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Copy number variations in Friesian horses and genetic risk factors for insect bite hypersensitivity

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Abstract

Background: Many common and relevant diseases affecting equine welfare have yet to be tested regarding structural variants such as copy number variations (CNVs). CNVs make up a substantial proportion of total genetic variability in populations of many species, resulting in more sequence differences between individuals than SNPs. Associations between CNVs and disease phenotypes have been established in several species, but equine CNV studies have been limited. Aim of this study was to identify CNVs and to perform a genome-wide association (GWA) study in Friesian horses to identify genomic loci associated with insect bite hypersensitivity (IBH), a common seasonal allergic dermatitis observed in many horse breeds worldwide.

Results: Genotypes were obtained using the Axiom® Equine Genotyping Array containing 670,796 SNPs. After quality control of genotypes, 15,041 CNVs and 5350 CNV regions (CNVRs) were identified in 222 Friesian horses. Coverage of the total genome by CNVRs was 11.2% with 49.2% of CNVRs containing genes. 58.0% of CNVRs were novel (i.e. so far only identified in Friesian horses). A SNP- and CNV-based GWA analysis was performed, where about half of the horses were affected by IBH. The SNP-based analysis showed a highly significant association between the MHC region on ECA20 and IBH in Friesian horses. Associations between the MHC region on ECA20 and IBH were also detected based on the CNV-based analysis. However, CNVs associated with IBH in Friesian horses were not often in close proximity to SNPs identified to be associated with IBH.

Conclusions: CNVs were identified in a large sample of the Friesian horse population, thereby contributing to our knowledge on CNVs in horses and facilitating our understanding of the equine genome and its phenotypic expression. A clear association was identified between the MHC region on ECA20 and IBH in Friesian horses based on both SNP- and CNV-based GWA studies. These results imply that MHC contributes to IBH sensitivity in Friesian horses. Although subsequent analyses are needed for verification, nucleotide differences, as well as more complex structural variations like CNVs, seem to contribute to IBH sensitivity. IBH should be considered as a common disease with a complex genomic architecture.

Keywords: Copy number variations, Friesian horse, Genome-wide association study, Insect bite hypersensitivity

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Background

Genome sequence diversity can be present in various forms ranging from single-nucleotide polymorphisms (SNPs) to structural variants such as copy number variation (CNV). CNVs, a term that refers to a change in the number of copies of a genomic segment, are responsible for more sequence differences between individuals than SNPs and are considered to be a major source of inter-individual genetic variation contributing to differences in phenotypes (e.g. [1, 2]). Like SNPs, CNVs can be used to identify associations with genetic diseases and other important complex traits. CNVs explain variable penetrance of Mendelian and polygenic diseases and are responsible for variation in phenotypic expression of complex traits [e.g. 1, 2]. Considerable advances regarding CNVs have been made during the last decade [2].

Several studies identified copy number variations in horses using different techniques [3–12]. Part of these studies tried to establish associations between CNVs and a specific trait, a disease or even gene expression [3, 5–12]. Most of these studies found either no association or inconclusive associations as the number of horses with phenotypic information or with specific CNVs were limited. For example, a 62 kb duplication on *Equus caballus* (ECA) chromosome 10 seemed to be related to recurrent laryngeal neuropathy [5]. However, this duplication was detected in two unphenotyped parents and only 10 out of 234 cases (representing three breeds), but in none of the 228 controls [5]. The number of horses (between 4 to 70) studied by Doan and colleagues [3], Ghosh and colleagues [6], Wang and colleagues [11], Ghosh and colleagues [7], Park and colleagues [10] and McQueen and colleagues [8] was likely too few to be able to establish associations between CNVs and the investigated complex phenotypes. Pawlina-Tyszko and colleagues [12] identified an association between CNVs and equine sarcoids as these structural variants were overrepresented in sarcoid cells compared to unaltered skin tissue samples from 16 horses. Metzger and colleagues [9] performed a CNV-based genome-wide association (GWA) study in 717 horses from 17 breeds and showed that three CNV regions on ECA1, ECA8 and ECA9 were significantly associated with equine body size. To our knowledge, significant associations between CNVs and a specific trait in horses using a genome-wide approach have only been detected by Metzger and colleagues [9], an achievement likely accomplished because they investigated a large sample ($n = 717$) and their trait of interest (body size) may not have been as complex as most of the diseases investigated to date (reflected by a substantial heritability of the trait). Many common and relevant diseases affecting equine welfare and other traits of importance have not yet been tested based on structural variants.

One such disease is insect bite hypersensitivity (IBH), a seasonal allergic dermatitis observed in many horse breeds worldwide. The hypersensitive reaction to bites of *Culicoides* spp. causes an intense itch that results in self-inflicted trauma. Common clinical symptoms are hair loss, thickened skin, scaling and even open wounds (e.g. [13]). The welfare and commercial value of affected horses is therefore seriously reduced. Moreover, owners from affected horses suffer economic losses mostly due to an attempt to alleviate the itch and treat clinical symptoms.

The aetiology of IBH is multifactorial in origin and involves both environmental and genetic factors. Both heritability estimates (e.g. [14, 15]) and previous GWA studies [16–19] showed that the inheritance of IBH is truly polygenic in nature. Several breed-specific loci were identified, while across-breed associations with IBH were located on ECA7, 9, 11 and 20 [16–18]. Several candidate genes have been examined for an association with IBH in various breeds. However, associations with IBH were not often established or were inconsistent across breeds [20–22]. In contrast, the major histocompatibility complex (MHC, or equine lymphocyte antigens (ELA) in horses) class I and II regions on ECA20 were repeatedly associated with IBH in several horse breeds [21–25] and with allergen-specific immunoglobulin E levels against two moulds [26].

Most GWA studies in horses used SNPs, while the identification of potential associations between specific traits and CNVs has been limited [5–12]. To our best knowledge, a potential association between IBH and CNVs has never been studied and CNVs have not yet been identified in Friesian horses. As CNVs are the largest source of genetic variation identified in the horse genome so far [3, 6], CNV-based GWA studies could facilitate the identification of associations between our trait(s) of interest and additional genetic variation that is otherwise undetectable in SNP-based GWA studies. Moreover, genomic regions associated in CNV-based GWA studies may reveal more complex structures underlying phenotypic variation. Therefore, the aim of this study was to identify CNVs and to perform a CNV-based GWA study to identify genomic loci associated with IBH in Friesian horses. A SNP-based GWA study was performed as well to allow for comparison. Identification of genomic regions and genes associated with IBH will increase our knowledge on the aetiology and will allow for more effective selection aimed at decreasing IBH prevalence.

Methods

Phenotypes and horses

Data were gathered through an online inquiry containing questions about the horse, the farm and IBH. Questions

concerned among other things clinical symptoms, recurrence of symptoms, application of preventive measures and success of preventive measures that were applied. Where necessary, questions were clarified with figures and pictures of horses depicting clinical symptoms on various parts of the body and of different severity. Development of the inquiry was performed in close collaboration with a veterinary expert on diagnosing IBH from the Veterinary Faculty of Utrecht University. Through the inquiry, the necessary information was obtained to discriminate cases and controls. Cases were defined as Friesian horses showing the typical seasonal appearance of IBH clinical symptoms. In case preventive methods were applied, cases were defined as Friesian horses that will develop symptoms when these methods would cease. Controls were defined as Friesian horses free of clinical symptoms. However, in several controls (11.6%) preventive methods were applied (e.g. a blanket to protect the horse from any kind of biting insects). Owners of these horses indicated that, when the preventive methods would cease, the horse would not develop symptoms.

A strict protocol was followed to select the horses for genotyping to increase the reliability of the phenotype. Controls were carefully selected to ensure exposure to *Culicoides* spp. Controls were therefore located on a farm where at least one case was present (82.6% of the controls). Not all controls could be collected from farms with cases present, and the remaining controls were collected from IBH high-risk regions in the Netherlands (e.g. [27]). Although age at onset of IBH varies greatly [e.g. [14, 16]], average age at onset is considered to be between 2 and 4-years-of age. Controls were therefore required to be at least 4-years-of-age and at least one to two years at risk of developing IBH clinical symptoms. Cases showed symptoms preferably during two or more seasons (90.8% of the cases; Additional file 1) and their status was confirmed by a veterinarian (60.0% of the cases). Moreover, both cases and controls were preferably located for two or more years on their current farm to ensure a constant management (91.4% of the horses).

Data consisted of 280 Friesian horses, of which 142 were cases and 138 were controls. All horses originated from the Netherlands. Age and sex distribution was similar for cases and controls (Additional file 1). Also, cases and controls descended from a similar number of ancestors, where 40 sires had both case(s) and control(s) among their offspring (66% of the data).

DNA extraction, quality and genotyping

DNA was extracted from hair samples that were collected with written permission of the horse's owner. DNA was extracted following the standard protocol for hair roots using the NucleoSpin® Tissue kit from Bioke (Macherey-Nagel). Concentration of DNA was measured using the Infinite® M200 and normalized to 10 ng/μl using Tecan EVO®. Lack of degradation of DNA was confirmed by electrophoresis, carried out using a 1-ll sample on 1.5% multi-purpose agarose gels in Tris–borate–EDTA buffer at 120 V.

Genotypes were obtained using the Axiom® Equine Genotyping Array containing 670,796 SNPs. Part of the quality control was performed with Axiom™ Analysis Suite 1.1 using the default settings of the *Best Practices Workflow*. *OTV* (Off Target Variants) *Caller* was used to perform a post-processing analysis to identify miscalled clustering and to re-label horses in this cluster as OTV (for 6083 SNPs). Gender and computed gender based on homozygosity of the X-chromosome matched for all horses. Subsequent quality control was performed using PLINK software v1.07 [28, 29]. SNPs with call-rate < 90% and MAF < 5% were discarded. All horses passed the call-rate threshold of ≥90%.

The pedigree (**A**, according to Colleau [30]) and genomic relationships (**G**, according to VanRaden [31]) among horses was calculated with calc_grm software [32]. Four horses caused large discrepancies between pedigree and genomic relationships; their genotype data was therefore discarded. The multidimensional scaling plot (Additional file 2) showed that the horses belonged to one cluster. After all quality checks were performed, the final genotype dataset to perform a SNP GWA study contained 276 horses – 141 cases and 135 controls – and 307,075 SNPs (45.8% of all SNPs present on the array).

SNP-based GWA study

The significance level of differences in allele frequencies between cases and controls per SNP was calculated using a χ^2-test with 1df using the *assoc* and *adjust* commands in PLINK software v1.07 [28, 29]. Genomic Control corrected *P*-values were presented. The conservative Bonferroni corrected significance level was 1.63×10^{-7} ($= {}^{\alpha}/_{n}$, where α was the desired significance level being 0.05 and n was the number of SNPs ($n = 307,075$) that were tested).

CNV calling

To compute values requested for CNV calling, the files denominated '*summary*', '*calls*' and '*confidences*' that were built during SNP genotyping in Axiom™ Analysis Suite 1.1 were used. These files were applied into PennCNV [33–35] function '*generate_affy_geno_cluster.pl*' to generate canonical clusters values [36] for each SNP on the array. Subsequently, the canonical clusters were used into the '*normalize_affy_geno_cluster.pl*' function, which estimated Log R Ratio (LRR) and population frequencies

of the B allele (BAF) values for each of the 276 analysed horses. GC content around each SNP (1 Mb window) was estimated using the BEDTools '*nuc*' function [37] that generated the GCModel file. The GCModel file was needed to adjust LRR values for genomic waves by the '*genomic_wave.pl*' function in PennCNV (for genomic waves bias details, see [33]). To infer BAF values for each SNP, we applied the '*compile_pfb.pl*' function on all 276 analysed horses. The file containing these BAF values was needed to call CNVs with a hidden Markov model algorithm in PennCNV [33–35] with the '*detect_cnv.pl*' function using default parameters. All called CNVs were filtered based on minimum length (≥1 kb, technical reasons related to SNP density), LRR SD (≤0.3) and BAF drift (≤0.02). After quality control, CNV information from the 31 autosomal horse chromosomes and 222 horses (111 cases and 111 controls) remained for the subsequent analysis. Most horses (45 out of 54) were removed based on high BAF drift.

CNV region identification and association

Individual CNVs of the 222 Friesian horses were merged into CNVRs, i.e. genomic regions in the horse genome covering CNVs that overlapped by at least 1 bp [38] using the *CNVR* building process in CNVRuler [39]. To minimize the possibility of overestimating the size of CNVRs, low-density areas were trimmed: the area covered by < 10% of the total contributing CNVs within a CNVR was removed using the *recurrence* option.

A CNV-based case-control association analysis for IBH – based on all CNVs identified in Friesian horses – was performed with CNVRuler [39]. The performed analysis tested for an association between CNVs at a specific CNVR and the disease status (case or control). Separate *P*-values were generated for gains and losses using logistic regression including one principal component as covariates to adjust for population stratification (second and third principal component were not significant, data not shown). In the end, three association analyses were performed e.g. considering only gains, only losses or both gains and losses. That is, the number of cases with a particular gain (a CNV with more than 2 copies within a specific CNVR) was compared to the number of controls with this gain. The second analysis only considered losses (a CNV with less than 2 copies within a specific CNVR), where again the number of cases with a particular loss was compared to the number of controls with this loss. In the analysis concerning both gains and losses, we compared the number of cases having a CNV (either gain or loss) within a specific CNVR with the number of controls having a CNV within this specific CNVR. We did not relate the number of copies to for instance the severity of IBH, among other things because often many horses had the same number of copies.

We graphically represented all copy number events and respective frequencies in grid of panels implemented in the Bioconductor R package *gtrellis* [40]. Due to space limitation all information shown is based on genomic windows of 1 Mb.

Gene mapping, enrichment and ontology analysis

The overlap between the CNVRs identified in Friesian horses and genes annotated in the horse genome (*Ensembl* – BioMart: http://www.ensembl.org/biomart/martview/ and [41]; EquCab2) was determined and classified into upstream, downstream, equal, encompassing or inside. The enrichment of overlapping genes was performed based on human orthologues available in BioMart. The use of human orthologues is justified due to the still incomplete annotation of genes on the equine genome [6, 9]. Based on these orthologues, the *ClusterProfiler* Bioconductor R package with the *enrichKEGG* function [42] was used to identify KEGG pathways that were overrepresented in CNVRs.

CNV validation by qPCR

Validation of low frequent CNVs was performed by quantitative real-time PCR (qPCR) (Additional file 3). Low frequent CNVs were selected, as these CNVs are more likely to be false positives compared to CNVs shared by more horses. We selected 12 individual low frequent CNV calls based on incidence, size and state, to sample CNVs from the whole distribution of size and state. Concerning incidence, unique (private) CNVs and those present in 2 horses (all with exactly the same breakpoints) were selected to represent two classes of low frequent CNVs. Concerning size, for each frequency class, 6 CNVs were selected based on the size distribution. A CNV was selected from the minimum, first quadrant, mean, median, third quadrant and maximum part of the distribution. Concerning state, in each frequency class we ensured the inclusion of at least one CNV belonging to each of the common states (0n, 1n and 3n) (Additional file 3).

To determine the quality of DNA samples to be validated, the amount of dsDNA was measured with Qubit® Fluorometer. Subsequently, for each sample we used 4 different concentrations to determine primer efficiency: 15 ng, 7.5 ng, 3.8 ng and 1.9 ng of DNA. Reactions were assembled in a final volume of 12.5 μl, containing 3.75 μl DNA, 6.25 μl 2X reaction buffer MESA Blue from Invitrogen™, 1.25 μl forward primer (2 μM) and 1.25 μl reverse primer (2 μM). Samples with a CNV in a specific region and diploid (2n) reference samples were tested with the designed primersets (Additional file 3). Measurements were performed with the Applied Biosystems® 7500 real-time PCR system. Cycle thresholds ($\log_2$ Ct) were corrected for primer efficiency. ΔCt was

calculated as Ct from the sample with a specific CNV minus Ct of the diploid (2n) reference sample [43]. The reference sample was given by a random horse with 2n state. As it is challenging to determine the exact state of a CNV with data from SNP-arrays, we considered a CNV validated when its state (loss or gain) was equal.

Identification of novel CNVRs

We determined the overlap between CNVRs identified in the Friesian horse population and equine CNV(R)s already published in scientific literature [3–9, 11, 12]. It allowed us to identify CNVRs that had not been discovered so far (i.e. = novel CNVRs) and CNVRs that are shared across breeds. CNVRs among studies were considered to overlap when at least 1 bp was in common and were considered the same event (that is, the same CNVR) when the mutual overlap was > 70%.

Results

SNP-based GWA study

The SNP-based GWA study showed a clear association between a region on ECA20 and IBH in Friesian horses (Fig. 1). Regions with a suggestive association (*P*-value < 0.0001) were identified on ECA2, 9, 10 and 11. In total 2 SNPs passed the Bonferroni corrected significance level (Additional file 4). The SNP most significantly associated with IBH (*P*-value = 7.41×10^{-7}) and of good quality was AX-103894624 located on ECA20:31,245,645 (Additional file 5). Estimated odds ratio was 2.62 (allele substitution effect $\beta = ln(OR) = 0.96$). The IBH-associated allele frequency was 0.46 in cases and 0.25 in controls.

CNV and CNVR detection

In total, 15,041 CNVs were detected in 222 horses of which 85.5% were gains and 14.5% were losses (Fig. 2, Additional file 6). Number of CNVs per horse ranged from 18 to 262 (mean = 67.8; median = 53.5). Number of SNPs per CNV ranged from 3 to 483 (mean = 15.1; median = 8). Size of the CNVs ranged from 1017 bp to 2.73 Mb (mean = 61.7 kb; median = 29.8 kb). Most CNVs were detected on ECA20, where chromosomal coverage was 11.5% (Additional file 6). CNVs were detected on all autosomes (Additional file 6).

Overlapping CNVs were concatenated into 5350 CNVRs (Additional files 6 and 7). CNVRs encompassing both losses and gains in different horses corresponded to 3.2% (Additional file 6), while only losses corresponded to 2.1% and only gains to 94.7% of all identified CNVRs. Size of the CNVRs ranged from 123 bp to 1.04 Mb (mean = 46.8 kb; median = 29.3 kb). Although the minimum length of a CNV was set to 1 kb, several CNVRs turned out to be < 1 kb due to trimming of low-density areas as described in the Methods section. Chromosomal coverage (percentage of a chromosome covered by CNVRs) was highest for ECA17 (16.2%), followed by ECA26 (16.0%) and ECA12 (15.1%). We did not find a relation between the number of SNPs on a chromosome and chromosomal enrichment for CNVRs (Additional file 6). Coverage of the total genome by CNVRs was 11.2%. In total 1949 CNVRs (36.4%) were shared by at least 2 horses. Mean size of shared CNVRs (79.3 kb) was larger compared to mean size of unique CNVRs (private, 28.2 kb) and 49.2% of CNVRs involved genes (Additional files 6 and 7).

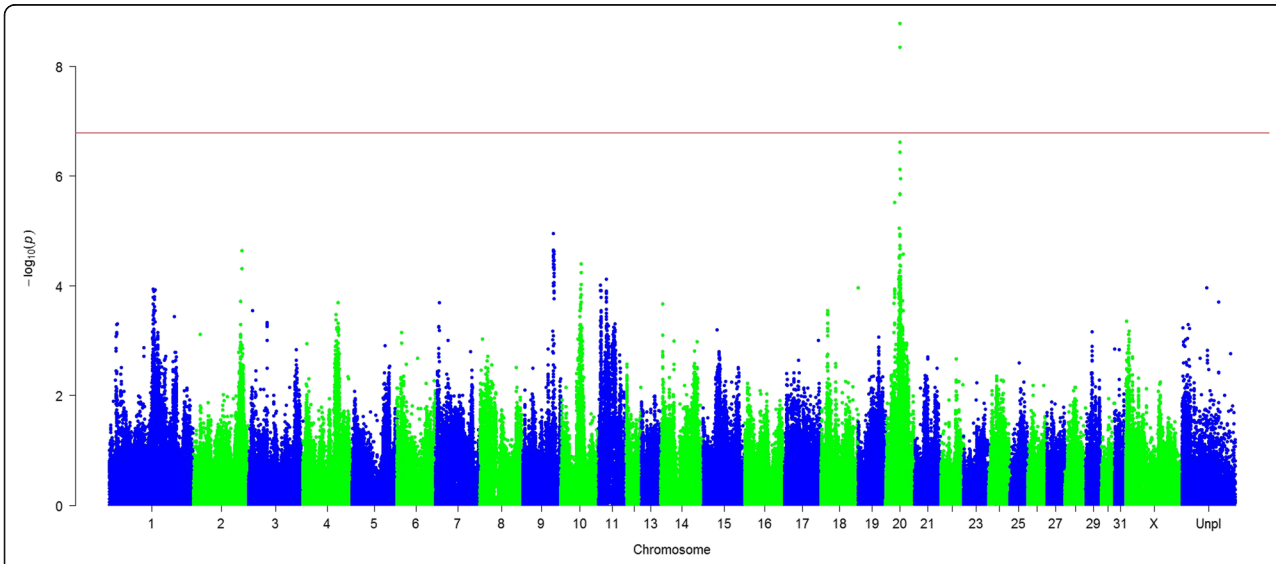

Fig. 1 Manhattan plot of insect bite hypersensitivity in Friesian horses based on a SNP-based genome-wide association study. Significance level based on allele frequency differences between cases ($n = 141$) and controls ($n = 135$) using a χ^2-test (1df). The horizontal red line is the Bonferroni corrected significance level (*P*-value = 1.63×10^{-7})

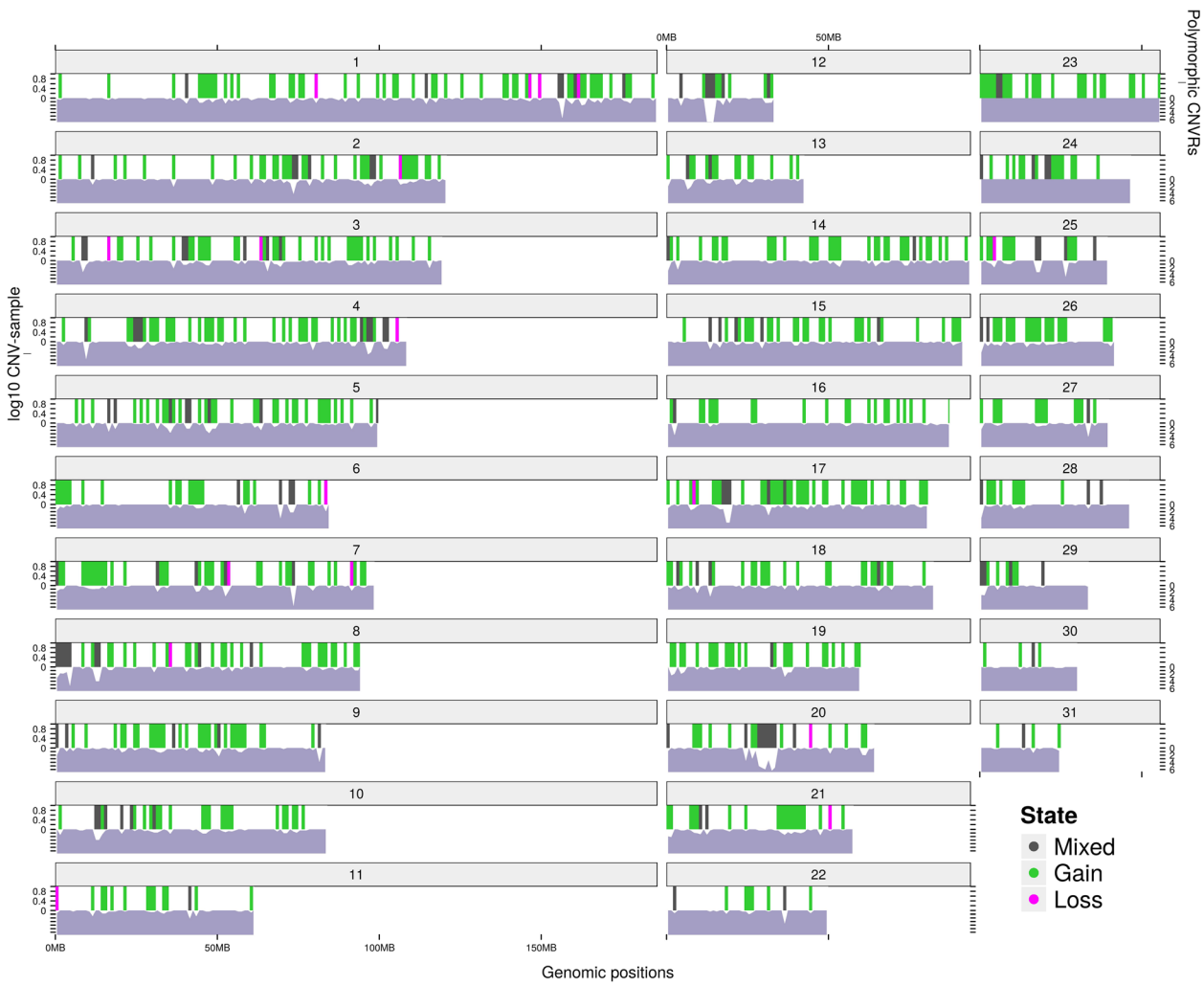

Fig. 2 Distribution of polymorphic CNVRs in the Friesian horse genome. Somatic horse chromosomes containing CNV regions with their frequency. The grey bars represent regions harbouring losses and gains concomitantly, the green bars exclusively gains and the magenta bars exclusively losses. The genomic interspersed regions without CNVRs are represented in white. Polymorphic CNVRs: the bars indicate the CNVRs with at least 3 CNVs mapped in 3 different horses (i.e. 1 % of frequency). log10 CNV-sample: the $\log_{10}$ of the number of horses containing a CNV event throughout the genome, indicated as a distribution in white

Association of CNVs with IBH

In total 19 CNVs were associated with IBH (*P*-value < 0.05, Table 1, Additional file 8), but did not reach genome-wide significance. CNVs within a specific CNVR on ECA10 (12,948,489 to 13,075,518 bp) had the lowest *P*-value in the association test (*P*-value = 0.0003), was 127,030 bp in size and included both gains and losses. A CNV within this specific CNVR was observed in 25 cases (15 gains and 10 losses) and 6 controls (all gains) with an odds ratio (OR) of 5.92 (Table 1). CNVs (gains) within a specific CNVR on ECA20 had the lowest *P*-value in the association test based only on gains (*P*-value = 0.001; 30,624,048 to 30,689,273 bp) and was 65,226 bp in size. A CNV (gain) within this specific CNVR was observed in 60 cases and 35 controls with an OR of 2.65 (Table 1). CNVs (losses) within a specific CNVR on ECA20 (30,743,179 to 30,775,429 bp) had the lowest *P*-value in the association test based only on losses (*P*-value = 0.008) and was 32,251 bp in size. A CNV (loss) within this specific CNVR was observed in 18 cases and 5 controls with an OR of 2.45 (Table 1). Individual CNVs within the CNVRs with the lowest *P*-value in the association tests are visualized in Additional file 9. Candidate genes located within CNVRs in which CNVs were associated with IBH are presented in Table 1.

The CNV-based GWA study results showed that IBH in Friesian horses is associated with CNVs within specific CNVRs on several autosomes. In total 7 out of 19 CNVRs in which CNVs were associated with IBH were

Table 1 Results of a CNV-based case-control association analysis of insect bite hypersensitivity in Friesian horses

						CNV frequency										
						Case			Control							
CNVR ID	ECA[a]	Start (bp)	End (bp)	Size (bp)	State	Gain	Loss	Total	Gain	Loss	Total	*P*-value Gain	*P*-value Loss	*P*-value	OR[b]	Gene[c]
CNVR_1144_1	4	23,383,774	23,505,444	121,671	Gain	1	0	1	9	0	9	0.033		0.0332	$0.10^{0.01–0.84}$	
CNVR_1296_1	4	79,687,222	79,957,793	270,572	Gain	11	0	11	26	0	26	0.009		0.0092	$0.36^{0.17–0.78}$	SPAM1
CNVR_2035_1	8	3,638,239	3,783,874	145,636	Mixed	7	2	9	1	1	2	0.046	0.580	0.0367	$5.40^{1.11–26.3}$	IGLV8–61, ENSECAG00000014327
CNVR_2452_1	10	12,948,489	13,075,518	127,030	Mixed	15	10	25	6	0	6	0.022	0.982	0.0003	$5.92^{2.24–15.7}$	AC011513.3, CEACAM1, CEACAM3-CEACAM8, PSG1-PSG9, PSG11
CNVR_2685_1	11	41,743,465	41,832,225	88,761	Mixed	8	5	13	2	2	4	0.055	0.265	0.0245	$3.82^{1.19–12.3}$	
CNVR_2758_1	12	19,366,263	19,527,441	161,179	Gain	14	0	14	2	0	2	0.004		0.0038	$9.86^{2.09–46.5}$	OR4D10, OR4D11, ENSECAG00000008771
CNVR_2979_1	14	64,375,388	64,516,633	141,246	Gain	3	0	3	11	0	11	0.040		0.0395	$0.25^{0.07–0.94}$	
CNVR_4066	20	20,271	503,862	483,592	Mixed	1	0	1	7	1	8	0.064	0.987	0.0450	$0.11^{0.01–0.95}$	
CNVR_4114_1	20	24,230,088	24,296,409	66,322	Mixed	6	0	6	19	0	19	0.010		0.0100	$0.28^{0.10–0.74}$	BTN3A1, BTN3A2, BTN3A3
CNVR_4120_1	20	26,392,472	26,531,248	138,777	Gain	25	0	25	46	0	46	0.003		0.0034	$0.41^{0.23–0.74}$	ENSECAG00000001951, ENSECAG00000019806, ENSECAG00000002019, ENSECAG00000002140, ENSECAG00000002328, ENSECAG00000000865
CNVR_4132_1	20	29,805,544	29,821,078	15,535	Gain	23	0	23	9	0	9	0.007		0.0074	$3.11^{1.36–7.12}$	
CNVR_4140_1	20	30,493,889	30,523,455	29,567	Mixed	34	8	42	56	4	60	0.003	0.248	0.0159	$0.52^{0.30–0.88}$	MICA, MICB
CNVR_4141_1	20	30,624,048	30,689,273	65,226	Gain	60	0	60	35	0	35	0.001		0.0006	$2.65^{1.52–4.61}$	ENSECAG00000017324, ENSECAG00000002838
CNVR_4142_1	20	30,743,179	30,775,429	32,251	Mixed	14	18	32	11	5	16	0.422	0.008	0.0092	$2.45^{1.25–4.79}$	ENSECAG00000019095
CNVR_4155_1	20	32,467,872	32,582,288	114,417	Mixed	3	10	13	7	19	26	0.231	0.086	0.0285	$0.44^{0.21–0.92}$	
CNVR_4271_1	21	10,784,937	10,861,217	76,281	Mixed	8	1	9	3	0	3	0.077	0.987	0.0464	$4.20^{1.02–17.3}$	
CNVR_4537	23	32,331,580	32,716,916	385,337	Gain	1	0	1	9	0	9	0.034		0.0342	$0.11^{0.01–0.85}$	ENSECAG00000026512, ENSECAG00000009426
CNVR_4747[d]	26	250,337	434,632	184,296	Mixed	1	8	9	8	8	16	0.048	0.966	0.0332	$0.12^{0.01–0.98}$	
CNVR_4992_1[d]	28	4428	376,070	371,643	Mixed	3	11	14	12	13	25	0.066	0.620	0.0092	$0.21^{0.05–0.83}$	ENSECAG00000001173, ENSECAG00000003651

Significance level (*P*-value) based on CNV frequency differences between cases ($n = 111$) and controls ($n = 111$) using logistic regression and 1 principal component as covariate, including CNVR identification (CNVR_ID), chromosome, start and end position of CNVR (in base pairs), state (gain, loss or complex), odds ratio (OR) and candidate genes based on *Ensembl* IDs and human orthologues

[a]*Equus caballus* chromosome

[b]Odds ratio of the CNV with 95% confidence interval in superscript

[c]*SPAM1* = sperm adhesion molecule 1 (OMIM:600930), *IGLV8–61* = immunoglobulin lambda variable 8–61 (OMIM:147240), *AC011513.3* = ENSG00000267881, *CEACAM1* = carcinoembryonic antigen-related cell adhesion molecule 1 (OMIM:109770), *CEACAM3* = carcinoembryonic antigen-related cell adhesion molecule 3 (OMIM:609142), *CEACAM4* = carcinoembryonic antigen-related cell adhesion molecule 4 (ENSG00000105352), *CEACAM5* = carcinoembryonic antigen-related cell adhesion molecule 5 (OMIM:114890), *CEACAM6* = carcinoembryonic antigen-related cell adhesion molecule 6 (OMIM:163980), *CEACAM7* = carcinoembryonic antigen-related cell adhesion molecule 7 (ENSG00000007306), *CEACAM8* = carcinoembryonic antigen-related cell adhesion molecule 8 (OMIM:615747), *PSG1* = pregnancy-specific beta-1-glycoprotein 1 (OMIM:176390), *PSG2* = pregnancy-specific beta-1-glycoprotein 2 (OMIM:176391), *PSG3* = pregnancy-specific beta-1-glycoprotein 3 (OMIM:176392), *PSG4* = pregnancy-specific beta-1-glycoprotein 4 (OMIM:176393), *PSG5* = pregnancy-specific beta-1-glycoprotein 5 (OMIM:176394), *PSG6* = pregnancy-specific beta-1-glycoprotein 6 (OMIM:176395), *PSG7* = pregnancy-specific beta-1-glycoprotein 7 (OMIM:176396), *PSG8* = pregnancy-specific beta-1-glycoprotein 8 (OMIM:176397), *PSG9* = pregnancy-specific beta-1-glycoprotein 9 (OMIM:176398), *PSG11* = pregnancy-specific beta-1-glycoprotein 11 (OMIM:176401), *OR4D10* = olfactory receptor family 4 subfamily D member 10, *OR4D11* = olfactory receptor family 4 subfamily D member 11, *BTN3A1* = butyrophilin subfamily 3 member A1 (OMIM:613593), *BTN3A2* = butyrophilin subfamily 3 member A2 (OMIM:613594), *BTN3A3* = butyrophilin subfamily 3 member A3 (OMIM:613595), *MICA* = major histocompatibility complex class I chain-related gene A (OMIM:600169), *MICB* = major histocompatibility complex class I chain-related gene B (OMIM:602436)

[d]Odds ratio of CNV gain is mentioned

located on ECA20 encompassing the MHC class I, II and III region. These CNVRs identified on ECA20 did correspond to the associated region on the same chromosome identified with the SNP-based GWA study (Table 1 and Additional file 5).

Biological pathways affected by CNVRs

The biological pathways enriched in the identified CNVRs were mainly related to sensory perception, metabolism and immunity (Fig. 3). The pathway with lowest *P*-value was *Olfactory transduction* (*hsa04740*, *P*-value = 1.4×10^{-22}) followed by *Natural killer cell mediated cytotoxicity* (*hsa04650*, *P*-value = 1.9×10^{-8}) and *Autoimmune thyroid disease* (*hsa05320*, *P*-value = 0.0006). The pathways that we identified were in agreement with previous CNV studies in horses and in other mammals (e.g. [2, 6]), that identified these olfactory receptors, metabolism and immunity related genes as CNV hotspots.

CNV validation and identification of novel CNVRs

Validation of low frequent CNVs by qPCR indicated that state and copy number of 50% of private CNVs were exactly validated. Also, 66% of the CNVs shared by two horses were validated, where state of the CNV was validated in 50% and state and copy number were validated in 16.7% (Additional file 3).

When looking at the CNVs within 19 CNVRs in Friesian horses significantly associated with IBH, 17 of these regions were also found in one or more horse populations presented in the literature (Additional file 10). Out of these 17 regions, only one was considered a different event as the overlap between the CNVR identified in Friesian horses and the region presented in literature was smaller than 70% (50.2%, CNVR4271; Additional file 10). In total 16 out of 17 CNVRs were considered the same event (that is, the same CNVR) as the region presented in literature. For all these 16 CNVRs, the region identified in Friesian horses completely overlapped with CNV(R)s presented in literature (Additional file 10).

In total 3105 out of 5350 CNVRs were novel (58.0%), that is, so far only identified in the Friesian horse population. 42.0% of the CNVRs were also found in one or more horse populations presented in literature (that is, at least 1 bp overlap).

Discussion

To date, CNVs have been shown to explain the largest part of genetic variation in genomes of many species including the horse [3, 6]. As such, CNVs are likely to further contribute to our understanding of the equine genome and its expression. Although the efficiency of CNV identification using SNP arrays is lower compared to CNV focussed arrays (e.g. CGH array), the results of

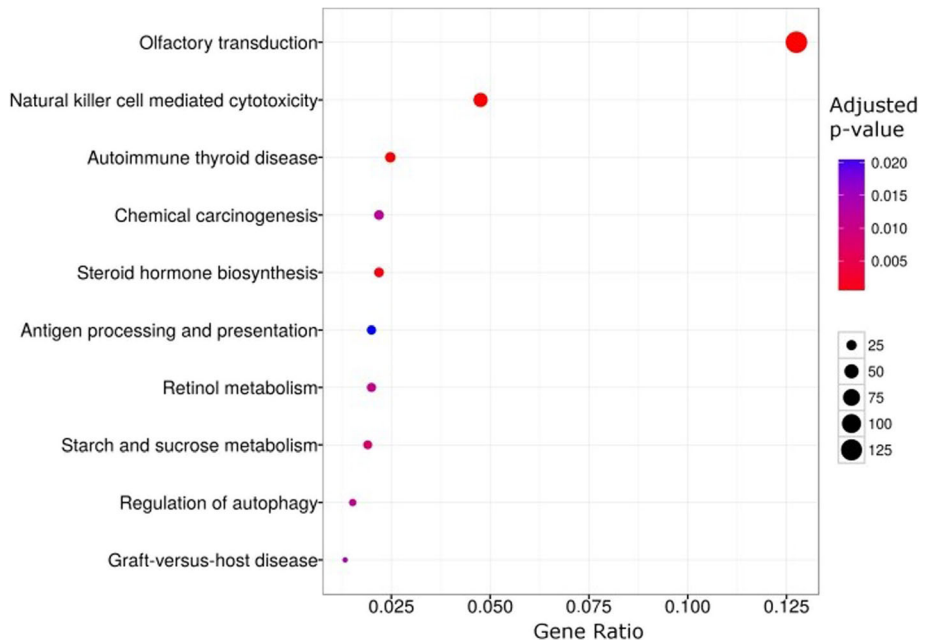

Fig. 3 Biological pathways enriched in CNVRs detected in Friesian horses. Biological pathways enriched in CNVRs detected in 222 Friesian horses. To compose the gene enrichment, we used human orthologues (*Ensembl* – BioMart, [41]) of genes overlapping CNVRs. The y-axis indicates the enriched KEGG pathway. The x-axis the ratio between the number of analysed genes and the number of genes in each KEGG pathway presented on the y-axis. The 'Adjusted *p*-value' heatmap represents the enrichment *P*-value for each pathway corrected for false discovery rate (FDR, [60]). The circle size represents the number of genes in each pathway

the current analyses were comparable to previously published equine CNV studies that predominantly utilized CNV focussed arrays. The chromosomes with the highest CNV distribution (that is, number of CNVs) in this study were ECA1, 12, and 20, the same chromosomes as identified by Dupuis and colleagues [5], Doan and colleagues [3], Metzger and colleagues [9], Ghosh and colleagues [6] and Wang and colleagues [11]. Chromosomes with the greatest coverage by CNVRs were ECA12, 17, and 26, with multiple previous studies also identifying ECA12 [3, 6, 9, 11]. The fraction of the genome covered by CNVRs in our study (11.2%) was higher than previously published by Doan and colleagues [3, 4], Dupuis and colleagues [5], Ghosh and colleagues [6] and Wang and colleagues [11], yet similar to the genome coverage obtained by Metzger and colleagues [9] using PennCNV and 50 k SNP genotyping data. However, CNV and CNVR metrics (e.g. number and size) from equine CNV studies are highly diverse. Ghosh and colleagues [6] composed a CNV dataset for the horse genome based on all published equine CNV data at that time [3–6, 9, 11, 44]. Noticeably, CNV data were included irrespective of the CNV discovery platform used, the analytical methods applied, and the (sometimes limited) number of breeds and individuals. Differences in CNV metrics between the studies also arose from experimental differences [6]. Indeed, the number of CNVs identified in the same horse population differed greatly depending on the CNV detection algorithm that was used, whereas the overlap in detected CNVs was also limited [9]. The composite CNV dataset contains 1476 CNV regions (CNVRs). The majority of these CNVRs were study-specific, while 20% were shared between two or more studies [6].

Recently, data from more contemporary studies were added [7, 8, 10, 12] and indicated that 58.0% of the CNVRs identified in Friesian horses can be considered novel CNVRs. However, Friesian horses are genetically somewhat more inbred and distant to the other investigated breeds as indicated by previous research on genetic diversity in several horse breeds using Short Tandem Repeat loci [45] and SNP data (unpublished data). Therefore, novel CNVRs could be expected and may reflect, in part, variants associated with specific adaptations or with breeding goal traits in Friesian horses. Nonetheless, validation of CNVs is needed in order to conclude if identified CNVs should be considered true structural variants. The state (gain or loss) of low frequent CNVs (private or shared by two horses only) of various lengths was validated for 58.3% of the tested CNVs. Both state and copy number were validated for 33.3% of the tested CNVs. Metzger and colleagues [9] validated state and copy number of 91.7% CNVs through qPCR, but their choice of CNVs to be validated was non-random as only common CNVs associated with body size were selected. Although no attention was paid to incidence, size and state, Pawlina-Tyszko and colleagues [12] randomly selected 12 CNVs using qPCR. Identical validation results were obtained, as both state and copy number were validated for 33.3% of the tested CNVs and state was validated for 58.3%. Validation, characterization and sharing of identified structural variants like CNVs is encouraged as a way to increase our understanding of the equine genome and its expression.

Many traits of interest in horses, as well as common and relevant diseases affecting equine welfare have not been tested in light of structural variants. Only a limited number of studies [3, 5–12] have attempted to detect associations between CNVs and a specific trait, a disease or gene expression in horses. In many studies, no association or inconclusive associations were found, likely due to the number of horses with the phenotype or the specific CNV having been (very) limited. To our best knowledge, we were the first to perform a CNV-based case-control association study for IBH. Most CNVs within specific CNVRs that we found to be associated with IBH were also identified in other horse population(s) [3–9, 11], increasing the likelihood of these CNVs being true structural variants. Several CNVs within or surrounding the major histocompatibility complex region (MHC, or ELA in horses) on ECA20 were associated with IBH in Friesian horses. Some of these CNVs contained MHC class I associated genes (e.g. *BTN3A3*) involved in T cell mediated immunity. Similarly, the SNP-based GWA study and all previous studies on IBH using SNP data to date have identified this region [16, 18, 19, 23]. However, we obtained the lowest *P*-values thus far likely due to an increase in power caused by the large number of inbred horses that were investigated with the high density SNP array combined with a strict protocol to select cases and controls.

Furthermore, serological research established an association between MHC antigens and IBH [24, 25]. MHC class I and II evoke immune responses by recognizing many foreign molecules, whereas MHC class III encodes for complement components and specific cytokines (e.g. TNF-α, [46–48]. The MHC region contains genes that have critical functions in immunity, in both the adaptive and innate immune systems (e.g. [46, 49]). In vertebrates, the MHC region is the most gene-dense region of the genome (e.g. [50, 51]). Genes within this region are extremely polymorphic. In an attempt to explain this unusually high diversity, several hypotheses founded on disease-based and reproductive mechanisms have been formulated (as reviewed by [52, 53]). One such hypothesis is the overdominance (or heterozygote advantage) hypothesis, which states that MHC diversity is maintained by balancing selection for millions of years reflecting "the long-standing battle for supremacy

between our immune system and infectious pathogens" (as expressed by [51]). Loss of diversity in these genes seems to reduce the ability to cope with diseases [54] with associations between MHC polymorphisms and fitness having been detected (as reviewed by [52, 53]). Although our knowledge on MHC, its polymorphisms, diversity, and expression continues to increase, there is still much to elucidate. Results of the SNP- and CNV-based GWA studies suggest that the MHC region is associated with IBH. Recently Lanz and colleagues [55] showed that IBH and airway hyperreactivity in horses were associated and discussed the potential of a (partly) common immunogenetic background. The regions identified to be associated with IBH (including MHC) might therefore underlie other equine hypersensitivities as well. Our CNV-based GWA study reflects a first attempt in associating IBH with variants other than SNPs. While validation is still needed to verify the contribution of these CNVs to IBH, it appears that not only nucleotide differences contribute to IBH sensitivity but more complex, structural variations like CNVs also play a role.

Genetic variation is what makes each individual unique and can determine a unique sensitivity to a specific disease. An abundance of SNPs has been identified in many species, and SNPs associated with specific diseases and other phenotypes have been found. Besides the MHC class I, II and III region on ECA20, promising regions likely to hold gene(s) contributing to IBH sensitivity were located on ECA9, 10 and 11 (based on the SNP-based GWA study and [16–19]). However, more in depth research (e.g. sequencing) is needed to be able to identify and validate variations contributing to IBH sensitivity. During the last decade, CNVs proved to be a prevalent form of variation in many species, ultimately responsible for a substantial proportion of the total genetic variability in populations that contributes to a variety of phenotypes. The importance of CNVs has been recognized in modulating gene expression and disease phenotype with associations having been established for autoimmune diseases [56], asthma [57], and obesity [58, 59]. Both CNV and SNP analyses identified an association between the MHC region on ECA20 and IBH (this study and [16–19]). However, CNVs associated with IBH in Friesian horses were not often in close proximity to SNPs previously identified to be associated with IBH. Although this study comprises a first attempt to associate structural variants in the equine genome with IBH, the associated CNVs potentially represent additional regulators of IBH providing further evidence that IBH is a multifactorial polygenic disease with a complex genomic architecture.

Conclusions

In conclusion, CNVs were identified in a sample of the Friesian horse population and results were comparable to previously published equine CNV research. Characterization and sharing of identified CNVs within various horse breeds, including the Friesian horse breed, contributes to our knowledge on structural variants in horses and enhances our understanding of the equine genome and its phenotypic expression. Results of the association analyses showed that not only do nucleotide differences appear to contribute to IBH sensitivity, but more complex, structural variations like CNVs are also highly likely to be involved. IBH can therefore be considered as a multifactorial polygenic disease with a complex genomic architecture.

Additional files

Additional file 1: Characteristics of the investigated Friesian horses. Characteristics of the investigated cases concerning preventive methods and the observed, seasonality of clinical symptoms. And characteristics of the investigated cases and controls, where mean age, number of males and females, and pedigree of investigated cases, controls and of the total investigated Friesian horse population are presented. (DOCX 13 kb)

Additional file 2: Multidimensional scaling plot of 276 genotyped Friesian horses. Multidimensional scaling plot of 276 genotyped Friesian horses calculated with *cluster* and *mds-plot* commands in PLINK software v1.07 [28, 29] using autosomal SNPs. (DOCX 31 kb)

Additional file 3: CNVs randomly selected based on incidence and size validated through qPCR. CNVR identification, chromosome (ECA), start and end position (in bp) and size of the CNVs in the investigated Friesians horse sample is presented, including information on whether the CNV concerned a private (present in 1 horse) or shared (present in 2 horses; the exact same breakpoints were observed) CNV. The designed primers, state of the CNV and results of the qPCR are given. (DOCX 14 kb)

Additional file 4: Regional association plot (ECA20) of insect bite hypersensitivity in Friesian horses. Significance level based on allele frequency differences between cases ($n = 141$) and controls ($n = 135$) using a χ^2-test (1df). The horizontal red line is the Bonferroni corrected significance level (P-value $= 1.63 \times 10^{-7}$). (DOCX 27 kb)

Additional file 5: SNPs most significantly associated with insect bite hypersensitivity in Friesian horses. SNPs most significantly associated with insect bite hypersensitivity including chromosome, position (in basepairs), SNP name, P-value, odds ratio (OR) and allele frequency in cases and controls. SNPs marked grey passed the Bonferroni corrected significance level (P-value $= 1.63 \times 10^{-7}$). (DOCX 14 kb)

Additional file 6: Chromosomal distribution, characteristics and enrichment of detected CNVs and CNVRs. Number of CNVs and CNVRs detected per *Equus caballus* chromosome (ECA), including detection, state, content, mean size (in base pairs), coverage (in base pairs), chromosomal distribution ($= \frac{number\ of\ CNVs\ per\ chromosome}{total\ number\ of\ CNVs} \times 100\%$), chromosomal coverage ($\frac{CNVR\ coverage\ per\ chromosome}{length\ of\ chromosome} \times 100\%$) and SNP coverage in base pairs ($= \frac{length\ of\ chromosome}{number\ of\ SNPs\ per\ chromosome}$). (DOCX 24 kb)

Additional file 7: 5350 CNVRs detected by CNVRuler based on 15,041 CNVs identified by PennCNV in 222 Friesian horses. 5350 CNVRs detected by CNVRuler based on 15,041 CNVs identified in PennCNV [33–35] in 222 Friesian horses. Information that is presented includes CNVR identification (CNVR_ID), *Equus caballus* chromosome (ECA), start position (in bp), end position (in bp), size (in bp), copy number state, number of horses (N) with CNVR and whether the CNVR includes a gene(s) and is present in 1 horse (private) or more (shared). The gene(s) located within the CNVR were identified using human orthologues. Start and end position of the genes annotated in the horse genome are presented, including *Ensembl* IDs and whether the CNVR is upstream, inside, downstream of the gene or encompasses the gene. (XLSX 603 kb)

Additional file 8: Genome-wide CNV and SNP association plots of insect bite hypersensitivity in Friesian horses. Genome-wide CNV and SNP

association plots of IBH in 222 Friesian horses. The $-\log_{10}$ *P*-value is plotted against the chromosomal location (start position) of CNVs within a specific CNVR and each SNP tested across all chromosomes. The horizontal red line indicates a *P*-value of 0.05 for CNVs within a specific CNVR and the Bonferroni corrected significance level (*P*-value = 1.63×10^{-7}) for SNPs. Transparent vertical bars are included to be able to compare the GWA results. A) CNV association plot based on an analysis taking into account both gains and losses. B) CNV association plot based on an analysis taking into account gains only. C) CNV association plot based on an analysis taking into account losses only. D) SNP association plot, for comparison purposes. (DOCX 147 kb)

Additional file 9: Visualization of individual CNVs within the CNVRs with the lowest *P*-value in the association tests. Visualization of individual CNVs within the CNVR on ECA10:12,948,489-13,075,518 (association test including both gains and losses), ECA20:30,624,048-30,689,273 (association test including gains only) and ECA20:30,743,179-30,775,429 (association test including losses only). Each row represents one horse and the X-axis is the position on the chromosome. The black line marks the location of the CNVR. Blue lines represent controls, red lines cases. A dotted line represents a deletion (state = 0n), a striped line represents a CNV with state equals 1n and a solid line represents a duplication (3n). (DOCX 50 kb)

Additional file 10: Overlap between CNVs within specific CNVRs associated with IBH in Friesian horses and CNV(R)s already published in literature. Overlap between CNVs within specific CNVRs associated with IBH ($n = 19$) and CNV(R)s already published in literature. CNVR identification, chromosome (ECA), start and end position (in bp) and size (in bp) of the CNVR is presented. It is indicated how the CNVR in Friesian horses overlapped with the CNV(R)s already published in literature (classification) and how great the overlap of the CNVR in Friesian horses was with literature (in percentage and bp). (DOCX 16 kb)

Abbreviations
CNV: Copy number variation; CNVR: Copy number variation region; ECA: *Equus caballus*; GWA: Genome-wide association; IBH: Insect bite hypersensitivity; MHC: Major histocompatibility complex; SNP: Single-nucleotide polymorphisms

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Availability of data and materials
The CNVRs identified in this study are available in Additional file 7. Otherwise, the dataset analysed during the current study are not publicly available as owners gave their written consent only to use the data for the current study on IBH.

Authors' contributions
AS, VHS, RPMAC and BJD carried out the identification of CNVRs and genome-wide association study and drafted the manuscript. BWD performed laboratory activities including qPCR and helped to draft the manuscript. AS, BDV, LF, AS, SB, NB, SJ, GL and BJD participated in the design and coordination of the study. BDV, LF, AS, SB, NB and GL helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Hair samples from both cases and controls were collected with a written informed consent of the horse's owner. It was considered that there was no need for an Animal Care and Ethics Committee approval according to the Dutch law after consultation with the Animal Experiment Expert from Wageningen University & Research. The Animal Welfare Officer thought it was not an animal experiment as referred to in the Dutch Act on Animal Experiments.

Consent for publication
Not applicable.

Competing interests
Richard PMA Crooijmans is a Deputy Section Editor for BMC Genetics. As for the rest, there are no competing interests.

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Additional file 1 – Characteristics of the investigated Friesian horses

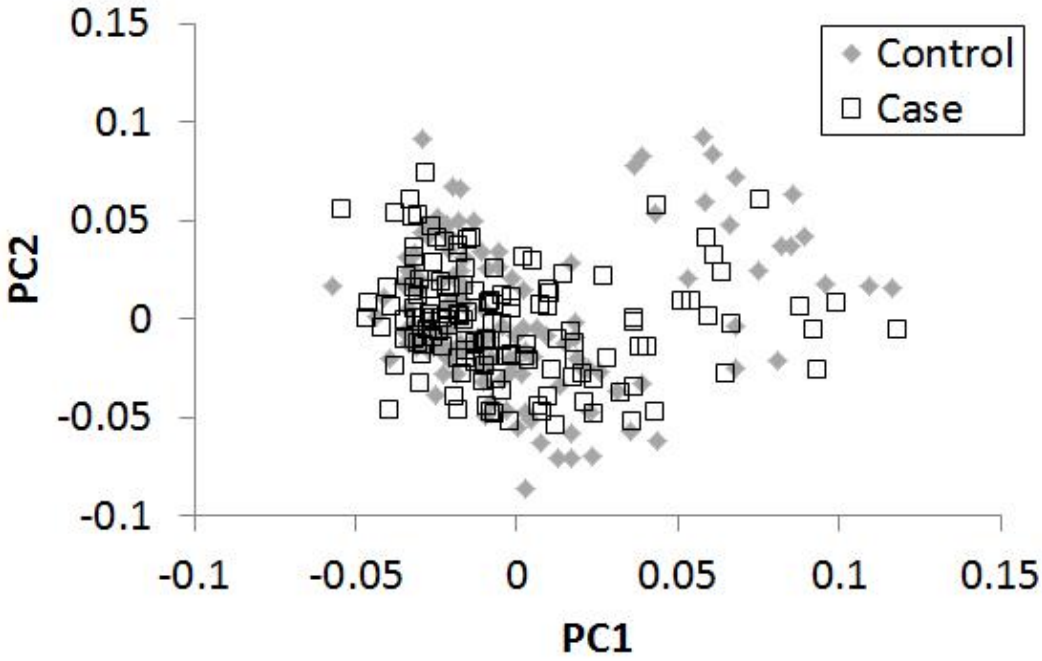
Characteristics of the investigated cases concerning preventive methods and the observed, seasonality of clinical symptoms.

	Percentage
Preventive measures applied	
Yes	92.9%
No	7.1%
Most commonly applied preventive measures	
Eczema blanket	81.7%
Application oil/cream	48.1%
Anti-insect spray	31.3%
Applied measures successful	
Yes	77.9%
No	22.1%
Number of seasons affected	
1 season	9.2%
≥2 seasons	90.8%
Appearance symptoms	
Spring	71.6%
Summer	26.2%
Other	2.2%
Regression of symptoms	
Fall	48.2%
Winter	50.4%
Other	1.4%
Severity of symptoms over years	
Remains equal	62.0%
Differs per year	17.1%
Increases	14.0%
Other	6.9%
Severity of clinical symptoms	
Mild	62.7%
Moderate	24.5%
Severe	12.8%
Most commonly observed symptoms	
Itch	91.1%
Scaling	57.4%
Hair loss	56.4%
Most commonly affected locations	
Mane	91.2%
Tail	76.5%
Head	38.2%
Ears	32.4%

Characteristics of the investigated cases and controls. Mean age, number of males and females, and pedigree of investigated cases, controls and of the total investigated Friesian horse population are presented.

Trait	Case	Control	Total
Number of horses	142	138	280
Age, years			
Mean (SD)	11.9 (5.3)	11.8 (5.0)	11.8 (5.1)
Range	3 – 26	4 – 24	3 – 26
Sex			
Female	114	116	230
Male	28	22	50
Pedigree			
Number of sires	82	71	113
Number of dams	132	128	241

Additional file 2 – Multidimensional scaling plot of 276 genotyped Friesian horses
Multidimensional scaling plot of 276 genotyped Friesian horses calculated with *cluster* and *mds-plot* commands in PLINK software v1.07 [28, 29] using autosomal SNPs.



Additional file 3 – CNVs randomly selected based on incidence and size validated through qPCR

CNVR identification, chromosome (ECA), start and end position (in bp) and size of the CNVs in the investigated Friesians horse sample is presented, including information on whether the CNV concerned a private (present in 1 horse) or shared (present in 2 horses; the exact same breakpoints were observed) CNV. The designed primers, state of the CNV and results of the qPCR are given.

CNVR ID	ECA	Start	End	Size	Frequency[1]	Selection[2]	State[3]	FORWARD primer	REVERSE primer	qPCR validation
CNVR_455	2	6,604,846	6,633,079	28,234	1	Private	3n	CTCATCCTTCCCGATTCAAG	TGACTGAAATTGGTGGGTCA	state and copy number validated
CNVR_549	2	57,911,942	58,295,805	383,864	1	Private	3n	ACCACATGGGATCTGGGTTA	AGCTGAGGATGGATGAATGG	not validated, 1n
CNVR_1313	4	85,131,970	85,133,058	1,089	1	Private	3n	CAGGCAGAAGAAAAGCAACC	CCATCTCCCATGACTCCATT	not validated, 2n
CNVR_1474	5	42,938,000	42,974,425	36,426	1	Private	3n	GAGGAGGAGGATGTGCTGAG	GCGGCCTATCATGTCTCATT	not validated, 1n
CNVR_1906	7	31,308,295	31,322,017	13,723	2	Shared	1n	GCTGCATCAATGAGTGAGGA	ACACATACGTCCATGCCTGA	not validated, 3n
CNVR_2278	9	21,363,205	21,374,842	11,638	1	Private	1n	CATAGGCCCCTGTTTCTTGA	CTGAGGACATTGCCTTCCAT	state and copy number validated
CNVR_2742_1	12	12,628,967	12,638,616	9,650	2	Shared	3n	GTGTATGGGCTCCTGTGGTT	AGTGAGGAGGATGGAGCTGA	not validated, 2n
CNVR_2947	14	50,371,500	50,444,641	73,142	2	Shared	3n	CATACGCTTGTCCACCACAC	CCACTCTGGTGAGGGATGTT	state and copy number validated
CNVR_3021	14	76,161,461	76,177,420	15,960	2	Shared	3n	ACCAACACTCCCTCCATCAG	AGACCGCAAAGGCTGTAGAA	state (gain) validated, 5n
CNVR_3214	15	65,900,256	65,921,640	21,385	1	Private	0n	TGAATTGGACTTGGGGAGAG	TTGCCAGGGTCTGGTTCTAC	state and copy number validated
CNVR_3332	16	46,179,071	46,180,799	1,729	2	Shared	3n	GGCGACAGTTTCCAAGGTTA	TGTACAGCAAGGCTCCTGAA	state (gain) validated, 4n
CNVR_4758	26	2,838,859	2,851,887	13,029	2	Shared	0n	CCCTCAAGGCACCACTAGAA	GCAGCATCCATACTCCCTGT	state (loss) validated, 1n

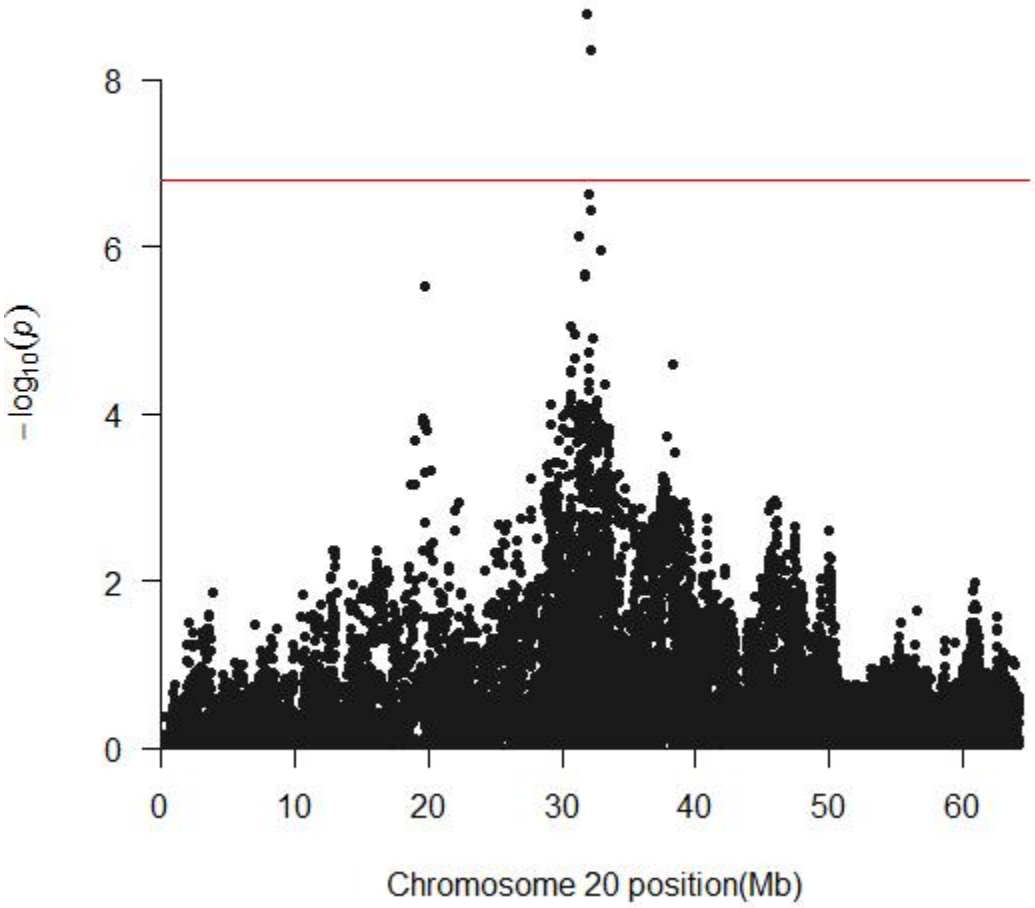
[1]Frequency is the frequency of CNVs concerning the private and shared CNVs.

[2]CNV was present in 1 horse (private) or 2 horses (shared; exact same breakpoints).

[3]0n = deletion of two copies (null), 1n = deletion of one copy (A,B), 3n = single copy duplication (AAA,AAB,ABB,BBB).

Additional file 4 – Regional association plot (ECA20) of insect bite hypersensitivity in Friesian horses

Significance level based on allele frequency differences between cases (n = 141) and controls (n = 135) using a χ^2-test (1df). The horizontal red line is the Bonferroni corrected significance level (P-value = 1.63×10^{-7}).



Additional file 5 - SNPs most significantly associated with insect bite hypersensitivity in Friesian horses

SNPs most significantly associated with insect bite hypersensitivity including chromosome, position (in basepairs), SNP name, *P*-value, odds ratio (OR) and allele frequency in cases and controls. SNPs marked grey passed the Bonferroni corrected significance level (*P*-value = 1.63×10^{-7}).

					Allele frequency	
ECA[a]	Position (bp)	SNP name	*P*-value[b]	OR[c]	Cases	Controls
2	105,754,941	AX-103202148[d,e]	2.33×10^{-05}	$2.359^{1.613-3.449}$	0.409	0.227
9	67,850,426	AX-104986198	2.35×10^{-05}	$0.336^{0.206-0.550}$	0.093	0.233
9	68,034,922	AX-104179213	2.36×10^{-05}	$0.354^{0.222-0.565}$	0.106	0.252
9	68,079,384	AX-103384306	1.13×10^{-05}	$0.303^{0.180-0.512}$	0.080	0.224
9	68,250,373	AX-104536698	2.36×10^{-05}	$0.354^{0.222-0.565}$	0.106	0.252
9	68,299,439	AX-103728993	2.24×10^{-05}	$0.356^{0.224-0.566}$	0.110	0.258
20	19,706,668	AX-104183365	3.04×10^{-06}	$5.437^{2.600-11.37}$	0.164	0.035
20	30,658,219	AX-103232956[e]	8.99×10^{-06}	$2.395^{1.657-3.460}$	0.461	0.263
20	31,013,680	AX-104729989[e]	1.14×10^{-05}	$2.298^{1.613-3.274}$	0.519	0.319
20	31,042,472	AX-102963215[e]	2.11×10^{-05}	$2.368^{1.620-3.460}$	0.449	0.256
20	31,245,645	AX-103894624	7.41×10^{-07}	$2.621^{1.819-3.776}$	0.464	0.248
20	31,652,366	AX-103664602	2.19×10^{-06}	$2.420^{1.707-3.430}$	0.518	0.307
20	31,655,194	AX-103592064	2.13×10^{-06}	$2.419^{1.707-3.427}$	0.518	0.307
20	31,935,993	AX-104352898[d,e]	1.65×10^{-09}	$3.865^{2.516-5.938}$	0.406	0.150
20	32,060,910	AX-103144182	1.86×10^{-05}	$0.449^{0.317-0.637}$	0.317	0.508
20	32,075,365	AX-104028000[d,e]	2.37×10^{-07}	$2.983^{2.000-4.448}$	0.408	0.188
20	32,150,140	AX-103022378[d,e]	3.66×10^{-07}	$2.965^{1.979-4.443}$	0.385	0.174
20	32,179,520	AX-104642494[d,e]	4.42×10^{-09}	$4.377^{2.681-7.145}$	0.321	0.098
20	32,290,762	AX-103805671	1.24×10^{-05}	$0.427^{0.296-0.614}$	0.286	0.484
20	32,941,949	AX-104318994[e]	1.11×10^{-06}	$0.400^{0.282-0.569}$	0.308	0.527

[a]*Equus caballus* chromosome.

[b]*P*-value corrected for Genomic Control.

[c]Odds ratio of the allele substitution effect of the minor allele within the total population with 95% confidence interval in superscript.

[d]SNP quality is considered low based on its cluster plot.

[e]*P*-value <0.05 for testing Hardy-Weinberg Equilibrium.

Additional file 6 – Chromosomal distribution, characteristics and enrichment of detected CNVs and CNVRs

Number of CNVs and CNVRs detected per *Equus caballus* chromosome (ECA), including detection, state, content, mean size (in base pairs), coverage (in base pairs), chromosomal distribution ($= \frac{number\ of\ CNVs\ per\ chromosome}{total\ number\ of\ CNVs} \times 100\%$), chromosomal coverage

	Chromosome characteristics			CNV characteristics		CNVR characteristics										
							Detection[1]		State			Content[2]				
ECA[3]	Length (bp)[4]	SNPs	SNP coverage (bp)	Number	Distribution (%)	Number	Shared	Private	Gain	Loss	Complex	Genic	Intergenic	Mean size (bp)	Coverage (bp)	Coverage (%)
1	185,838,109	61,449	3,024	1,097	7.3	452	150	302	432	14	6	244	208	47,801	21,605,974	11.6
2	120,857,687	52,366	2,308	736	4.9	367	128	239	351	7	9	152	215	35,142	12,896,969	10.7
3	119,479,920	26,730	4,470	626	4.2	284	110	174	268	9	7	126	158	51,833	14,720,499	12.3
4	108,569,075	24,670	4,401	751	5.0	289	109	180	278	5	6	163	126	55,862	16,144,118	14.9
5	99,680,356	23,166	4,303	613	4.1	225	89	136	210	3	12	151	74	53,593	12,058,519	12.1
6	84,719,076	45,517	1,861	515	3.4	236	60	176	228	5	3	112	124	32,662	7,708,322	9.1
7	98,542,428	22,233	4,432	616	4.1	197	86	111	181	9	7	119	78	56,351	11,101,068	11.3
8	94,057,673	20,926	4,495	617	4.1	192	74	118	179	3	10	92	100	53,946	10,357,714	11.0
9	83,561,422	19,272	4,336	472	3.1	214	83	131	202	7	5	89	125	46,469	9,944,316	11.9
10	83,980,604	19,970	4,205	413	2.7	197	74	123	184	7	6	99	98	47,075	9,273,687	11.0
11	61,308,211	14,029	4,370	185	1.2	92	31	61	87	4	1	65	27	48,163	4,430,979	7.2
12	33,091,231	8,787	3,766	1,091	7.2	80	29	51	65	5	10	49	31	62,517	5,001,346	15.1
13	42,578,167	10,388	4,099	199	1.3	56	28	28	51	2	3	40	16	62,621	3,506,783	8.2
14	93,904,894	21,712	4,325	490	3.3	214	83	131	212	0	2	98	116	51,999	11127767	11.9
15	91,571,448	21,091	4,342	371	2.5	200	72	128	193	3	4	94	106	43,054	8,610,802	9.4
16	87,365,405	20,257	4,313	276	1.8	162	48	114	156	3	3	80	82	35,129	5,690,941	6.5
17	80,757,907	18,576	4,347	715	4.7	264	121	143	257	2	5	84	180	49,673	13,113,694	16.2
18	82,527,541	18,767	4,397	549	3.6	233	80	153	220	4	9	120	113	48,520	11,305,251	13.7
19	59,975,221	14,259	4,206	403	2.7	156	56	100	154	1	1	67	89	43,403	6,770,897	11.3
20	64,166,202	37,029	1,733	1,724	11.5	184	65	119	154	6	24	84	100	31,034	5,710,226	8.9
21	57,723,302	13,496	4,277	365	2.4	151	63	88	146	2	3	58	93	43,259	6,532,039	11.3
22	49,946,797	11,707	4,266	160	1.1	84	18	66	79	2	3	41	43	32,746	2,750,674	5.5
23	55,726,280	12,737	4,375	355	2.4	119	49	70	117	0	2	61	58	59,755	7,110,823	12.8
24	46,749,900	10,834	4,315	287	1.9	95	35	60	84	4	7	47	48	52,543	4,991,545	10.7
25	39,536,964	9,320	4,242	270	1.8	66	26	40	58	2	6	48	18	53,314	3,518,751	8.9
26	41,866,177	10,036	4,172	288	1.9	135	50	85	131	2	2	54	81	49,487	6,680,781	16.0
27	39,960,074	9,632	4,149	263	1.8	111	40	71	106	2	3	38	73	48,734	5,409,432	13.5
28	46,177,339	10,675	4,326	241	1.6	119	40	79	114	2	3	63	56	45,319	5,392,948	11.7
29	33,672,925	8,362	4,027	175	1.2	59	18	41	54	0	5	31	28	47,727	2815910	8.4
30	30,062,385	7,507	4,005	93	0.6	68	18	50	67	0	1	38	30	37,748	2566878	8.5
31	24,984,650	6,415	3,895	85	0.6	49	16	33	48	0	1	24	25	35,832	1,755,771	7.0
Total	2,242,939,370	611,915		15,041		5,350	1,949	3,401	5,066	115	169	2,631	2,719		250,605,424	

$= \frac{CNVR\ coverage\ per\ chromosome}{length\ of\ chromosome} \times 100\%$) and SNP coverage in base pairs ($= \frac{length\ of\ chromosome}{number\ of\ SNPs\ per\ chromosome}$).

[1]When a CNVR was detected in 2 or more horses, it was considered shared; when a CNVR was detected in 1 horse only, it was considered private.

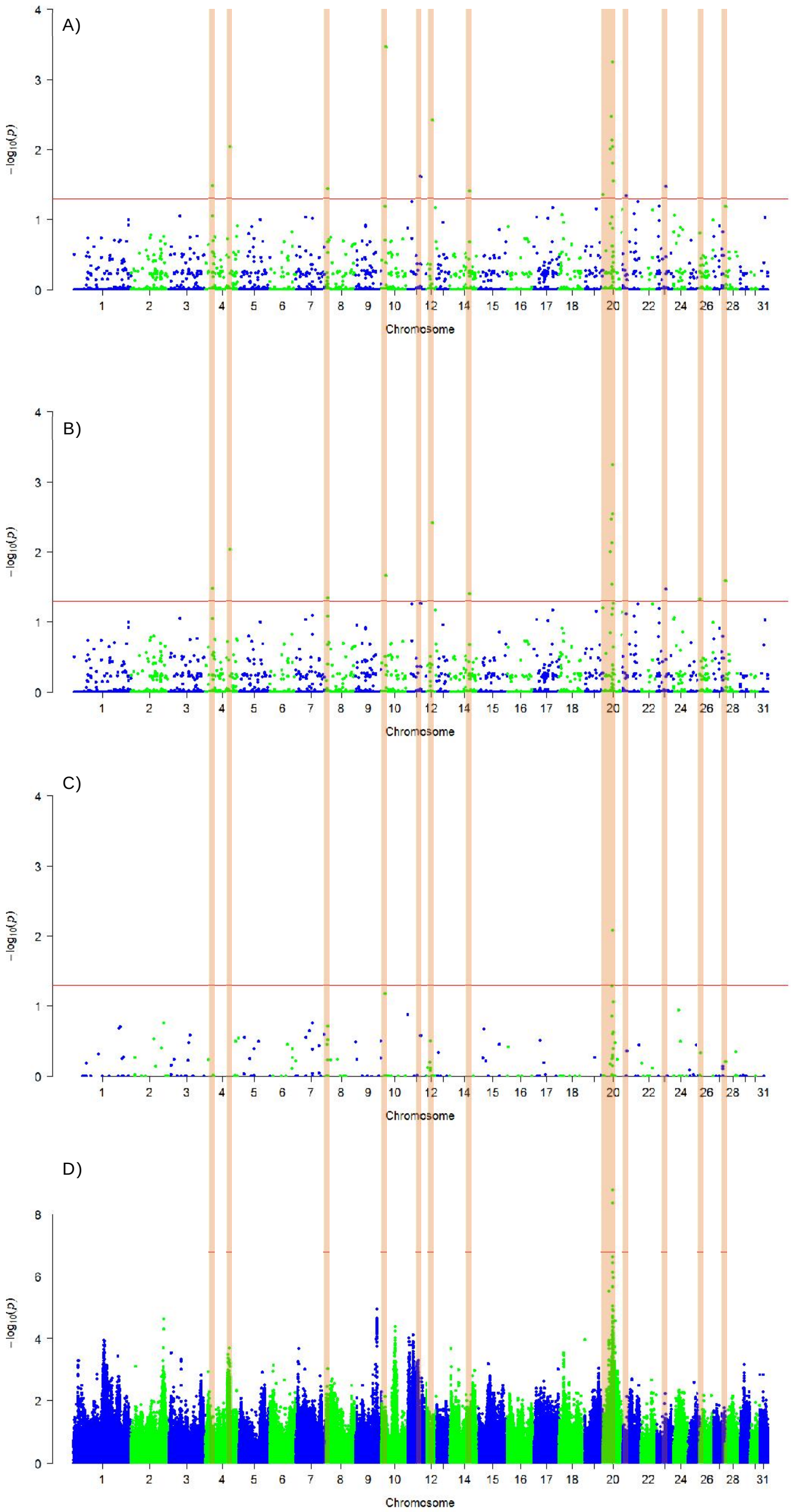
[2]A CNVR was considered genic when at least a part of the CNVR and gene overlapped.

[3]*Equus caballus* chromosome.

[4]Length of chromosomes in basepairs was retrieved from *Ensembl* (http://www.ensembl.org/Equus_caballus/Info/Index).

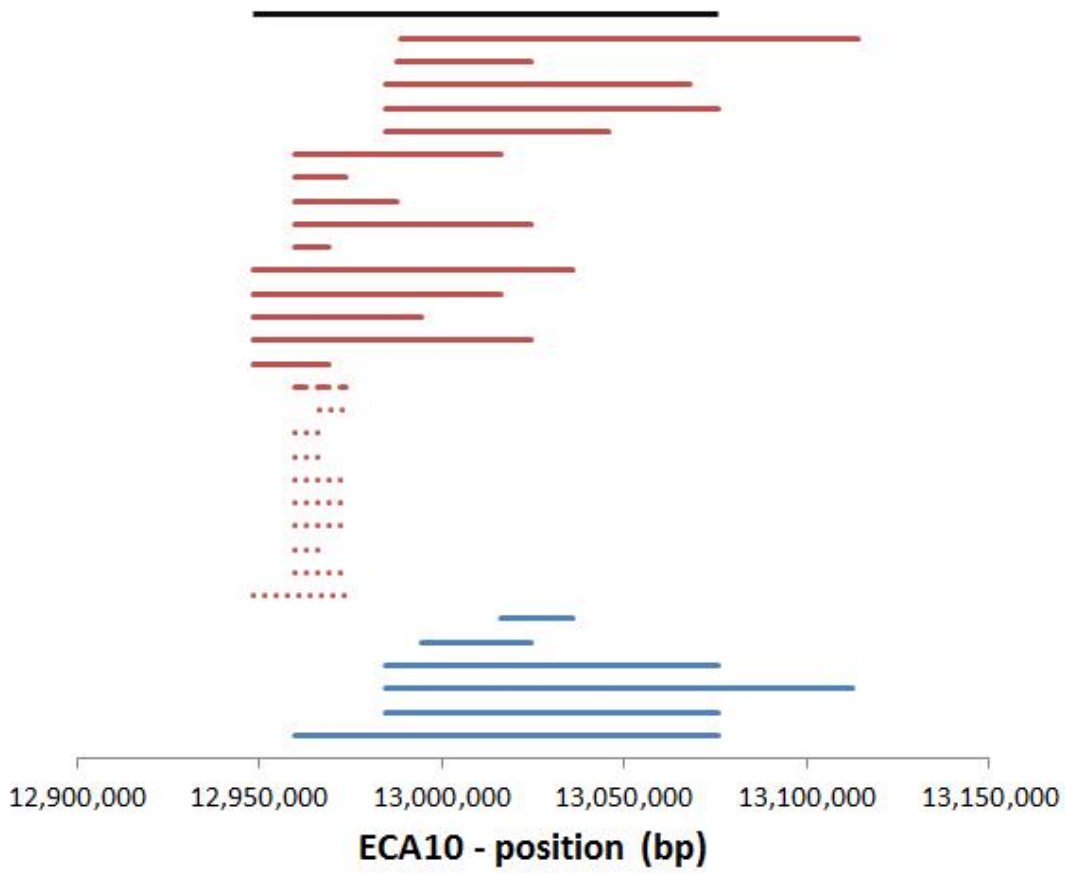
Additional file 8 – Genome-wide CNV and SNP association plots of insect bite hypersensitivity in Friesian horses

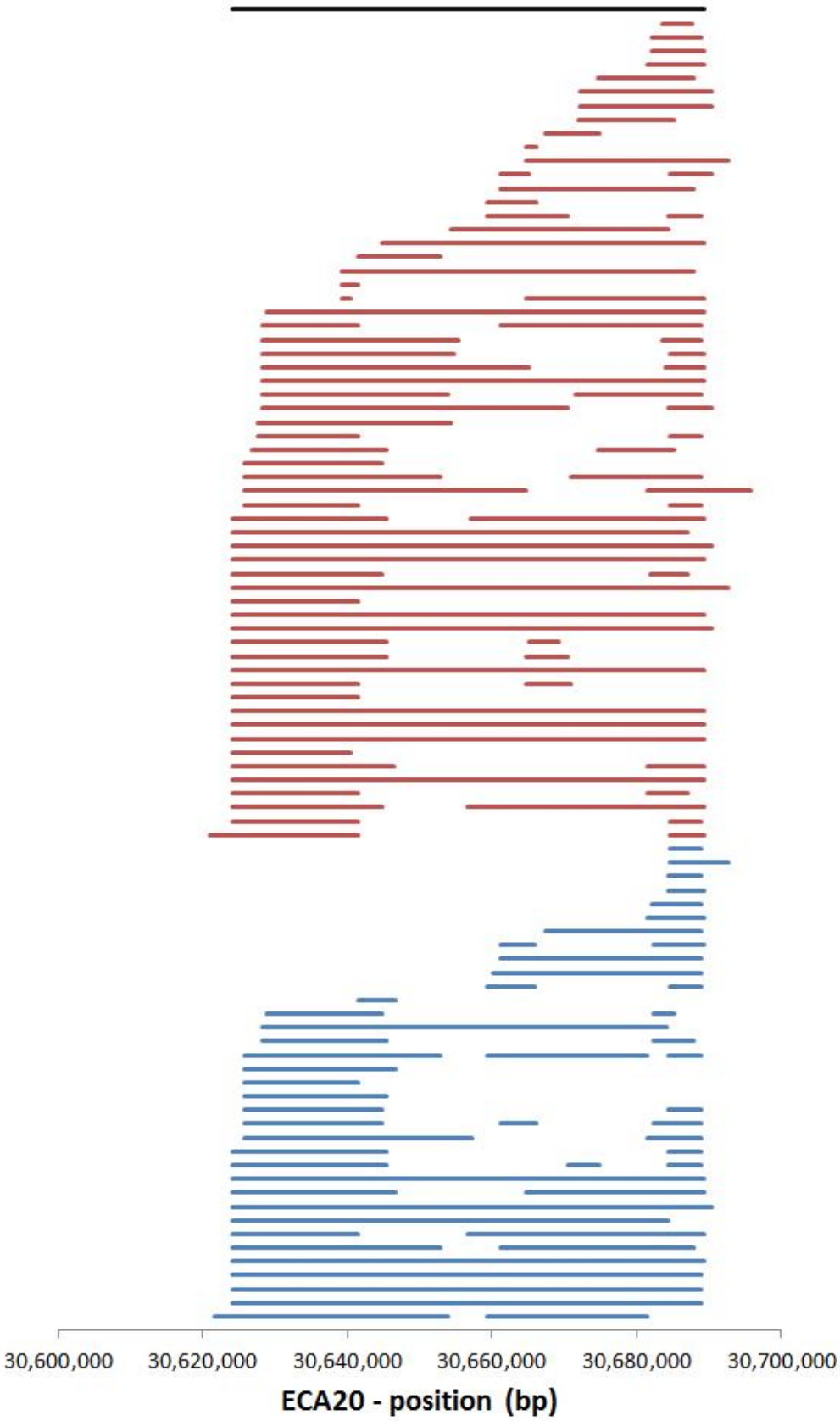
Genome-wide CNV and SNP association plots of IBH in 222 Friesian horses. The $-\log_{10}$ *P*-value is plotted against the chromosomal location (start position) of CNVs within a specific CNVR and each SNP tested across all chromosomes. The horizontal red line indicates a *P*-value of 0.05 for CNVs within a specific CNVR and the Bonferroni corrected significance level (*P*-value = 1.63×10^{-7}) for SNPs. Transparent vertical bars are included to be able to compare the GWA results. A) CNV association plot based on an analysis taking into account both gains and losses. B) CNV association plot based on an analysis taking into account gains only. C) CNV association plot based on an analysis taking into account losses only. D) SNP association plot, for comparison purposes.

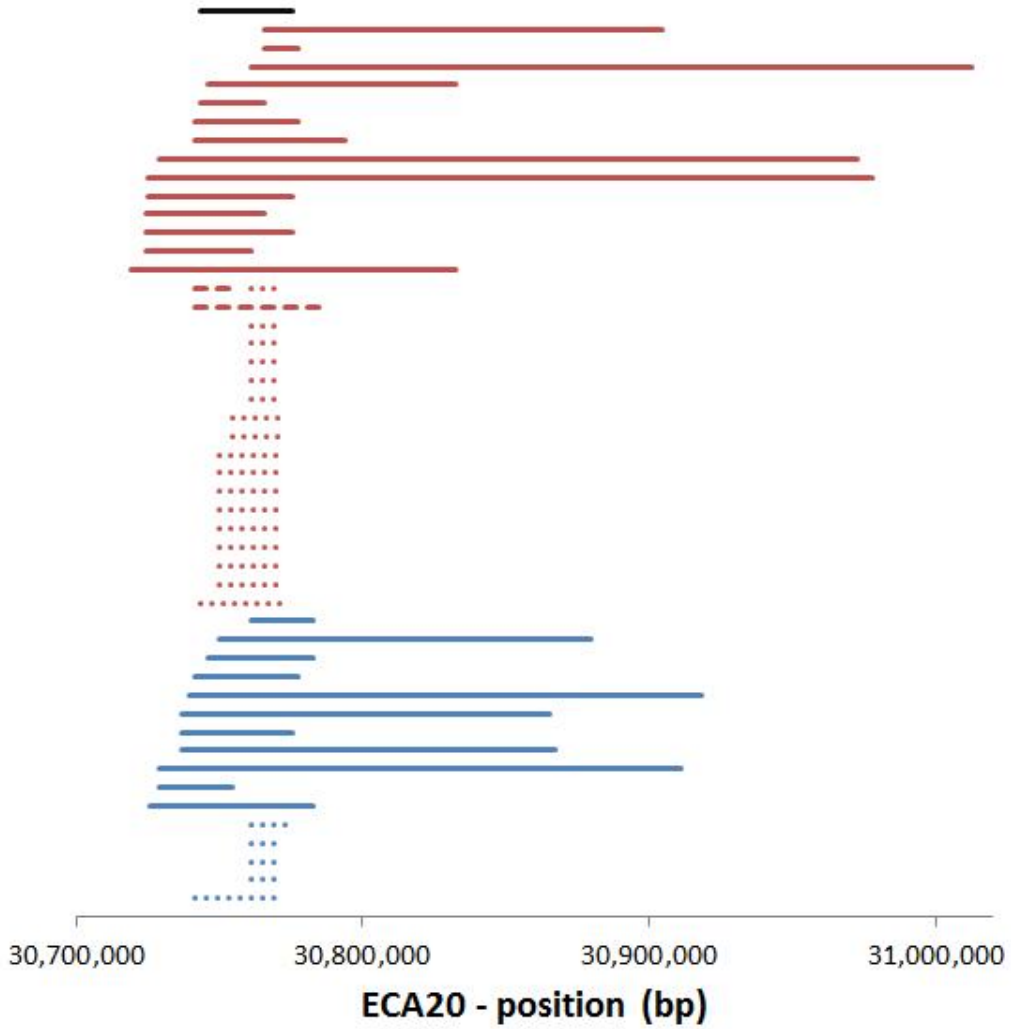

A)
B)
C)
D)
$-\log_{10}(p)$
Chromosome
1 2 3 4 5 6 7 8 9 10 12 14 15 16 17 18 20 22 24 26 28 31

Additional file 9 – Visualization of individual CNVs within the CNVRs with the lowest *P*-value in the association tests

Visualization of individual CNVs within the CNVR on ECA10:12,948,489-13,075,518 (association test including both gains and losses), ECA20:30,624,048-30,689,273 (association test including gains only) and ECA20:30,743,179-30,775,429 (association test including losses only). Each row represents one horse and the X-axis is the position on the chromosome. The black line marks the location of the CNVR. Blue lines represent controls, red lines cases. A dotted line represents a deletion (state = 0n), a striped line represents a CNV with state equals 1n and a solid line represents a duplication (3n).




30,600,000
30,620,000
30,640,000
30,660,000
30,680,000
30,700,000
ECA20 - position (bp)


30,700,000
30,800,000
30,900,000
31,000,000
ECA20 - position (bp)

Additional file 10 – Overlap between CNVs within specific CNVRs associated with IBH in Friesian horses and CNV(R)s already published in literature

Overlap between CNVs within specific CNVRs associated with IBH (n = 19) and CNV(R)s already published in literature. CNVR identification, chromosome (ECA), start and end position (in bp) and size (in bp) of the CNVR is presented. It is indicated how the CNVR in Friesian horses overlapped with the CNV(R)s already published in literature (classification) and how great the overlap of the CNVR in Friesian horses was with literature (in percentage and bp).

CNVR ID	ECA	Start	End	Size	Classification[1]	Overlap, %[2]	Overlap, bp
CNVR_1144_1	4	23,383,774	23,505,444	121,671	Encompass[c,d,f,g]	100.0%	121,671
CNVR_1296_1	4	79,687,222	79,957,793	270,572	Encompass[f]	100.0%	270,572
CNVR_2035_1	8	3,638,239	3,783,874	145,636	Encompass[a,b,c,d,f,g,h]	100.0%	145,636
					Downstream[e]	32.8%	47,729
CNVR_2452_1	10	12,948,489	13,075,518	127,030	Encompass[a,b,c,d,g,h]	100.0%	127,030
CNVR_2685_1	11	41,743,465	41,832,225	88,761	Encompass[a,b,c,f,g,h]	100.0%	88,761
					Downstream[h]	0.9%	770
CNVR_2758_1	12	19,366,263	19,527,441	161,179	Encompass[a,b,c,d,e,f,h]	100.0%	161,179
					Upstream[e]	0.6%	924
CNVR_2979_1	14	64,375,388	64,516,633	141,246	Encompass[a,c,f,g]	100.0%	141,246
CNVR_4066	20	20,271	503,862	483,592	Novel CNVR		
CNVR_4114_1	20	24,230,088	24,296,409	66,322	Encompass[a,c,d,e,f,h]	100.0%	66,322
					Inside[e,h]	2.1%[h]	1,401
						12.4%[h]	8,201
						12.7%[h]	8,439
						53.1%[e]	35,186
					Downstream[e,h]	64.3%[e]	42,632
						67.0%[h]	44,419
						94.2%[h]	62,456
CNVR_4120_1	20	26,392,472	26,531,248	138,777	Encompass[a,b,c,d,f,h]	100.0%	138,777
					Inside[e,h]	1.0%[h]	1,401
						1.3%[h]	1,751
						33.7%[h]	46,700

						37.1%[e]	51,512
						38.6%[e]	53,626
						51.6%[h]	71,635
						65.0%[e]	90,197
						87.5%[h]	121,484
					Upstream[e]	82.1%	113,946
						88.0%	122,098
CNVR_4132_1	20	29,805,544	29,821,078	15,535	Novel CNVR		
CNVR_4140_1	20	30,493,889	30,523,455	29,567	Encompass[a,b,d,e,g,h]	100.0%	29,567
					Inside[f]	2.9%	860
					Upstream[e]	97.1%	28,709
CNVR_4141_1	20	30,624,048	30,689,273	65,226	Encompass[a,b,d,f,g,h]	100.0%	65,226
					Inside[h]	38.6%	25,160
					Upstream[h]	29.0%	18,904
CNVR_4142_1	20	30,743,179	30,775,429	32,251	Encompass[a,b,d,f,g,h]	100.0%	32,251
CNVR_4155_1	20	32,467,872	32,582,288	114,417	Encompass[f]	100.0%	114,417
					Upstream[a,b,c,d,g,h]	98.2%	112,330
CNVR_4271_1	21	10,784,937	10,861,217	76,281	Downstream[a,b]	50.2%	38,274
CNVR_4537	23	32,331,580	32,716,916	385,337	Encompass[g,h]	100.0%	385,337
CNVR_4747_1	26	250,337	434,632	184,296	Encompass[f]	100.0%	184,296
					Inside[h]	1.8%	3,401
						8.6%	15,903
					Upstream[c,h]	6.8%[h]	12,503
						8.6%[h]	15,903
						58.4%[c,h]	107,621
CNVR_4992_1	28	4,428	376,070	371,643	Encompass[b,g,h]	100.0%	371,643
					Inside	0.5%[h]	1,751
						0.9%[h]	3,451
						6.7%[h]	24,898
						25.9%[e]	96,427
					Upstream[h]	74.6%	277,430
					Downstream[h]	16.4%	61,010
						24.5%	91,107

[1]Encompass = CNVR in Friesian horses is fully captured by the region presented in literature, inside = the region presented in literature is fully captured by the CNVR in Friesian horses, upstream = CNVR in Friesian horses starts upstream of the region presented in literature and ends upstream of the region presented in literature, downstream = CNVR in Friesian horses starts downstream of the region presented in literature and ends downstream of the region presented in literature. Reference to literature: [a]Doan and colleagues [3]; [b]Doan and colleagues [4]; [c]Dupuis and colleagues [5]; [d]Ghosh and colleagues [6]; [e]Ghosh and colleagues [7]; [f]McQueen and colleagues [8]; [g]Metzger and colleagues [9]; [h]Wang and colleagues [11].

[2]The percentage of CNVR in Friesian horses that overlaps with the region presented in literature.