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Molecular and Population Analysis of Natural Selection on the Human Haptoglobin Duplication

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SUMMARY

Haptoglobin binds free haemoglobin that prevents oxidative damage produced by haemolysis. There is a copy number variant (CNV) in the haptoglobin gene (*HP*) consisting of two alleles, *Hp1* (no duplication), and *Hp2* (1.7kb duplication involving two exons). The spread of the *Hp2* allele is believed to have taken place under selective pressures conferred by malaria resistance. However, molecular evidence is lacking and *Hp* did not emerge in genomewide SNPs surveys for evidence of selection. In Europe, there is geographical constancy of *Hp2* frequency, indicative of absence of clinal pressures and that modern day European alleles represent a "snapshot" of their out-of-Africa migrations. In this work we test for signatures of natural selection acting on the *Hp* CNV in a sample from the UK population (Avon Longitudinal Study of Parents and Children, ALSPAC). We present here heterozygosity decay, pairwise F_{ST} values observed between ALSPAC and 301 populations from all five populated continents, extended haplotype homozygosity analyses involving the CNV and 80 SNPs surrounding the CNV ~500kb in each direction, and linkage disequilibrium and pairwise haplotypic analyses involving 160 SNPs on chromosome 16q22.1. Taken together, our results represent the first molecular analysis of natural selection in the *Hp* CNV genetic region.

Keywords: Haptoglobin, natural selection, copy number variant, ALSPAC

Introduction

Haptoglobin is an acute-phase binding protein that scavenges free haemoglobin in the plasma: this prevents oxidative damage following intravascular haemolysis (Garby & Noyes, 1959) and also directs haem and iron to specific recycling pathways via the CD163 receptor. In humans, the haptoglobin gene (*HP*) is located on chromosome 16q22.1. *HP* is a copy number variant (CNV) gene with two main alleles: *Hp*1 and *Hp*2. Whilst the *Hp*1 allele features no duplication and 4 exons, the *Hp*2 allele contains a 1.7kb duplication, originated by a non-homologous recombination event (Maeda et al., 1984). This event gives a total of 6 exons, the fifth and sixth being identical to exons 3 and 4 of *Hp*1 (Yang et al., 1983). The *HP* CNV is functional, with the genotypes producing different peptides which form dimers/polymers. *Hp*1/1 expression results in a dimer, and products of *Hp*1/2 and *Hp*2/2 result in varying sizes of polymers. These proteins bind to haemoglobin with differing affinities (Okazaki et al., 1997). Additionally, *HP* allele products may differ in functional affinity with the CD163 receptor, for the removal of haptoglobin–haemoglobin complexes through endocytosis (Kristiansen et al., 2001).

The *HP* gene has become polymorphic in human populations. The phenotypic distribution of *HP* in northwestern European populations is: *Hp*1/1 16%, *Hp*1/2 48% and *Hp*2/2 36%, corresponding to allele frequencies of *Hp*1 at 0.4 and *Hp*2 at 0.6 (Langlois and Delanghe, 1996). Furthermore, the proportions of *HP* genotype are highly variable between populations worldwide (Carter & Worwood, 2007).

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There is growing evidence that *HP* genotype has a role in disease aetiology, due to the differential effect of products on the binding and removal of free haemoglobin, which may damage renal tissue and aggravate bacterial infection (Carter & Worwood, 2007). Several studies have suggested a protective role for *Hp*2 with several infectious diseases [(McDermid & Prentice, 2006) for review], but it has also been reported that *Hp*2/2 individuals are at higher risk of developing cardiovascular disease (Levy et al., 2002; Asleh and Levy, 2005; Levy et al., 2007). To date, however, results about the strength and direction of the association of *HP* genotype with cardiovascular disease and other diseases are conflicting (Carter & Worwood, 2007). Whether this association has come under the influence of natural selection is unknown.

Despite the reported associations of the *HP* genotype with several diseases, it is unclear what provided selective advantage for the spread of the *Hp*2 allele. One hypothesis to explain this is the conferral of malarial resistance by the *Hp*2 allele (Atkinson et al., 2007). Haptoglobin has demonstrable toxicity to malarial parasites *in vitro* (Imrie et al., 2004), and results from case-control studies suggest significantly lower prevalence of malaria in *Hp*2/2 and *Hp*1/2 individuals compared with those with *Hp*1/1 (Elagib et al., 1998). Also, evidence from a recent prospective cohort study showed increased protection from clinical malaria in *Hp*2/2 children, relative to the number of *Hp*1/2 and *Hp*1/1 patients, with a 30% reduction in recorded cases between genotypes (Atkinson et al., 2007). The authors suggest that higher anti-malarial protection offered by *Hp*2/2 allowed the genotype to spread in populations, but also that *Hp*1/1 may be sustained due to higher protection against other febrile processes, leading to the patterns of balanced polymorphism witnessed worldwide. However, the relationship between *HP* genotype, malaria resistance and possible selection remains controversial (Aucan et al., 2002; Bienzle et al., 2005). Furthermore, selection pressure provided by exposure to malaria is unlikely to have driven the persistence of the *Hp*2 allele in populations living at higher latitudes (McDermid and Prentice, 2006). In terms of molecular evolution, if the novel polymorphism (in this case, the haptoglobin duplication) offers an advantage for an organism to survive and reproduce, it will be a target of positive selection and not solely a product of genetic drift (Biswas & Akey, 2006).

In this work, we tested for molecular signatures of natural selection in *HP* in Europeans. To this end, we analysed *HP* CNV and SNPs densely distributed around *HP* from a random sample of 400 individuals from the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort (Golding et al., 2001). Ethical approval for all aspects of data collection was obtained from the ALSPAC Law and Ethics Committee (institutional review board 00003312). Written informed consent for the study was obtained.

Materials and Methods

HP CNV Typing

The haptoglobin duplication was typed using amplification ratio control system (ARCS, a liquid phase high-throughput assay for quantifying gene copy number that has been developed and validated in our lab (Guthrie et al., 2011). A short junction sequence which exists between exons 4 and 5 when the duplication is present was amplified using primers positioned on either side of it; thus no amplicons represented no duplication (1,1), one amplicon represented heterozygous duplication (1,2) and 2 amplicons represented homozygous duplication (2,2). A short amplicon in a stable copy number reference gene (*TP53*) was amplified simultaneously using another pair of primers, and after the first two cycles, amplification for both target and reference amplicons was driven by a single universal primer, thus stabilising the amplification kinetics of all amplicons and preserving ratiometry between target and reference. Following PCR, the samples were analysed using a LightTyper^{TM} (Roche Diagnostics GmbH, Roche Applied Science, 68298 Mannheim, Germany), which recorded the melting profiles of the DNA as temperature was increased from 45 °C to 80 °C. The haptoglobin amplicon was designed to have a melting temperature 4–5°C lower than the reference amplicon, resulting in a first derivative plot of the melting profile which showed two well separated peaks. The LightTyper melt file was analysed by Perl software written in-house which calculated peak heights as proxy for areas under the curve, and then generated cluster plots of the target peak height/reference peak height ratios. Junction nulls were handled separately, with absence of a target amplicon peak being output as zero. Samples which failed, or which amplified poorly resulting in unreliable clustering, were discarded; all others were assigned their *HP* CNV genotype.

SNPs

SNP data surrounding the *Hp* locus were obtained for the genotyped samples as part of a genome-wide association study of the ALSPAC cohort, the details of which have been published previously (Timpson et al., 2009). And 161 SNPs were included for analysis within ∼1 Mb in each direction of the *HP* gene at position 70649447 on chromosome 16, according to build 36.

LD Estimation

Haploview (Barrett et al., 2005) was used to estimate linkage disequilibrium (LD) measures between SNPs and the CNV in the sample. Additionally, we utilised the programme MIDAS (Gaunt et al., 2006) for analysis of interallelic association between diplotypes involving the SNPs and the CNV. MIDAS enables the characterization of LD and allelic frequencies observed for all four haplotypes in each pairwise LD.

Heterozygosity

Heterozygosity was estimated for SNPs in a 2Mb interval centred on from the *HP* CNV for HapMap samples from Europeans (CEU), Africans (YRI) Chinese (CHB) and Japanese (JPT) using data available in HapMap (http://hapmap.ncbi.nlm.nih.gov/). Heterozygosity was estimated using the formula

$$H = 1 - \sum_{i=1}^{m} f_i^2$$

where m is the number of alleles and f_i is the allele frequency of the ith allele.

F_{ST}

We estimated pairwise interpopulation diversity between 301 populations across the five continents, with allele frequency available for the *HP* CNV (Carter & Worwood, 2007). To this end, we computed the F_{ST} statistic according to Weir and Cockerham (1984).

Extended Haplotype Homozygosity

In order to test for the occurrence of positive selection acting around the haptoglobin CNV in Europeans, we analysed extended haplotype homozygosity (EHH) as previously described (Sabeti et al., 2002). EHH measures the decay of homozygosity at a core haplotype of interest. Phased haplotypes involving the CNV and 79 SNPs (spanning ~500 kb either side from the CNV) were obtained by the software PHASE v.2.0 (Stephens and Donnelly, 2003). We used the Sweep program for the identification of core haplotypes involving the CNV using the block definition from Gabriel et al. (Gabriel et al., 2002). Relative EHH (rEHH), accounting for different recombination rates across the genome, and significance values for both EHH and rEHH were also measured using the default parameters in Sweep.

Results

Of 400 samples analysed, 365 were successfully genotyped for the *HP* CNV. Clustering of the *HP* CNV raw data can be seen in Figure S1. Genotype and allele frequencies (Table 1) were similar to those in Northern European populations (Carter and Worwood, 2007). Using an online tool for Hardy-Weinberg Equilibrium (HWE) testing (http://www.oege.org/software/hardy-weinberg.html) (Rodriguez et al., 2009), the observed genotype frequencies were found to be consistent with HWE ($\chi^2 = 2.71$, $P = 0.10$).

Table 1 Haptoglobin Genotype and Allele Frequencies in 400 ALSPAC Samples.

	Genotype Frequency
Hp1/1	55
Hp1/2	154
Hp2/2	156
Fails	35

	Number	Allele frequency
Hp1	264	0.362
Hp2	466	0.638

Tagging SNPs

We checked for the existence of tagging SNP(s) for the duplication that could be used for the testing of selection. Three SNPs were in complete LD ($D' = 1$) with the CNV (rs152837, rs152828 and rs217180). However, individually the SNPs showed insufficient tagging power: $r^2 = 0.04$, 0.06 and 0.07, respectively. Insufficient tagging power was also observed for the remaining pairwise comparisons involving the CNV and each SNP (Table S1, Fig. 1). The maximum value of r^2 observed for all pairwise comparisons involving the CNV was 0.16 (mean ± S.E. = 0.026 ± 0.003). Subsequently an Expectation Maximization algorithm implemented in the Haplotype Trend Regression program (Zaykin et al., 2002) was applied to assess whether a combination of nearby markers could be used to tag the *Hp*2 allele. We analysed various combinations of SNP series, using the three SNPs above and also those in incomplete LD with the duplication. Neither the use of specific haplotype blocks nor "sliding" analysis across several markers resulted in increased tagging power for the duplication (data not shown). Difficulty in tagging copy number variants with SNPs has been widely encountered in complex genomic regions (Kehrer-Sawatzki, 2007), and no SNP or haplotype was found to offer effective tagging of the haptoglobin duplication.

Heterozygosity

Figure 2 shows reduced polymorphism around the *HP* CNV. This effect is evident in all four populations.

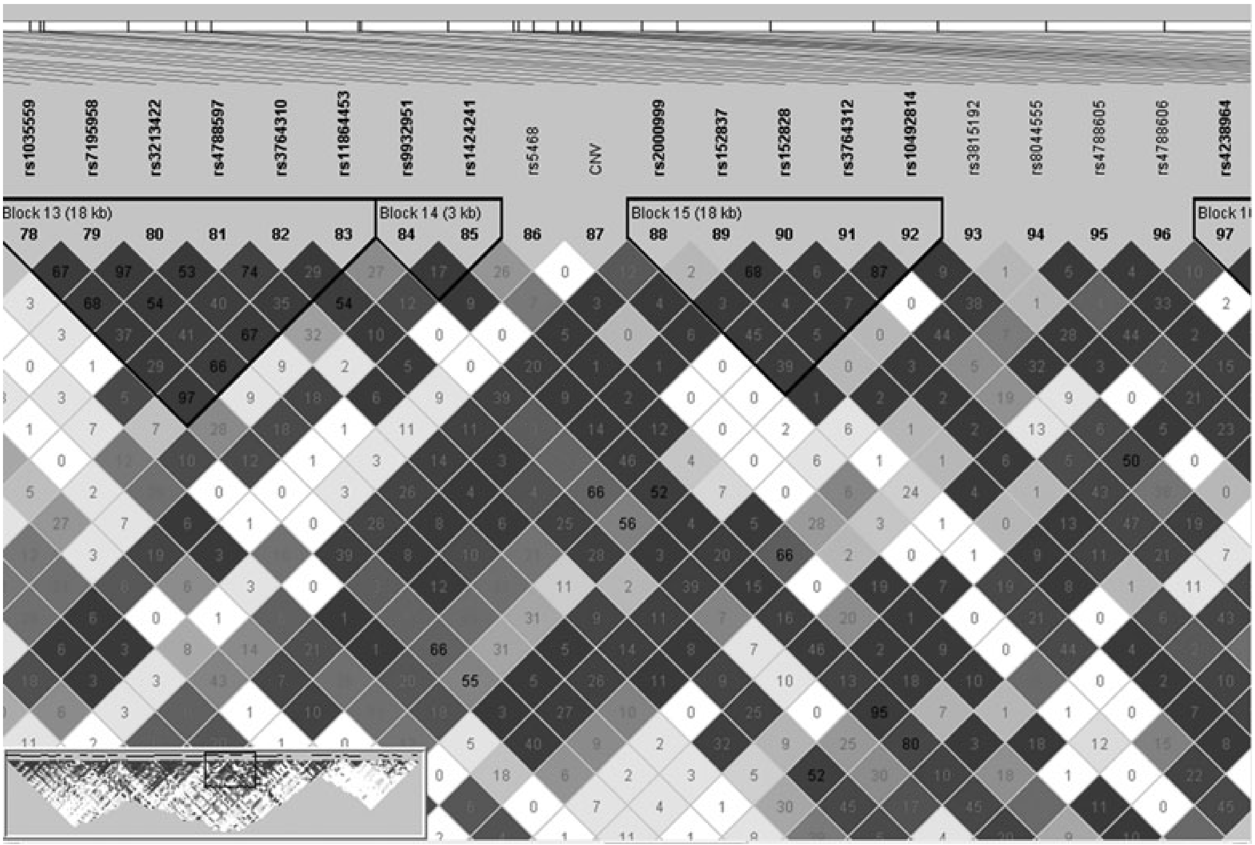


Figure 1 The r^2 values observed between the HP CNV (87) and neighbouring SNPs taken from Haploview.

F_{ST}

Table 2 shows descriptive statistics for F_{ST} values observed between the population analysed in this work (ALSPAC) and all other populations grouped by continent. Overall interpopulation genetic diversity (as measured by the mean F_{ST}), ranges from the smallest value of 0.0032 ± 0.0006 between ALSPAC and Europe populations to the largest value of 0.1443 ± 0.0180 between ALSPAC and Oceania populations. F_{ST} values for Europeans are comparable to those previously reported by Tian et al. (2009).

None of these mean values and none of the maximum F_{ST} values observed between ALSPAC and populations from all five continents was higher than 0.45 (a threshold previously used to define high F_{ST} candidate selection genes (Akey et al., 2002)).

Individual F_{ST} values for each pairwise comparison involving ALSPAC can be seen in Table S2. Table S1 shows a number of instances where $F_{ST} = 0$ (4 for Africa, 2 for America, 6 for Asia, 30 for Europe and 0 for Oceania) and $F_{ST} < 0.005$ (2 for Africa, 4 for America, 12 for Asia, 41 for Europe and 2 for Oceania) for pairwise comparisons involving ALSPAC.

Table 3 shows descriptive statistics of pairwise F_{ST} values observed for each pair of populations within each continent from published *HP* CNV frequencies (Carter and Worwood, 2007). In all continents with the exception of Europe, there are instances of $F_{ST} > 0.45$. Europe is the most homogeneous continent in relation to the *HP* CNV, as shown by the low mean F_{ST} value observed (mean $F_{ST} = 0.004$). This is in agreement with the absence of clinal pressures and is also consistent with the hypothesis that modern day Europeans represent a "snapshot" of their out-of-Africa migration with respect to the *HP* gene region.

Extended Haplotype Homozygosity

One core haplotype involving the *HP* CNV and the SNPs rs2000999 and rs152837 was observed. The allelic composition and frequencies of each haplotype were as follows: *Hp*1-G-A (36%), *Hp*2-G-A (36%), *Hp*2-A-A (21%) and *Hp*2-G-G (7%), markers in the order *HP* CNV, rs2000999 and rs152837. The non-duplication allele (*Hp*1) is known to be older in the population. The G and A alleles in rs2000999 and rs152837

