Mutations in *Pan* species are shaped by demographic history and harbor lineage-specific functions

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Abstract

Chimpanzees (*Pan troglodytes*) and bonobos (*Pan paniscus*), the *Pan* clade, are the closest living relatives of humans. They have experienced a rapid decline in population size during the last century, rendering them endangered. However, 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 features. Here, we test to which extent the demographic history influences the efficacy of purifying selection in these species, by making use of the most complete dataset of genomic variation in chimpanzees and bonobos to date. We find that small historical effective population sizes (N_e) correlate not only with small genetic diversity, but also with more homozygous deleterious alleles per individual and proportionately more deleterious changes at high frequencies compared to neutral ones. Furthermore, we investigate the putative genetic basis for the distinct phenotypic differences between chimpanzee and bonobo females by analyzing genes involved in female reproduction. We show that bonobo-specific non-synonymous changes are enriched in genes related to age at menarche in humans. This suggests that the prominent physiological differences in the female reproductive system between chimpanzees and bonobos might be explained, in part, by an enrichment of putatively adaptive changes in bonobo.

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) and4 share the most recent common ancestor with humans 5 to 13 Mya (Langergraber and Prüfer 2012; Prado-Martinez et al. 2013; De Manuel et al. 2016). The population history of bonobos is marked by multiple population bottlenecks in the past and a smaller long-term effective population size (N_e). 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 would 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, with central chimpanzee N_e being four times larger, and western chimpanzee N_e 1.4 times larger than the N_e of bonobos, which are the largest and smallest population sizes among chimpanzees, respectively (Kuhlwilm et al. 2016).

The two sister species have very different phenotypic traits and social dynamics. Chimpanzees show a clear hierarchy and frequent violence among males, while it has been suggested that bonobos show more egalitarian interactions (Nishida 1983; Goodall 1986; Furuichi 1987; Idani 1990), and female-female sexual interactions have been observed in bonobos but not chimpanzees (Kuroda 1980). Both bonobos and chimpanzee females display maximal or exaggerated sexual swellings in the perineal part around their ovulation period, which is a distinct feature of the *Pan* clade among the great apes (Nunn 1999). However, only bonobos 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). This has been suggested to be one reason why bonobos maintain peaceful social dynamics, by lowering the operational sex ratio (the temporal ratio of adult males to estrous females in the group, Randall Parish 1994; Furuichi 2011) and by strengthening female-female sexual interactions (as females are more attracted to other females that show sexual swellings, which builds female affiliative relationships and helps them have a strong social stance in their male-philopatric society, Ryu et al. 2015). Considering that the high social status of bonobo females is suggested to be a driving

force of selection on non-aggressive males (Hare et al. 2012), the prolonged sexual swelling can be understood as a key aspect of the bonobo-specific nature. However, these interesting traits in female reproduction, which have been suggested to be derived in the bonobo lineage (Wrangham 1993), have not yet been associated to genetic changes. On the physiological level, using 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 sexual development (Behringer et al. 2014). We suggest that these physiological differences have a genetic ground, and identifying the genetic changes responsible for these traits would help us better comprehend the evolution of *Pan* species. Derived alleles in a lineage at high frequencies are the obvious candidate changes for such lineage-specific traits. Particularly deleterious changes, based on the level of conservation across different taxa and known consequences, might be relevant since they have an increased potential for functional impact.

On the other hand, 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 (number of heterozygous genotypes per individual; Lohmueller et al. 2008; Kidd et al. 2012; Torkamani et al. 2012; Hodgkinson et al. 2013) and the rate of random fixation of derived alleles is higher (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. However, for estimating 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 Polymorphisms (SNPs) in a single genome are deleterious (Subramanian and 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 and Kimura 1984). Such 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 these slightly deleterious SNPs at high frequencies in a population with relaxed purifying selection

pressure than in one with more efficient purifying selection. 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). Previous studies have 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 from the genomes of various great ape species, including eastern and Nigerian-Cameroon chimpanzees, 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 known subspecies, particularly the central and western chimpanzee populations. Furthermore, including bonobos into this picture would improve the inferences.

In this study, we examine 59 chimpanzee and 10 bonobo genomes (De Manuel et al. 2016), first, for analyzing patterns of an accumulation of putatively deleterious derived alleles, with the goal to test how their different demographic histories have shaped the distribution of such alleles. We stratified the alleles into heterozygous and homozygous genotypes per individual, and across functional categories, also in LoF mutations, which are the most disruptive type of deleterious changes. Second, we analyze changes on the bonobo lineage and the chimpanzee lineage. We prioritize these by using non-synonymous changes, and assess the enrichment in genes associated to different phenotypes in humans. 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.

Results and discussion

Neutrality index in populations

We focused on 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 and 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. A value lower than one might indicate an excess of fixed non-synonymous alleles and imply positive selection. We find that the NI 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 S2). This could be explained by a reduced efficacy of purifying selection in this population as the result of a low N_e (Eyre-Walker 2006). In bonobos, the NI is higher than in non-western chimpanzees (1.36). The excess of potentially deleterious polymorphism in western chimpanzees is not exclusive for protein-coding changes, but exists also in different categories of non-coding sites in functional elements, such as 5'UTRs, and upstream and downstream regions of genes, compared to neutral changes across the genome (Fig. 1B, Table S2). This analysis increases power by including a larger number of sites in the genomes, and suggests that, also on non-coding loci, the efficacy of purifying selection is low in the populations with a small N_e in the past.

Distributions of deleterious changes

We analyze the population-wise site frequency spectrum (SFS) of deleterious derived alleles (Fig. 2C), using eight individuals from each population. Deleterious alleles in Fig. 2C were defined by C-score (Kircher et al. 2014), but patterns are generally similar when using other methods (Figs. S1-S5). The central chimpanzee population, which had the largest N_e in the past, shows a very high proportion of singletons (48%) and a lower proportion of deleterious derived changes in non-singleton sites than the other chimpanzee populations (56%-64%). Conversely, the western chimpanzee population, which had a very small N_e, carries the smallest proportion of singletons (37%), but these singletons have the highest ratio of deleterious to neutral sites (1.25) than any other population (1.06-1.16). Together, these results agree with better efficacy to remove the deleterious alleles in larger populations, as observed previously across great apes (Cagan et al. 2016).

The pattern in bonobos is similar to western chimpanzees: The proportion of deleterious alleles at high frequencies is large compared to the chimpanzee populations. When stratifying alleles into singletons vs. non-singletons, the highest deleterious-to-neutral ratio in non-singletons is observed

in bonobos (0.96) when compared to the chimpanzee populations (0.90-0.93). This suggests that bonobos, which experienced a long-term small N_e and multiple bottlenecks since the split from chimpanzees (Prado-Martinez et al. 2013), have accumulated proportionately more deleterious alleles at high frequencies than any chimpanzee population. All these observations agree well with previous reports for modern humans including archaic populations, dogs and rice (Castellano et al. 2014; Kirk E Lohmueller 2014; Marsden et al. 2016; Liu et al. 2017), which demonstrated that a smaller N_e correlates with a higher proportion of deleterious alleles at high frequencies, reflecting the relaxation of purifying selection.

Another way to assess the efficacy of purifying selection is to look at the effect of population size on the individual mutational load. Although there is an ongoing discussion on this matter (Charlesworth 2013; Kirk E. Lohmueller 2014; Simons et al. 2014; Kim and Lohmueller 2015), population size and genetic drift seem to influence the distribution of changes in homozygous positions. We estimated the mutational load, defined as the number of loci with putatively deleterious derived alleles per individual. We stratified them into two different classes, by counting either only heterozygous loci or only homozygous loci. When only heterozygous loci 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 loci are considered, the mutational load of western chimpanzees sharply increases (Fig. 3B), and becomes significantly higher than that of central chimpanzees ($P < 0.001$; Wilcoxon rank test). Slightly deleterious alleles could more often reach high frequencies and be observed in homozygosity in populations with small N_e, since purifying selection would be less efficient. Regarding the other chimpanzee populations, the mutational load shows a gradient that correlates negatively with their long-term N_e (De Manuel et al. 2016) in homozygous sites ($r=-0.85$ with $p=0.07$, Spearman correlation test), and positively in heterozygous sites (although not formally significant: $r=0.75$ with $p=0.15$). This pattern is broadly similar throughout other classes of sites, including synonymous changes, in agreement with previous observations (Lohmueller et al. 2008) (Figs. S6-S11). We note that the pattern of homozygous mutational load appear less clear in C-score and GERP ($r=-263$ with $p=0.738$ and $r=0.136$ with $p=0.864$ respectively, Spearman correlation test), which might be the result of biases due to the reference chimpanzee genome being a western chimpanzee. However, the distributions of the heterozygous and homozygous mutational loads of central chimpanzees are different from the

other three chimpanzee populations in GERP score (P<0.0001 and p=0.009 respectively) and C-score (p<0.0001). We conclude that the population history generally affects the way derived changes are distributed across homo- and heterozygous sites in the genome.

Protein-truncating variants

We furthermore assessed patterns in protein-truncating variants in each population. These loss of function (LoF) changes may be considered as most likely disruptive for protein function and hence a conservative measure for evaluating the efficacy of purifying selection. 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. In this study, 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. We also could include the western and central chimpanzee populations, which had the smallest and largest historical population sizes among the chimpanzee populations, respectively.

In our data, the patterns of mutational load in LoF mutations, defined as the number of LoF derived alleles per individual, follow the patterns in 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 individuals carry between 61 and 118 heterozygous LoF alleles, and between 547 and 560 homozygotes. 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 cannot directly compare the number of homozygous sites to those in modern humans because in (Lek et al. 2016) alleles different from the reference genome were considered. This measures only polymorphisms within the human lineage, but excludes fixed alleles. 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 2.7-fold as many fixed LoF mutations in bonobos than in central chimpanzees. However, LoF singletons are three times more often observed in

central chimpanzees than western chimpanzees, while the other chimpanzee populations are similar to the western chimpanzees. Only 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). A reduced efficacy of purifying selection in western chimpanzees due to their lower population size might cause such a relative excess. This effect is particularly pronounced in singletons (Fig. 3C), suggesting that LoF mutations are more often tolerated in western chimpanzees.

These observations support the effect of a low N_e in western chimpanzees and bonobos, leading to increased fixation of disruptive variants and a relative excess of disruptive variants over neutral variants, 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. We conclude that in *Pan* species the effective population size does indeed have an effect on purifying selection in LoF mutations, in agreement with the observations above and in (Cagan et al. 2016).

An overview of lineage-specific changes

We assessed lineage-specific single nucleotide changes (SNCs), which we define here as alleles which are fixed or observed at very high frequency (>=90%) in one lineage, while fixed ancestral in the other lineage. In doing so, we avoid omitting loci because of sequencing errors or gene flow between populations when defining almost-fixed changes on either the bonobo or the chimpanzee lineage. 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 chimpanzees, while this is three-fold more when using 10 bonobos and 59 chimpanzees (Table S3). These observations clearly reflect that bonobos, which experienced a long-term small N_e in the past, have a much larger number of almost fixed SNCs compared to chimpanzees, as an effect of drift.

These SNCs (additional supplementary material) are candidates for functional changes explaining lineage-specific traits. We assume that, among those SNCs, deleterious ones are more 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 thus suggest that genes with lineage-specific deleterious SNCs are good candidates to underlie lineage-specific traits, and deserve further investigation. We assessed the deleteriousness of the lineage-specific SNCs as a proxy for functional and phenotypic consequences, using six different deleteriousness measures: Grantham score, C-score, GWAVA, SIFT, PolyPhen-2 and GERP score (Methods). We present a catalog of genes with the lineage-specific deleterious SNCs for protein-coding changes, and 50 most deleterious SNCs in genome-wide inferences (Table S4, S5). 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* (Nystad et al. 2014) and *MAP4* (Daisuke et al. 2009), while six are involved in male reproduction: *ACSBG2* (Fraisl et al. 2006), *GALNTL5* (Takasaki et al. 2014), *NME8* (Sadek et al. 2001), *WBP2NL* (Wu et al. 2007), *WDR63* (Hozumi et al. 2008) and *ZFHX3* (Hering et al. 2014). In chimpanzees, we identified only one gene related to female reproduction (*TOP2A*, Tubbs et al. 2009; Konecny et al. 2010) and one gene involved in male reproduction (*FANCL*, Wong 2003). Except for *LVRN* and *FANCL*, these genes are expressed in both *Pan* lineages. We also found that, in both species, multiples genes carry lineage-specific deleterious SNCs related to immunity, optic, heart and nervous system (Table 2).

Protein-truncating variants might have even stronger phenotypic effects. We find nine such SNCs in bonobos, one stop loss (*ACSM4*) and stop gains in *C12orf75*, the olfactory receptor *OR4N2*, *CABLES1*, *SIGLEC7*, *SEMA5B*, *B3GALNT1*, the breast cancer antigen *CEP85L* (Scanlan et al. 2001) and *TAAR2* (Table S6). On the other hand, chimpanzees carry only two LoF changes (*ATP5L2* and *HEPACAM2*). To explore the putative effect of lineage-specific SNCs on phenotypes, we explored the NHGRI-EBI GWAS Catalog (MacArthur et al. 2017), containing data for 2,385 traits. 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".

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 S7, S8, with the gene expression level in tissues of chimpanzees and bonobos (Brawand et al. 2011)).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 S9). *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, the following genes were described to show signatures of positive selection (Cagan et al. 2016): *MUC13* and *ADGB* for all chimpanzees (ELS test), as well as *TPGS2* (HKA test) and *TGS1* (Fay and Wu's H test) in western chimpanzees. These non-synonymous changes might thus have mediated adaptation in these respective lineages.

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 S10). On the other hand, in the chimpanzee lineage, we find 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 S10).

Genes associated to female reproduction

One of the most prominent differences between chimpanzees and bonobos is found in the female reproductive system, where female bonobos have prolonged maximal sexual swellings, which female chimpanzees do not have. We hypothesize that SNCs on the bonobo lineage influencing the female reproductive system might underlie the phenotypic differences of the two species. Previously inferred genome-wide signatures of strong positive selection (Cagan et al. 2016) found no enrichment in female reproduction genes. However, this could be due to the limited power of these methods to detect positive selection early after the divergence of the two species up to 2

Mya. Our Gene Ontology enrichment test did not show an enrichment, which could be due to the absence of proper Gene Ontology categories, or due to polygenic changes in reproductive traits in the absence of strong signatures of monogenic adaptation.

