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Original study

The equine *DNAH3* gene: SNP discovery and exclusion of an involvement in recurrent airway obstruction (RAO) in European Warmblood horses

Mostafa Shakhsi-Niaei[1], Jolanta Klukowska-Rötzler[1], Cord Drögemüller[1], Vincent Gerber[2] and Tosso Leeb[1]

[1]Institute of Genetics, Vetsuisse Faculty, University of Bern, Bern, Switzerland, [2]Swiss Institute of Equine Medicine, Vetsuisse Faculty, University of Bern and ALP-Haras, Bern, Switzerland

Abstract

Recurrent airway obstruction is one of the most common airway diseases affecting mature horses. Increased bronchoalveolar mucus, neutrophil accumulation in airways, and airway obstruction are the main features of this disease. Mucociliary clearance is a key component of pulmonary defense mechanisms. Cilia are the motile part of this system and a complex linear array of dynein motors is responsible for their motility by moving along the microtubules in the axonemes of cilia and flagella. We previously detected a QTL for RAO on ECA 13 in a half-sib family of European Warmblood horses. The gene encoding *DNAH3* is located in the peak of the detected QTL and encodes a dynein subunit. Therefore, we analysed this gene as a positional and functional candidate gene for RAO. In a mutation analysis of all 62 exons we detected 53 new polymorphisms including 7 non-synonymous variants. We performed an association study using 38 polymorphisms in a cohort of 422 animals. However, after correction for multiple testing we did not detect a significant association of any of these polymorphisms with RAO ($P>0.05$). Therefore, it seems unlikely that variants at the *DNAH3* gene are responsible for the RAO QTL in European Warmblood horses.

Keywords: horse; allergy; recurrent airway obstruction; dynein; association

Abbreviations: ATP: adenosine-5'-triphosphate; DNAH: dynein heavy chain; ECA: equine chromosome; HOARSI: horse owner assessed respiratory signs index; PCD: primary ciliary dyskinesia; QTL: quantitative trait locus; RAO: recurrent airway obstruction; SNP: single nucleotide polymorphism; T_m: melting temperature

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Corresponding author:
Tosso Leeb; email: tosso.leeb@vetsuisse.unibe.ch
Vetsuisse Faculty, Institute of Genetics, University of Bern, Bremgartenstrasse 109a, 3001 Bern, Switzerland

Introduction

RAO or heaves is one of the most common airway diseases affecting mature horses. RAO is characterised by bronchospasms and increased mucus and neutrophil accumulation in the airways. It occurs following exposure of susceptible horses to antigens and endotoxins present in hay and stable dust (Cunningham & Dunkel 2008, Léguillette 2003). Because of many similarities of this disease to human asthma it is used as an animal model of human asthma (Deaton 2006, Leclere *et al.* 2011).

The prevalence of RAO is estimated at 10-20 % of adult horses living in cold and temperate climates. There is no breed or sex predisposition for RAO (Leclere *et al.* 2011). RAO is a complex trait controlled by environmental and genetic factors. In a Warmblood population there was evidence for the presence of one or more major genes and the heritability in a study cohort exposed to hay feeding was estimated to approach 100 %. Thus hay-feeding may be the only significant environmental factor influencing this disease (Gerber *et al.* 2009).

Mucociliary clearance is a key component of pulmonary defense mechanisms. It involves the regulation of ion transport by the airway epithelium, mucus secretion, and ciliary function (Knowles & Boucher 2002). Cilia and flagella contain motile microtubules with a »9+2«structure, in which two central singlet microtubules are surrounded by nine outer doublet microtubules. A complex linear array of dynein motors is responsible for their motility (Brokaw 2009, Hayashi & Shingyogi 2008).

Cytoplasmic and axonemal dyneins are involved in the cytoplasmatic movement of organelles and the bending of cilia and flagella, respectively (Bartoloni *et al.* 2001). The DNAH proteins assemble with intermediate and light chains into large multiprotein complexes to form inner and outer dynein arms (Holzbaur & Vallee 1994, DiBella & King 2001). The inner and outer dynein arms slide on the outer doublet microtubules by hydrolysing ATP.

Defects in the motile cilia are responsible for the most prominent ciliopathy in mammals, called primary ciliary dyskinesia. One aspect of PCD is respiratory disease due to impaired mucociliary clearance. Additional features of PCD are sperm immobility and randomisation of left-right asymmetry (Afzelius 1976, Rossman *et al.* 1980). The different features of PCD are now clearly explained by mutations involving various subunits of the axonemal structures (Lee 2011).

Due to their importance for mucociliary clearance the genes encoding dynein subunits can be considered functional candidates for RAO. In a previous microsatellite-based linkage study we detected a QTL for RAO in a half-sibling family of European Warmblood horses with a peak of about 4 Mb extending from 24-28 Mb on ECA 13 (Swinburne *et al.* 2009). The *DNAH3* gene expressed in trachea, testis and brain (Maiti *et al.* 2000) is located on ECA 13 at 26.4-26.6 Mb in the peak region of the detected QTL. Therefore, we analysed the equine *DNAH3* gene as a positional and functional candidate gene and performed an association study in a cohort of European Warmblood horses.

Material and methods

Animals and phenotypes

For variant detection we selected two RAO-affected and two non-affected half-sibling offspring of a previously described European Warmblood family, in which the QTL on ECA 13 was detected (Swinburne *et al.* 2009). We selected these four horses based on their phenotypes and marker haplotypes at the QTL.

For the association analysis we used 464 European Warmblood horses that were unrelated at the grandparental level. More than 60 % (281) of the sampled horses were from Switzerland and Germany. Additional horses were from Belgium, the Czech Republic, Denmark, France, Hungary, Ireland, Latvia, The Netherlands, Poland, Portugal, Russia, Slovakia, and Sweden. Cases and controls were matched according to countries in order to minimise the stratification. A multidimensional scaling plot of genome-wide SNP data from these horses did not result in different clusters between cases and controls (data not shown).

We determined the phenotypes in our sample population according to the »Horse Owner Assessed Respiratory Signs Index« (Ramseyer *et al.* 2007, