Spearman correlation test) and positive in heterozygous sites ($R=0.97$, $p=0.004$), using Grantham score. This pattern is broadly similar throughout other classes of sites, including synonymous changes, in agreement with previous observations (Lohmueller et al. 2008) (Figs. S9-S14). This pattern appears less clear using C-score and GERP ($R=0.05$, $p=0.93$ and $R=0.2$, $p=0.74$, respectively for homozygous mutational load, Spearman correlation test). This might be due to the reference chimpanzee genome used in these methods being a western chimpanzee, as that could lower these scores for the derived changes in western chimpanzees. Still, the distributions of the heterozygous

and homozygous mutational load of central chimpanzees are different from the other three chimpanzee populations in C-score and GERP ($P < 0.0001$ and $P = 0.009$ respectively; Wilcoxon rank test). Simulated data also appears to show mutational load positively correlating with the N_e in heterozygous loci, and negatively in homozygous loci (Fig. S18), even though the statistical significance is rather weak (R=0.86 p=0.33 and R=-0.86 p=0.33 respectively both in deleterious and neutral changes, Spearman correlation test).

Protein-truncating variants

We also assessed the patterns in protein-truncating variants in each population by estimating the mutational load in loss-of-function (LoF) mutations, defined as the number of LoF derived alleles per individual. These SNCs may be considered as most likely disruptive for protein function and hence a straightforward measure for evaluating the efficacy of purifying selection. In our data, the patterns of mutational load in LoF mutations follow the patterns in other categories of deleterious mutations (Fig. 3B). With higher N_e, the load tends to increase in heterozygous sites (r=0.786 with p=0.115). On average, chimpanzee populations carry between 61 and 118 heterozygous LoF alleles, and between 547 and 560 as homozygotes (Fig. 3C). In comparison, the average mutational load of LoF mutations in modern human populations is 85 for heterozygous sites (Lek et al. 2016), which is in agreement with previous observations of a slightly higher number of heterozygous LoF mutations in chimpanzees than humans, but smaller than in gorillas (Xue et al. 2015; de Valles-Ibáñez et al. 2016). We do not directly compare the number of homozygous sites to those in modern humans, because in (Lek et al. 2016) only polymorphisms within the human lineage were used, while in our analysis, not only polymorphisms but also fixed differences between the two *Pan* species were measured (de Manuel et al. 2016).

When considering the same numbers of individuals per population, we observe twice as many fixed LoF mutations in western chimpanzees, and a 2.7-fold increase of fixed LoF mutations in bonobos, compared to central chimpanzees (data not shown). Conversely, the number of LoF singletons is three times higher in central chimpanzees than western chimpanzees, while the other chimpanzee populations are similar to

the western chimpanzees, as expected by their background levels of genetic diversity. However, in western chimpanzees, the proportion of LoF mutations to neutral mutations is higher in polymorphisms than in fixed variants ($P < 0.01$; G-test). Also, the proportion of polymorphic LoF to neutral sites is higher in western chimpanzees than central chimpanzees ($P = 0.012$, G-Test). This effect is particularly pronounced in singletons (Fig. 3C), suggesting that LoF mutations are more often tolerated in western chimpanzees compared to the other populations, which could be due to less efficient purifying selection.

An overview of lineage-specific changes

We assessed lineage-specific SNCs for their predicted functional effect, since these are candidates for functional changes explaining lineage-specific traits. In Table 1, we provide an overview of these SNCs, stratified by annotation category, when using ten individuals each from the bonobo and central chimpanzee population. This shows that bonobos have, on average, about two-fold more lineage-specific changes than central chimpanzees. Not surprisingly, this ratio is even higher when using 10 bonobos and the 59 chimpanzees (Table S4).

We assessed the deleteriousness of the lineage-specific SNCs as a proxy for functional and phenotypic consequences, using the six deleteriousness measures described above (Methods). This produced a catalog of genes with lineage-specific deleterious SNCs among protein-coding changes, and of genes carrying the 50 most deleterious lineage-specific SNCs in genome-wide inferences across these measures (Table S5, S6), in total affecting 78 genes in chimpanzees and 244 genes in bonobos. In bonobos, five of these genes are, according to the literature, involved in female reproduction: *ABCA13 (Nymoen et al. 2015)*, *ESPL1* (Gurvits et al. 2017), *KIF14 (Singel et al. 2014)*, *LVRN* (Singel et al. 2014) and *MAP4 (Nystad et al. 2014)*, while six are involved in male reproduction: *ACSBG2* (Daisuke et al. 2009)*, GALNTL5* (Fraisl et al. 2006)*, NME8* (Takasaki et al. 2014)*, WBP2NL* (Sadek et al. 2001), *WDR63* (Wu et al. 2007) and *ZFHX3* (Hozumi et al. 2008). In chimpanzees, we identified only one gene related to female reproduction (*TOP2A*, Hering et al. 2014) and one gene involved in male reproduction (*FANCL*, Wong 2003). These genes are expressed in both *Pan* lineage cell lines (Table S9, S10), which implies that those changes might