In order to approach a deeper exploration of genes associated with polygenic traits, we performed a systematic enrichment screen (Methods) of genome-wide association studies (MacArthur et al. 2017), using a more permissive set of derived non-synonymous SNCs at high frequency (frequency > 90%) in one population, which were almost fixed ancestral in the other population (frequency < 10%), allowing for gene flow between populations. The analysis is based on the nearest genes to the associated loci in humans, since most associated human SNPs 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 S11), among which "Menarche (age at onset)" suggests that there is indeed an enrichment of non-synonymous changes in genes affecting this female reproduction-related trait. Furthermore, we find an enrichment of such SNCs in genes associated to "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 female reproduction. 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 (Day et al. 2017). Here, we observe a significant proportion of menarche-associated genes with such bonobo-specific non-synonymous SNCs (P = 0.0025, G-Test, Table S12). This observation is more pronounced when considering the number of 73 SNCs (Table 2) falling 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). We do not observe an enrichment of such changes on the chimpanzee lineage (P = 0.48, G-Test). We confirmed that most of these genes (98%) are expressed in the available RNAseq data of chimpanzees and bonobos (Table S13), suggesting that they are functional in the *Pan* clade. We conclude that menarche-related genes seem to have

acquired more non-synonymous SNCs than expected on the bonobo lineage. 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 S13). We additionally find one nonsense SNC in *RPRD2* and one start codon SNC in *NKAIN2*.

Despite age at menarche being the investigated trait in humans, and differences in the age at the onset of menarche between chimpanzees and bonobos (Behringer et al. 2014), 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 the strongest candidates. Our results are in agreement with suggestions that the prominent physiological differences between chimpanzee and bonobo female sexual swellings are derived features in bonobos (Wrangham 1993), and 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 and Fruth 2000; Furuichi 2011), which can be understood as typical for bonobo-specific behavior and sociality. Since related traits show changes on the bonobo lineage as well (*e.g.* behavior- and cognition-related genes), these should also be investigated in future studies.

Conclusions

We made use of 10 bonobo and 59 chimpanzee genomes in this study, which is the most comprehensive genomic data to date for *Pan* species, including all four known subspecies of chimpanzees. Based on these, we investigated the efficacy of purifying selection in relation to the demographic history of populations, by using deleterious derived alleles. Our results show that the small effective population size in the past is a good proxy of a reduced efficacy of purifying selection in the *Pan* clade.

We also investigated the possible genetic basis for physiological differences between the two *Pan* species. We demonstrate that non-synonymous changes on the bonobo lineage are significantly enriched among genes associated to age at menarche in humans. We interpret this as evidence that bonobos have acquired functional changes in female reproductive system-related genes, which partially explain their phenotypic differences to chimpanzees. In our knowledge, this is the first time that this complex trait in female bonobos has been approached using genetic data. Bonobos are an understudied species in population genetics, hence fine-scaled studies of their population structure and sequencing of more individuals is desirable. Together with more knowledge about the genetic underpinning of the female reproductive system, this will lead to better understand their distinct features.

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 at high coverage, including four chimpanzee populations: 18 central, 20 eastern, 10 Nigerian-Cameroon and 11 western chimpanzees (Table S1). All genomes were mapped to the human genome assembly *hg19* (ENSEMBL GRCh37.75), and previously published genotype calls were used (De Manuel et al. 2016). We applied the ENCODE 20mer uniqueness filter, and required all the heterozygous loci to be allele balanced, where the ratios of the raw reads are between 0.25 and 0.75, in order to exclude potential sites with biases and contamination. Across 69 individuals, the initial VCF file contained 36,299,697 loci in total. After all the 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 the human allele was only seen in one read in one individual, but a derived allele in at least five reads and all the other individuals, selecting individuals with less than 10,000 such positions across the genome, and then selected the individuals with the highest coverage from the remaining samples, leaving eight individuals per population (Table S1). Sites without high-quality genotypes were not considered for these

analyses. Furthermore, where stated, a subset of ten central chimpanzees for a fair comparison with bonobos was used (Table S1). In the analysis of GWAS genes, 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 species 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.

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. "Population-specific derived changes" refers to the derived changes almost fixed (>=90%) in one population, where the other population appears to be fixed at the ancestral state.

Deleteriousness assessment

We assessed the number of recessive derived alleles, which we defined as deleterious non-synonymous changes. For the assessment of deleteriousness, we used the following methods: Grantham scores, C-score, GWAVA, SIFT, PolyPhen-2 and GERP scores. Using these different measures allows us to ensure our results are robust to the method used. The Grantham Score (Grantham 1974; Li et al. 1984) represents only the physical/chemical properties of amino acid 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. GWAVA (Ritchie et al. 2014) predicts the functional impact of genetic variants, based on genome-wide properties. SIFT (Kumar et al. 2009) predicts the deleteriousness of amino acid changes, based on the degree of conservation inferred from sequence alignments. 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. Finally, GERP (the 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. 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/).

Data analysis

Gene annotation was done using ENSEMBL VEP v83 (McLaren et al. 2016). Neutral loci were defined as described in (Gronau et al. 2011). 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. We used the software *FUNC* (Prüfer et al. 2007) to determine enrichment of Gene Ontology terms. We ranked the genes with 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 with lineage-specific deleterious SNCs in each lineage, we used the Wilcoxon rank test. 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).

We retrieved genome-wide association data from the NHGRI-EBI GWAS Catalog (MacArthur et al. 2017). For the enrichment test, we overlapped the associated genes with protein-coding SNCs on the chimpanzee and bonobo lineages, respectively. We used the permissive set of non-synonymous SNCs described above. For all genes for each trait in each study ("Disease trait"), we counted the number of non-synonymous SNCs on each lineage, and performed a significance test (G-test) against the total number of non-synonymous SNCs, with a P-value cutoff at 0.1. We also performed a G-test between the number of all non-synonymous SNCs on each lineage compared to the number of non-synonymous SNCs on each lineage falling in genes associated to the trait (P-value cutoff at 0.1), to determine whether or not the difference between the two species is significant. 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%), and counting the lineage-specific non-synonymous SNCs. 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. Finally, at least 10 loci need to be associated with a given trait in order to restrict the analysis to multigenic traits, and we report only significant traits with at least three genes carrying 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).

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Author Contribution

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

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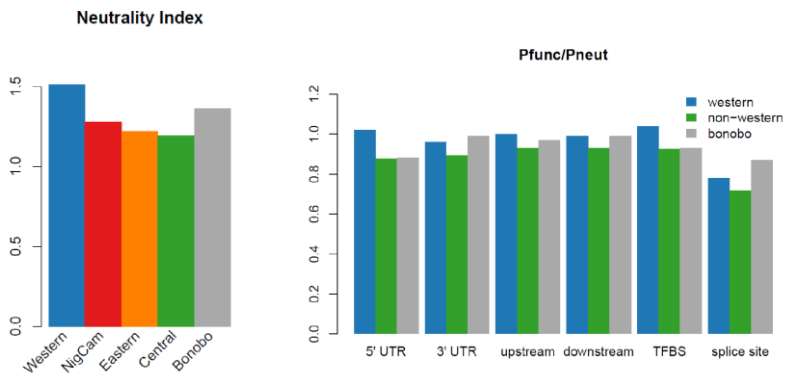

Figure 1. Patterns of functional and neutral alleles. A) Neutrality index in the five populations. B) Genome-wide ratio of functional to neutral polymorphism (P_{func}/P_{neut}) in different functional categories, for western chimpanzees, bonobos and the average of three non-western chimpanzee populations.

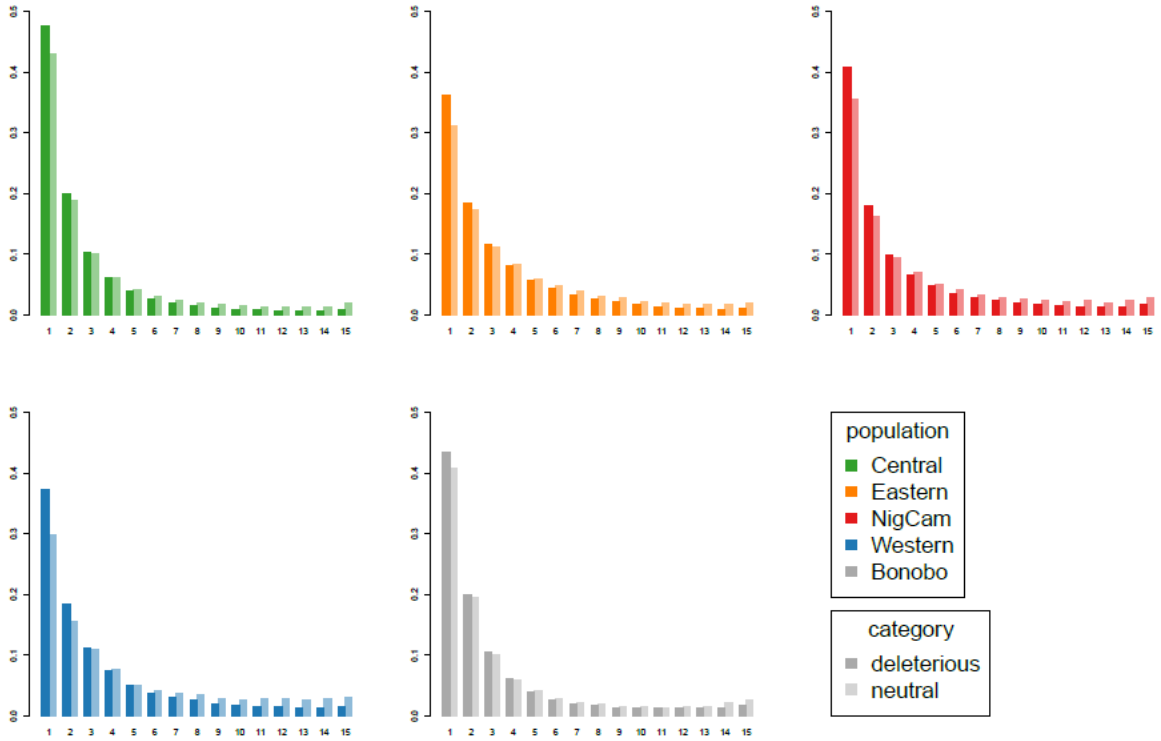

Figure 2. Site-Frequency-Spectra (SFS). SFS for deleterious derived alleles in each population, as defined by C-score (Kircher et al. 2014), in contrast to the SFS of derived alleles in neutral genomic regions, as defined by (Gronau et al. 2011). For a fair comparison, 8 individuals from each population were chosen for the analysis.

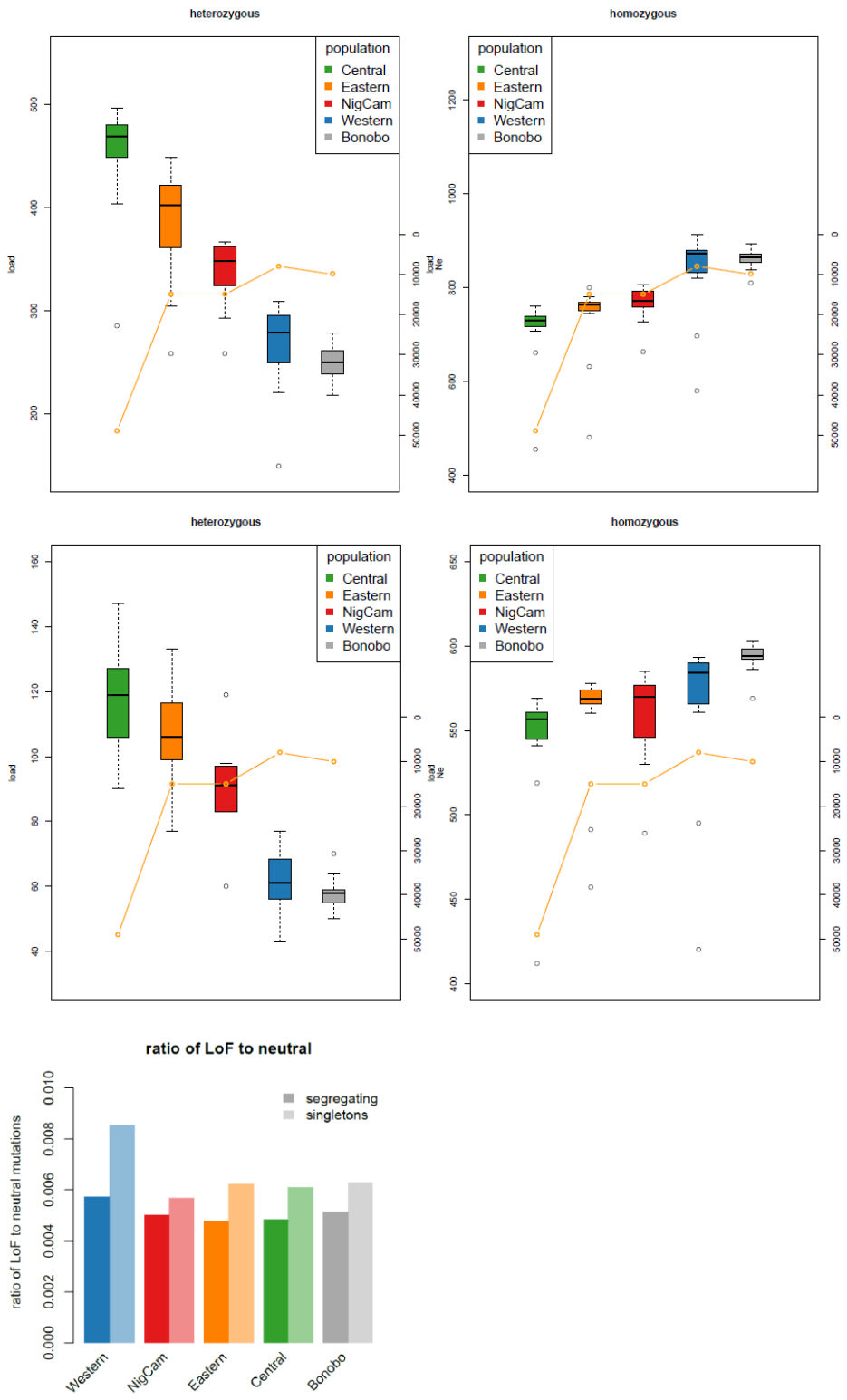

Figure 3. Mutational load and loss-of-function (LoF) variants. A) Individual mutational load in each population, considering only heterozygous sites (left). Mutational load considering only

homozygous sites (right). These are the counts of deleterious derived alleles in each individual in each population, as defined by SIFT. B) Individual number of LoF variants, considering only heterozygous sites (left). Individual number of LoF variants, considering only homozygous sites (right). C) Ratio of LoF variants to neutral variants in each population.

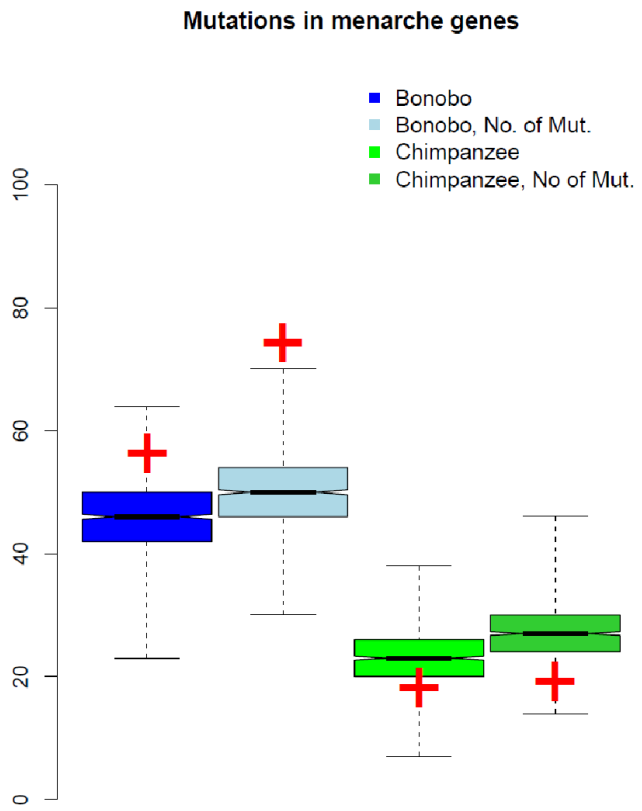

Figure 4. Enrichment of non-synonymous SNCs in genes associated with age at menarche in humans. The distributions of 1,000 random sets of 307 genes of similar coding length (±10%) as the menarche-associated genes are shown, with the observed values marked as red crosses. From the left, the number of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

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.

Table 2. Bonobo-specific non-synonymous changes in menarche-related genes.

Position	Gene	Mutation	Deleteriousness
1:14105326	PRDM2	T/A	SIFT=NA; PolyPhen=benign
1:154574654	ADAR	A/V	SIFT=deleterious; PolyPhen=benign
1:177930850	SEC16B	S/F	SIFT=deleterious; PolyPhen=probably_damaging
1:66067125	LEPR	S/A	SIFT=deleterious; PolyPhen=probably_damaging
1:98348908	DPYD	R/L	SIFT=deleterious; PolyPhen=benign
2:137639763	THSD7B	S/R	SIFT=tolerated; PolyPhen=benign
2:141027837	LRP1B	D/E	SIFT=deleterious; PolyPhen=probably_damaging
2:141113964	LRP1B	F/S	SIFT=tolerated; PolyPhen=possibly_damaging
2:141130669	LRP1B	R/Q	SIFT=NA; PolyPhen=benign
2:141215086	LRP1B	S/T	SIFT=NA; PolyPhen=benign
2:206911299	INO80D	E/D	SIFT=tolerated; PolyPhen=benign
2:206921468	INO80D	L/V	SIFT=tolerated; PolyPhen=benign
2:32631593	BIRC6	S/T	SIFT=NA; PolyPhen=probably_damaging
2:44942458	CAMKMT	A/T	SIFT=tolerated; PolyPhen=benign
2:48027770	MSH6	K/R	SIFT=tolerated; PolyPhen=benign
3:184049147	EIF4G1	E/K	SIFT=tolerated; PolyPhen=benign
3:44841903	KIF15	T/I	SIFT=tolerated; PolyPhen=benign
3:88205648	C3orf38	T/A	SIFT=tolerated; PolyPhen=benign
4:104066209	CENPE	K/E	SIFT=NA; PolyPhen=benign
4:104070295	CENPE	I/V	SIFT=NA; PolyPhen=benign
4:104082402	CENPE	L/V	SIFT=deleterious; PolyPhen=probably_damaging
4:104098148	CENPE	N/S	SIFT=tolerated; PolyPhen=benign
4:106156559	TET2	R/K	SIFT=tolerated; PolyPhen=benign
4:106156645	TET2	P/S	SIFT=tolerated; PolyPhen=benign
4:106196843	TET2	T/P	SIFT=tolerated; PolyPhen=benign
4:95129589	SMARCAD1	N/S	SIFT=tolerated; PolyPhen=benign
5:137713436	KDM3B	L/I	SIFT=deleterious; PolyPhen=possibly_damaging
5:137756422	KDM3B	N/S	SIFT=tolerated; PolyPhen=benign
6:101296499	ASCC3	H/L	SIFT=tolerated; PolyPhen=benign
6:128812928	PTPRK	H/R	SIFT=NA; PolyPhen=NA
6:148846475	SASH1	V/I	SIFT=tolerated; PolyPhen=benign
6:170628088	FAM120B	D/G	SIFT=tolerated; PolyPhen=benign
6:84315463	SNAP91	A/V	SIFT=tolerated; PolyPhen=possibly_damaging
7:27867388	TAX1BP1	P/H	SIFT=tolerated; PolyPhen=benign
8:1624699	DLGAP2	V/M	SIFT=deleterious;

			PolyPhen=probably_damaging
8:2820076	CSMD1	Q/H	SIFT=tolerated; PolyPhen=benign
8:35616858	UNC5D	R/S	SIFT=deleterious; PolyPhen=possibly_damaging
8:35648008	UNC5D	T/M	SIFT=tolerated; PolyPhen=possibly_damaging
8:53073977	ST18	I/V	SIFT=tolerated; PolyPhen=benign
8:87423991	WWP1	N/D	SIFT=tolerated; PolyPhen=benign
9:111798556	TMEM245	I/V	SIFT=tolerated; PolyPhen=benign
9:120475206	TLR4	G/E	SIFT=tolerated; PolyPhen=benign
9:6984389	KDM4C	H/Y	SIFT=deleterious; PolyPhen=possibly_damaging
9:7170033	KDM4C	T/I	SIFT=deleterious; PolyPhen=NA
10:102684190	FAM178A	R/C	SIFT=tolerated; PolyPhen=possibly_damaging
10:102684290	FAM178A	C/Y	SIFT=tolerated; PolyPhen=benign
10:13541765	BEND7	N/S	SIFT=tolerated; PolyPhen=benign
11:122829992	C11orf63	P/S	SIFT=tolerated; PolyPhen=benign
11:8474423	STK33	Q/E	SIFT=tolerated; PolyPhen=benign
12:108638308	WSCD2	P/Q	SIFT=tolerated; PolyPhen=benign
12:48073286	RPAP3	P/R	SIFT=tolerated; PolyPhen=benign
13:42763236	DGKH	A/V	SIFT=tolerated; PolyPhen=benign
14:100847730	WDR25	V/I	SIFT=tolerated; PolyPhen=benign
14:100847877	WDR25	R/C	SIFT=tolerated; PolyPhen=benign
14:101349312	RTL1	E/G	SIFT=tolerated; PolyPhen=benign
14:60950485	C14orf39	E/K	SIFT=deleterious; PolyPhen=possibly_damaging
14:94088581	UNC79	P/A	SIFT=tolerated; PolyPhen=benign
14:94107526	UNC79	I/V	SIFT=tolerated; PolyPhen=benign
15:48056211	SEMA6D	I/M	SIFT=tolerated; PolyPhen=benign
16:20380984	PDILT	E/K	SIFT=deleterious; PolyPhen=possibly_damaging
16:72832557	ZFHX3	G/R	SIFT=NA; PolyPhen=possibly_damaging
17:2202857	SMG6	P/L	SIFT=tolerated; PolyPhen=benign
17:2203679	SMG6	R/K	SIFT=tolerated; PolyPhen=benign
17:46309238	SKAP1	V/I	SIFT=tolerated; PolyPhen=NA
17:50212276	CA10	E/D	SIFT=tolerated; PolyPhen=benign
20:37263383	ARHGAP40	G/S	SIFT=deleterious; PolyPhen=probably_damaging
20:37275640	ARHGAP40	K/R	SIFT=tolerated; PolyPhen=benign
20:43532655	YWHAB	I/V	SIFT=deleterious; PolyPhen=benign
20:43532673	YWHAB	P/A	SIFT=tolerated; PolyPhen=benign
20:62859360	MYT1	A/V	SIFT=tolerated; PolyPhen=NA
22:50014212	C22orf34	G/R	SIFT=deleterious; PolyPhen=probably_damaging

X:109310617	TMEM164	V/I	SIFT=tolerated; PolyPhen=benign
X:8993603	FAM9B	L/F	SIFT=tolerated; PolyPhen=benign