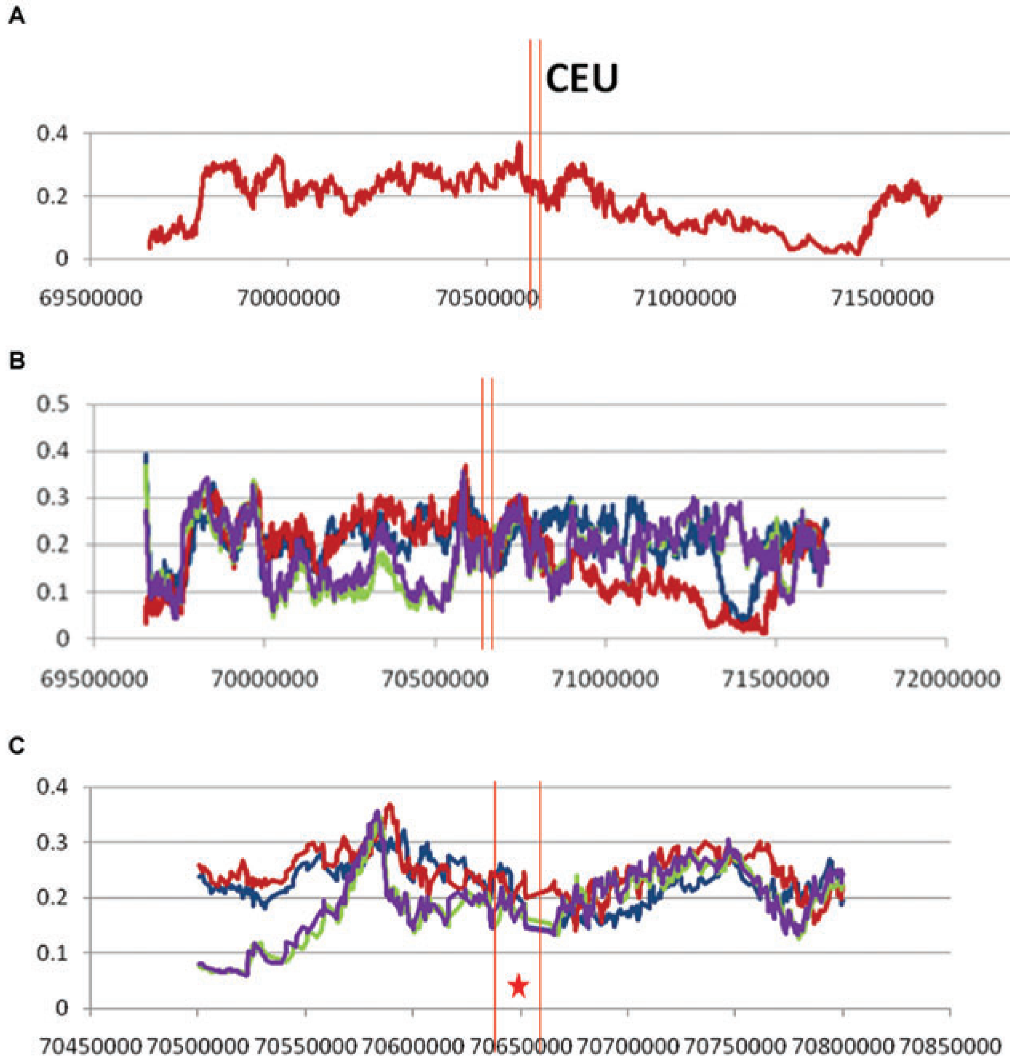


Figure 2 Polymorphism across ~2 Mb centred on the *HP* CNV observed in HapMap samples from Europeans (CEU), Africans (YRI), Chinese (CHB) and Japanese (JPT). A decay in heterozygosity around the *HP* CNV was observed in all population samples. (A) Pattern of heterozygosity variation observed in CEU for a ~2Mb interval centred on the *HP* CNV. (B) Pattern of heterozygosity variation observed in CEU, YRI, CHB and JPT for the same ~2 Mb interval. (C) Heterozygosity decay in a 300Kb region centred on the *HP* CNV. The two vertical lines represent the interval where *HP* is located. The asterisk in Figure 1c represents the location of the *HP* CNV.

are ancestral according to the chimpanzee allele as provided with the Sweep program. The frequency of the four haplotypes is therefore indicative of the age of each allele, with *Hp*1-G-A being the oldest haplotype and *Hp*2-G-G the youngest. Significant EHH ($P = 0.010$) was observed for the core haplotype *Hp*2-G-G, which displayed higher levels of EHH than expected among SNPs located ~500 Kb away from the duplication (Fig. 3). The addition of neighbouring SNPs to the core region did not change the results. The elimination of the *HP* CNV from the core region did not change the results.

LD and Haplotypes of HP CNV and SNPs

We have also characterised patterns of LD between the CNV and SNPs located up to ~1 Mb apart in either direction. Stratification of the pairwise comparisons according to the magnitude of LD ($D' \geq 0.75$ vs. $D' < 0.75$) showed a significantly lower allele frequency for those cases of higher LD (Table 4), in accordance with previous observations showing that rarer alleles show on average more LD than more common alleles (Zapata et al., 2001). In addition we found that, in general,

Table 2 Descriptive Statistics of Pairwise F_{ST} Values Observed between ALSPAC and each of the 301 Populations Across Five Continents with Information available for the *HP* CNV (Carter and Worwood, 2007).

	N	Minimum	Maximum	Mean	Std. Deviation
ALSPAC vs. Africa	41	0	0.3672	0.1046	0.0891
ALSPAC vs. America	42	0	0.3848	0.1032	0.1137
ALSPAC vs. Asia	99	0	0.1837	0.0291	0.0343
ALSPAC vs. Europe	91	0	0.0322	0.0032	0.0055
ALSPAC vs. Oceania	28	0.0025	0.3582	0.1443	0.0952

Table 3 Descriptive statistics of pairwise F_{ST} values observed between populations within each continent for the 301 populations with information available for the *HP* CNV (Carter and Worwood, 2007).

Continent	*N*	Minimum	Maximum	Average	S.D.
Africa	41	0	0.594	0.072	0.090
America	42	0	0.549	0.111	0.120
Asia	99	0	0.558	0.029	0.044
Europe	91	0	0.107	0.004	0.008
Oceania	28	0	0.652	0.124	0.159

the rarer allele in each SNP associates more frequently with *Hp*2 than with *Hp*1. Table 5 shows the 12 diplotypes (each involving the *HP* CNV and one SNP) with the highest levels of LD, as measured by D'. In total, 136 SNPs out of the 160 SNPs analysed showed higher haplotypic frequency for the haplotype "rare allele-*Hp*2" than for the haplotype "rare allele-*Hp*1" (Table S3). We computed the binomial probability of observing 136 or more successes (rare SNP allele associated with *Hp*2 in higher frequency) by using a Binomial Calculator (http://stattrek.com/Tables/Binomial.aspx). The specification of a probability of success on a single trial of 0.6 (allele frequency of *Hp*2), the number of trials (160) and the number of successes (136), rendered a binomial probability of $p = 3 \times 10^{-12}$. Therefore it seems unlikely that this effect is due to chance alone. The higher frequency of the haplotypes "rare allele-*Hp*2" than of haplotypes "rare allele-*Hp*1" is consistent with a non-random effect in the population leading to this pattern. This adds independently to the evidence from our EHH analysis suggesting a possible role of natural selection in the variation of haplotypes involving the *HP* CNV.

Allele Age

Table 6 shows age estimates computed for the *HP* CNV and for the 12 SNPs with high LD with the *HP* CNV. These neutral allele age estimates were based on a constant population size (Slatkin and Rannala, 2000). The *HP* CNV showed the oldest age. And 10 out of the 12 SNPs showed ages greater than 100,000 years, while two of them showed ages of 58,760 and 81,046 years, respectively. Considering that the out-of-Africa migration took place at around 50,000–100,000 years ago (Tishkoff and Verrelli, 2003), our results support the hypothesis that the SNPs associated with the *HP* CNV originated before the out-of-Africa migration. This assumes neutrality. However, allele ages for neutral alleles provide conservative upper bound for alleles which are under selection (Slatkin and Rannala, 2000). Taken together, these SNP data suggest the rapid emergence of the *Hp2* allele under 100,000 years ago and possibly more recently. This picture would be consistent with observations for other genotypes positively selected by malaria, and it contrasts with the picture of EHH and other features observed for lactase persistence, a "survival" genotype which has emerged in North Europe within the past 5,000–10,000 years (Bersaglieri et al., 2004).

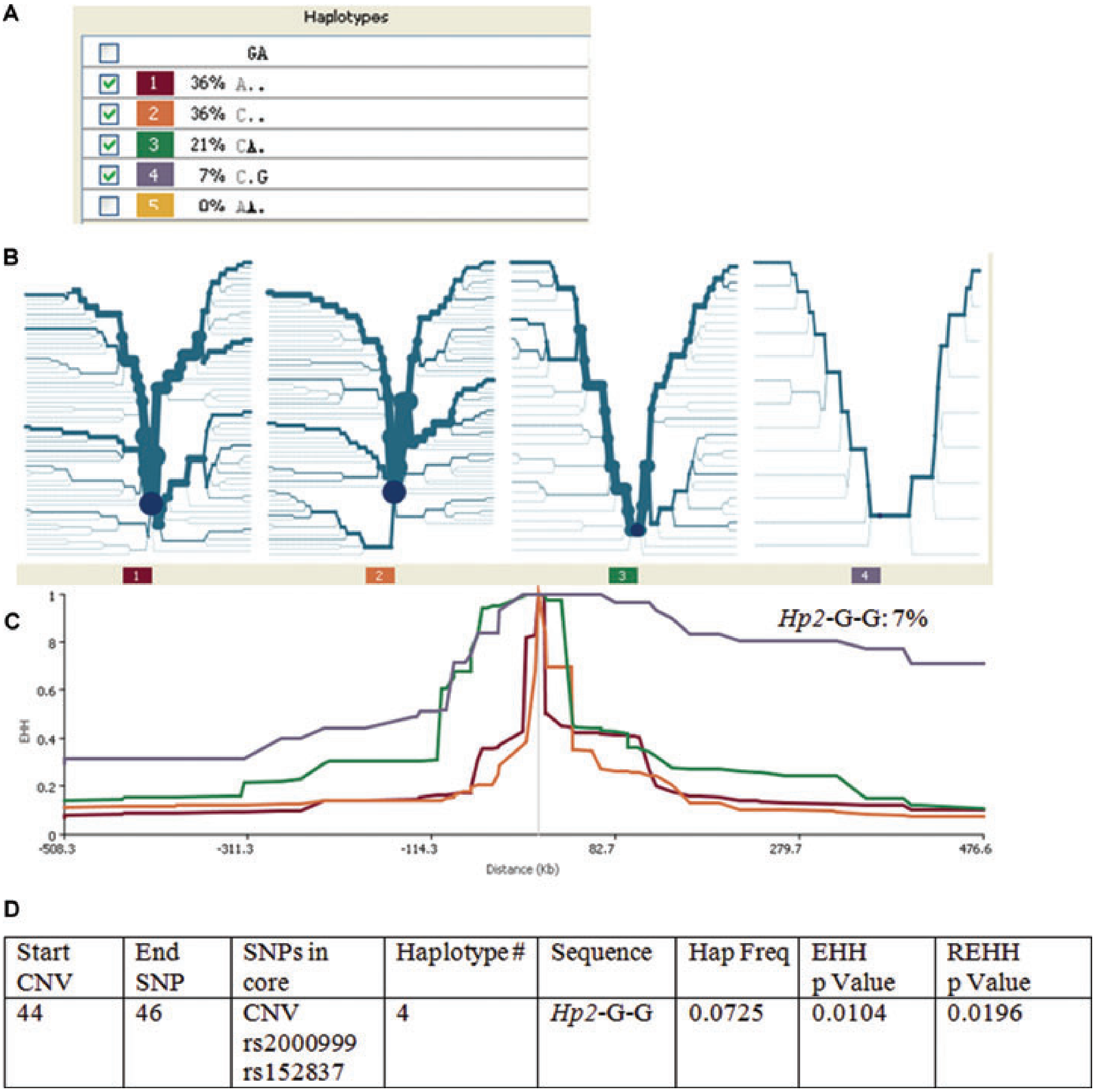
Discussion

In summary, our results from four different population and molecular approaches enable inferences about natural selection acting in the *HP* CNV region. We did not find strong evidence for natural selection on the *HP* CNV region, although both the reduction of heterozygosity in the *HP* CNV region and the F_{ST} values observed between populations across continents are in accordance with non-neutrality between populations. Our EHH results are in accordance with positive selection acting on the *HP* CNV region. However, this seems to be not completely explained by the *HP* CNV itself.

Our F_{ST} results showed a relatively constant frequency of the *HP* CNV in Europe compared with other continents. This suggests little selection in Europe, despite the reported existence of marsh fever (malaria) in marsh areas of Europe from the sixteenth to the nineteenth century (Dobson, 1994; Reiter, 2000). Marsh fever produced high levels of mortality, but the population attributable effect was small.

The absence of a tagging SNP for the *HP* CNV has implications for the interpretation of association studies. It might explain why Genome-wide Association studies that have been performed to date have not identified *HP* as a potential candidate gene.

The *Hp2* allele is newer than *Hp1 de facto*, according to the molecular evolution of this CNV. This is despite its higher frequency in populations, which would suggest that this allele is very old. Our new approach based on LD shows molecular evidence supporting the existence of a non-random effect consistent with natural selection. In particular, we have shown that there is a significant over-representation of rare SNP alleles associated with *Hp2*. This could be the result of a



Haplotypes

□			GA
☑	1	36%	A..
☑	2	36%	C..
☑	3	21%	CA.
☑	4	7%	C.G
□	5	0%	AA.

Start CNV	End SNP	SNPs in core	Haplotype#	Sequence	Hap Freq	EHH p Value	REHH p Value
44	46	CNV rs2000999 rs152837	4	*Hp2*-G-G	0.0725	0.0104	0.0196

Figure 3 EHH analysis of the *HP* CNV and neighboring SNPs. (A) Frequency of the four haplotypes defined by a core haplotype including the *HP* CNV. G and A are the ancestral alleles for SNPs rs2000999 and rs152837, respectively. In the first position, A corresponds to *Hp1* and C corresponds to *Hp2*; (B) Bifurcation diagrams for each of the four haplotypes. Division of the diagram reflects breakdown of LD; (C) EHH plotted for the core haplotype at ~500 kb in both directions from the core haplotype. The decay of EHH for haplotype *Hp2*-G-G is markedly different from the other haplotypes; (D) Significance of EHH and rEHH values for haplotype *Hp2*-G-G.

Table 4 ANOVA test comparing mean minor allele frequencies (MAF) between groups of SNPs stratified by level of disequilibrium with the *Hp*2 allele.

SNP	*N*	Mean q (95% CI)	S.D.	Minimum q	Maximum q
(a) D′ < 0.75	148	0.28 (0.26,0.30)	0.12	0.05	0.50
(b) D′ ≥ 0.75	12	0.13 (0.10,0.17)	0.06	0.04	0.22

*P < 0.001.
*Based on one way ANOVA.

Table 5 Haplotype frequencies for SNPs in high LD with the *HP* genotype. 10 SNPs show alleles associating more closely with the *Hp*2 allele (labels B-J and L), whilst two are more closely associated with the *Hp*1 allele (labels A and K).

	SNP code	Haplotype (*HP*, SNP)	Observed haplotype frequency	D'	r^2	Position on chromosome 16	Min. allele frequency
(A)	rs17665900	2,G	0.624	0.81	0.16	69915112	0.878
		2,A	0.015	−0.81			0.122
		1,G	0.255	−0.81			0.878
		1,A	0.107	0.81			0.122
(B)	rs1424241	2,C	0.475	−0.79	0.08	69966408	0.824
		2,T	0.162	0.79			0.176
		1,C	0.349	0.79			0.824
		1,T	0.013	−0.79			0.176
(C)	rs2000999	2,G	0.433	−0.89	0.12	69995594	0.786
		2,A	0.205	0.89			0.214
		1,G	0.353	0.89			0.786
		1,A	0.009	−0.89			0.214
(D)	rs152837	2,A	0.567	−1.00	0.04	70005252	0.929
		2,G	0.071	1.00			0.071
		1,A	0.362	1.00			0.929
		1,G	0.000	−1.00			0.071
(E)	rs152828	2,G	0.540	−1.00	0.06	70011387	0.901
		2,A	0.099	1.00			0.099
		1,G	0.362	1.00			0.901
		1,A	0.000	−1.00			0.099
(F)	rs217180	2,G	0.529	−1.00	0.07	70072130	0.890
		2,A	0.110	1.00			0.110
		1,G	0.362	1.00			0.890
		1,A	0.000	−1.00			0.110
(G)	rs12926250	2,G	0.529	−0.83	0.05	70100817	0.884
		2,T	0.109	0.83			0.116
		1,G	0.355	0.83			0.884
		1,T	0.007	−0.83			0.116
(H)	rs12928056	2,C	0.527	−0.84	0.05	70113075	0.882
		2,A	0.111	0.84			0.118
		1,C	0.355	0.84			0.882
		1,A	0.007	−0.84			0.118
(I)	rs11646048	2,A	0.439	−0.90	0.12	70179233	0.793
		2,G	0.200	0.90			0.207
		1,A	0.355	0.90			0.793
		1,G	0.007	−0.90			0.207
(J)	rs9940976	2,A	0.431	−0.82	0.11	70199327	0.778
		2,C	0.207	0.82			0.222
		1,A	0.347	0.82			0.778
		1,C	0.015	−0.82			0.222
(K)	rs726887	2,G	0.636	0.85	0.06	70239298	0.955
		2,T	0.004	−0.85			0.045
		1,G	0.319	−0.85			0.955
		1,T	0.041	0.85			0.045
(L)	rs212165	2,A	0.535	−0.82	0.05	70356964	0.889
		2,G	0.104	0.82			0.111
		1,A	0.354	0.82			0.889
		1,G	0.007	−0.82			0.111

Table 6 Age estimates of the *HP* CNV and 12 SNPs in high LD ($D' > 0.79$) with *HP* genotype

				Allele age estimates		
	SNP code	SNP allele	Min allele frequency (q)	Scaled time[1]	Generations[2]	Age (years)[3]
(A)	rs17665900	A	0.122	0.58	5844	116,875
(B)	rs1424241	T	0.176	0.74	7417	148,332
(C)	rs2000999	A	0.214	0.84	8388	167,761
(D)	rs152837	G	0.071	0.41	4052	81,046
(E)	rs152828	A	0.099	0.51	5069	101,386
(F)	rs217180	A	0.110	0.54	5443	108,850
(G)	rs12926250	T	0.116	0.57	5668	113,354
(H)	rs12928056	A	0.118	0.57	5712	114,241
(I)	rs11646048	G	0.207	0.82	8219	164,380
(J)	rs9940976	C	0.222	0.86	8587	171,748
(K)	rs726887	T	0.045	0.29	2938	58,760
(L)	rs212165	G	0.111	0.55	5488	109,760
(M)	*HP* CNV	1	0.362	1.15	11,531	230,616

[1] Scaled estimate based on formula by Slatkin and Rannala (Slatkin and Rannala, 2000).
[2] Based on the minimum populations size before recent modern human growth ($N = 10{,}000$).
[3] Assuming a generation span of 20 years.

"hitchhiking" effect with increasing allele frequency of the neutral SNPs close to the *HP* gene, which would result in a stronger association of rare SNP alleles with *Hp2* than with *Hp1*. This hitchhiking effect would have occurred before the migration into Europe when there was plausible selective pressure on *Hp2*. This is supported by our estimates of SNP allele ages that date back to before the migration into Europe. Comparison of our EHH results with published literature also gives an indication of a possible time span when selection took place. The lactase persistence allele, which emerged in North Europe within the past 5,000–10,000 years (Bersaglieri et al., 2004), shows a stronger and wider LD pattern than the one we observed for the *HP* region. Our age estimates, which assumed neutrality, for 12 SNPs suggest ~100,000 years. Therefore, a more conservative estimate for *HP* selection would range between 5,000 and 100,000 years.

The weak evidence for natural selection found in this work is more likely to reflect residual levels of an older selection phenomenon that is being diluted rather than being an active process of natural selection. This would be consistent with the relatively rare extended haplotype showing significant signatures of natural selection by the EHH test. The effect of malaria is negligible in modern European populations, although historically there may have been some selective pressure (Dobson, 1994; Reiter, 2000). In addition, if *Hp*2 does provide protection from chronic diseases and influence health in European populations, the effects should be prereproductive. It is more likely that all selection acting on the *Hp* CNV region occurred in Africa due to selection acting on malaria, and that current levels of selection in Europeans (as captured in our ALSPAC sample) are a "snapshot" of the genomic landscape originated by the out-of-Africa migration. Our findings are also consistent with positive selection due to malaria previously found for various blood protein genes (Kwiatkowski, 2005).

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Conflict of Interest Statement

All the authors declare that they have no conflicts of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Figure S1. ARCS assay for HP duplicon junction CNV.
Table S1. LD statistics for all pairwise comparisons involving the *HP* CNV and neighbouring SNPs in a 2 Mb interval centred on the *HP* CNV.
Table S2. Pairwise FST observed between ALSPAC and worldwide populations with HP CNV allelic frequencies described by Carter and Worwood (2007).
Table S3. Interallelic LD and descriptives for all pairwise comparisons involving the HP CNV and SNPs ∼2Mb apart centred on the HP CNV.

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