harbor functional differences. We also found that, in both species, multiple genes carry lineage-specific deleterious SNCs related to immunity, optic, heart and nervous system (Table S6, S7). Furthermore, we find nine protein-truncating variants in bonobos, and only two in chimpanzees (Table S8).

To explore the putative effect of lineage-specific SNCs on phenotypes, we explored the NHGRI-EBI GWAS Catalog (MacArthur et al. 2017). We find one lineage-specific derived SNC that is almost fixed in bonobos but absent in chimpanzees, which is polymorphic in humans and associated with the trait "economic and social preference". Chimpanzees carry the ancestral “risk allele" rs12606301-G. On the other hand, alleles that are at high frequency in chimpanzees, but absent in bonobos, are the human protective alleles rs17356907-G for breast cancer and rs3757247-A for both Vitiligo and type I diabetes, and the risk alleles rs1872992-G for "BMI-adjusted waist-hip ratio and physical activity measurement" and rs60945108-A for "physical activity".

Enrichment of deleterious and non-synonymous SNCs

To test if these lineage-specific deleterious SNCs are enriched in any particular gene family or pathway, we performed a formal Gene Ontology enrichment test. The results suggest that, on the bonobo lineage, among others, there is an enrichment in biological categories like “homophilic cell adhesion via plasma membrane adhesion molecules”, “steroid hormone mediated signaling pathway”, and “cell morphogenesis” (Table S12). On the other hand, in the chimpanzee lineage, we find an enrichment in categories such as “ionotropic glutamate receptor signaling pathway”, “positive regulation of GTPase activity”, and neuron-related categories like “positive regulation of axonogenesis” (Table S12).

In order to explore in further detail genes that are associated with polygenic traits, we performed a systematic enrichment screen for 2,385 traits from genome-wide association studies in humans (MacArthur et al. 2017), using a more permissive set of derived non-synonymous SNCs at high frequency (Methods). This analysis is based on the nearest genes to the associated site in humans, since most associated human SNCs are not segregating in the *Pan* dataset. We find 17 unique traits enriched for non-synonymous SNCs on the chimpanzee lineage, and five unique traits on the bonobo lineage (Table S13),

among them "Menarche (age at onset)". This suggests that in bonobos there is an enrichment of non-synonymous changes in genes affecting this female reproduction-related trait. Other categories for which we find an enrichment include "Cognitive performance", "Parkinson's disease", "Urinary albumin-to-creatinine ratio" and "Obesity-related traits", which might reflect changes in bonobos related to cognitive abilities and metabolism. Genes with non-synonymous SNCs on the chimpanzee lineage are enriched, among others, for associations to traits involving body mass index and height, as well as "Schizophrenia" and “Loneliness”.

The finding of an excess of lineage-specific non-synonymous SNCs in genes associated with age at menarche suggests that we can identify genetic changes that may underlie the physiological differences between the two *Pan* species in terms of some female reproduction traits. We further investigated this trait using the 307 protein-coding genes associated to age at menarche in the most recent, most comprehensive meta-analysis of this trait to date (MacArthur et al. 2017), which was not included in the GWAS database. Here, we observe a significant proportion of menarche-associated genes with bonobo-specific non-synonymous SNCs (P = 0.0025, G-Test, Table S14). This observation is even more pronounced when considering that 73 SNCs (Table S15) fall into these 55 candidate genes (P = 0.001, G-Test). This number of non-synonymous changes was not observed across 1,000 random sets of genes of similar length as the menarche-associated genes (Fig. 4). No enrichment of such changes is found on the chimpanzee lineage (P = 0.48, G-Test). We conclude that menarche-related genes seem to have acquired more non-synonymous SNCs than expected on the bonobo lineage.

Discussion

We made use of the genetic variation across 10 bonobo and 59 chimpanzee high-coverage genomes (Day et al. 2017), which is the most comprehensive genomic data to date for the two *Pan* species, including all four known subspecies of chimpanzees. We use the predicted effects of single-nucleotide variants to analyze differences in the distribution of deleterious alleles between populations, stratified by protein-coding, non-coding functional and loss-of-function SNCs. We present a catalog of SNCs that are lineage-

specific and determine associations to known functions in either species especially for non-synonymous variants, since these are likely to underlie phenotypic differences.

Effective population size influences the distribution of deleterious alleles

We investigated the efficacy of purifying selection in relation to the demographic history of populations, by making use of deleterious derived alleles. Our results show that the population-wise Neutrality Index is highest in the western chimpanzee population compared to the other chimpanzee populations and bonobos (Fig. 1A). We also observe that the proportions of deleterious derived alleles, in comparison to neutral derived alleles, are highest in the western chimpanzee population across different non-coding functional element categories (Fig. 1C). The ratios of deleterious derived allele frequencies to neutral derived allele frequencies using six different deleteriousness prediction methods (Fig. 2 and Fig. S2) show that in populations which experienced a long-term small N_e and population bottlenecks, proportionately more deleterious derived alleles segregate in the population. The proportion of deleterious derived allele frequencies in bonobos is higher at high frequencies compared to chimpanzees, which might be the consequence of a long-term small N_e and multiple population bottlenecks after the split from chimpanzees (de Manuel et al. 2016). At low frequencies, the proportion of deleterious derived allele frequencies is much higher in non-central chimpanzee populations compared to central chimpanzees, likely the consequence of a more recent small N_e and population bottlenecks in non-central chimpanzee populations (de Manuel et al. 2016). These observations are predicted by the nearly neutral theory (Won & Hey 2005; Prado-Martinez et al. 2013), *i.e.* slightly deleterious mutations, which would rather be selected against than be selected for by natural selection (Ohta 1972), but minor enough to be compensated by other mechanisms, could behave like neutral mutations and more easily become fixed in populations with small N_e. These results are also similar to the report that proportionately more deleterious derived alleles are observed in Neandertal exomes compared to modern human exomes, which diverged from each other within a similar time range as chimpanzee subpopulations did, and between exomes from Eurasian and African modern humans (Castellano et al. 2014; Prüfer et al. 2014). Proportionately more deleterious

derived alleles at low frequency in archaic humans and in Eurasians might be comparable to the patterns among chimpanzees, with a more recent experience of small N_e and population bottlenecks, allowing deleterious mutations to segregate. These results agree with a better efficacy to remove deleterious alleles in larger populations, as observed previously across great apes (Fu et al. 2012), and with previous reports for modern humans, dogs and rice (Cagan et al. 2016).

On the other hand, we compare the number of homozygous to heterozygous deleterious derived alleles in each individual across populations. This clearly shows a population-wise separation in the distribution of individual deleterious load (Fig. 3A). Heterozygous sites are strongly influenced by rare variants, and a proxy for the population diversity. Homozygous derived sites, on the other hand, are likely influenced by long-term N_e and population bottlenecks, and dominated by fixed or high frequency derived alleles. Although there is an ongoing discussion on this matter (Charlesworth 2013; Kirk E. Lohmueller 2014; Simons et al. 2014; Kim & Lohmueller 2015), population size and genetic drift seem to influence the distribution of changes in homozygous positions. This is interpreted as the influence of small N_e and population bottlenecks, which causes disproportionate shifts of deleterious derived changes to high frequencies in a population (Kirk E Lohmueller 2014; Simons et al. 2014). Our results on heterozygous sites suggest that the central chimpanzee population carries the largest genetic diversity, and on the homozygous sites that western chimpanzees carry the highest level of homozygous mutational load, similar to bonobos. We conclude that the population history generally affects the way derived changes are distributed across homo- and heterozygous sites in the genome in our closest living relatives. Interestingly, among chimpanzees, which are generally understood as a species with a large genetic diversity (Prado-Martinez et al. 2013), the western chimpanzee population appears to be rather similar to bonobos in their mutational load, which highlights its experience of small N_e and population drift similar to bonobos, after their split from the other chimpanzee lineages.

Previously, (de Valles-Ibáñez et al. 2016) analyzed the effect of population size on the deleterious burden based on LoF mutations in the great apes, suggesting it to be very weak. Here, we improve the inferences with a fine-scale analysis of the *Pan* clade and by increasing the number of individuals, which is critical

for using the same number of individuals in each population when we stratify by frequency. It is important that we could include the western and central chimpanzee populations, which had the smallest and largest historical population sizes among the chimpanzee populations, respectively (Kuhlwilm et al. 2016). The deleterious load in LoF mutations in our data agrees well with the patterns in other categories of deleterious alleles: Western chimpanzees carry more fixed and homozygous LoF alleles and a relative excess of LoF over neutral singletons. These observations generally support the effect of a low N_e in western chimpanzees and bonobos, while the higher N_e in central chimpanzees leads to a larger number of disruptive mutations in singletons, which are subsequently removed from the population by purifying selection. Based on these lines of evidence, from the genome-wide measures of neutrality to the proportions of deleterious changes across frequencies to mutational load and LoF mutations, and in agreement with the observations in (Cagan et al. 2016), we conclude that the small N_e in the past is a good proxy of a reduced efficacy of purifying selection in the *Pan* clade. We note that gene flow between chimpanzees and bonobos (de Manuel et al. 2016) might have an influence on our analysis. However, given the small extent of introgressed material (< 0.25% per individual), this seems unlikely (Nye et al. 2018). It will be necessary to study much larger cohorts of chimpanzees from the different subspecies to more directly compare our observations to humans, with sample sizes several orders of magnitude larger.

The genetic basis of lineage-specific phenotypes

Across all deleterious categories and all loci, bonobos carry a substantially larger number of lineage-specific SNCs. This reflects that in bonobos, which experienced a long-term small N_e in the past, due to genetic drift more alleles reached high frequencies or fixation. We assume that deleterious and protein-altering SNCs are likely to harbor functional consequences, possibly resulting in phenotypic changes. The concept of deleteriousness of an allele often represents conservation across species, since most new mutations are deleterious and thus removed from the population. Yet, novel functional changes would have arisen from such mutations. We present a catalog of genes with such changes, and suggest from literature that several of these in bonobos have functions in male and female reproduction, immunity or

the nervous system (Table S6).

The presence of putatively functional lineage-specific SNCs is not an immediate evidence for adaptive evolution. However, we note that several genes with non-synonymous lineage-specific SNCs (Table S9, S10) have been reported to show signatures of positive selection in bonobos (Cagan et al. 2016): *PCDH15*, *IQCA1*, *CCDC149* and *SLC36A1* in the Fay and Wu's H test, and *CFH* and *ULK4* in the HKA based test (Table S11). *PCDH15* (with two non-synonymous changes in bonobos, one of them predicted to be deleterious by SIFT and PolyPhen-2) is involved in retinal and cochlear function (Jacobson et al. 2008), and *ULK4* has been associated with schizophrenia and neuronal function (Lang et al. 2014). Among the genes with non-synonymous lineage-specific SNCs in chimpanzees, *MUC13* and *ADGB* were described to show signatures of positive selection (ELS test; Cagan et al. 2016).

One of the most prominent differences between chimpanzees and bonobos is found in female reproduction, with female bonobos having prolonged maximal sexual swellings, which female chimpanzees do not have, and female bonobos experiencing an earlier onset of their reproductive age. We hypothesized that SNCs on the bonobo lineage influencing the female reproductive system might underlie the phenotypic differences of the two species. Genome-wide signatures of strong positive selection (Cagan et al. 2016) found no enrichment in female reproduction-related genes, which could be due to the limited power of these methods to detect selection early after the divergence of the two species. However, we demonstrate that the trait "age at menarche" is among only five traits significantly enriched for bonobo-specific non-synonymous changes. Furthermore, the most complete dataset of genes associated to this trait shows an even stronger enrichment in bonobos, with 73 protein-coding SNCs in 55 genes (Table S15). Most of these genes (98%) are expressed in primary tissues of chimpanzees and bonobos (Table S15), suggesting that they are functional in the *Pan* clade. Among the 307 menarche-related genes, five (*ATE1*, *DLGAP1*, *CSMD1*, *LRP1B*, *TRPC6*) have been reported to show signatures of positive selection (Cagan et al. 2016), and one (*HLA-DQB1*) of balancing selection in bonobos. Among these, the Low Density Lipoprotein Receptor *LRP1B* carries three protein-coding changes, and *CSMD1* carries one (Table S15).

To our knowledge, this is the first time that this complex trait in female bonobos has been investigated

using genetic data, with the limitation that only ten bonobo genomes are available. Bonobos are an understudied species in population genetics, hence fine-scaled studies of their population structure and sequencing of more individuals would improve power in future studies. Despite age at menarche being the associated trait in humans, these genes encompass broader functions in the female reproductive system, rather than controlling only age at menarche. Hence, we interpret this as an enrichment of functional changes in female reproduction-related genes during the evolutionary history of bonobos on a polygenic scale, with *LRP1B* and *CSMD1* being good candidates to have the strongest influence. Our results are in agreement with suggestions that the prominent physiological differences between chimpanzee and bonobo female sexual swellings could be due to derived features in bonobos (Wrangham 1993), and suggest that it might have been adaptive on the bonobo lineage. These bonobo-specific non-synonymous changes in menarche-related genes deserve further investigation on the functional level, which would serve as the foundation for a better understanding of the female reproductive system. The sexual swelling in female bonobos has a profound influence on their biology and group dynamics (Hohmann & Fruth 2000; Furuichi 2011), which can be considered typical for bonobo-specific behavior and sociality. Since other relevant traits show an enrichment of changes on the bonobo lineage as well (*e.g.* in behavior- and cognition-related genes), these deserve further investigation in future studies.

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Author Contributions

TMB, AMA and MK conceived the project and wrote the paper. SH and MK analyzed data and wrote the paper.

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Figure 1. Patterns of functional and neutral alleles. A) Neutrality index in the five populations, with 95% confidence intervals across 22 chromosomes. B) Direction of selection-test in the five populations, with 95% confidence intervals across 22 chromosomes. C) Genome-wide ratio of functional to neutral polymorphism (P_{func}/P_{neut}) in different functional categories, for western chimpanzees, bonobos and the average of three non-western chimpanzee populations; with 95% confidence intervals across 22 chromosomes.

Figure 2. Ratio of Site-Frequency-Spectra (SFS) of deleterious to neutral derived alleles. Relative proportion of deleterious derived allele frequencies in each population, defined by GERP (Davydov et al., 2010) and POLYPHEN-2 (Adzhubei et al. 2010), respectively, compared to derived allele frequencies in neutral genomic regions, defined by (Gronau et al. 2011). For comparison, 8 individuals from each population were selected.

Figure 3. Mutational load and loss-of-function (LoF) variants. A) Individual mutational load in each population, considering only heterozygous sites (left), and only homozygous sites (right). These are the counts of deleterious derived alleles in each individual in each population, as defined by SIFT. Orange lines indicate the trend of N_e. Boxes and whisker diagrams represent lower quartile, median, and upper quartile. B) Individual numbers of LoF variants, considering only heterozygous sites (left), and only

homozygous sites (right). C) Ratio of LoF variants to neutral variants in each population.

Figure 4. Enrichment of non-synonymous SNCs in genes associated with age at menarche in humans. The distributions of the overlap of genes with non-synonymous SNCs with 1,000 random sets of 307 genes of similar coding length (±10%) as the menarche-associated genes are shown, with the observed values marked as red squares. From the left, the number of genes and SNCs with chimpanzee-specific changes, the number genes and SNCs with bonobo-specific changes. Lines represent median values, upper and lower deciles.

Table 1. Summary statistics of the total number of lineage-specific changes in each species.

Total number of derived changes	≥90%		100%	
	Bonobos	Chimpanzees	Bonobos	Chimpanzees
	1,267,164	618,150	1,193,455	520,330
Annotation category	Bonobos	Chimpanzees	Bonobos	Chimpanzees
3 prime UTR variant	11,983	5,923	11,352	5,012
5 prime UTR variant	1,861	912	1,758	794
Intergenic variant	488,378	240,355	458,804	202,127
Mature miRNA variant	10	0	7	0
Missense variant	2,714	1,329	2,557	1,175
Regulatory region variant	170,851	82,425	161,009	69,786
Start lost	9	7	9	5
Stop gained	30	10	27	10
Stop lost	14	5	14	4
Synonymous variant	3,420	1,719	3,239	1,450
TF binding site variant	1,213	583	1,140	481

Deleterious derived changes	Grantham	C-score	Gwava	SIFT	PolyPhen-2	GERP
Bonobos	162	107,064	2,316	214	70	15,486
Chimpanzees	74	21,517	1,056	102	49	2,365

NOTE. Number of alleles derived in one species that are fixed ancestral in the other species. ≥90% means higher than 90% allele frequency and 100%

means fixed in the population. The first three rows summarize the total numbers of annotated derived alleles in each category in each population. The bottom three rows summarize the numbers of deleterious derived alleles annotated with each method, which are in high frequency in each population.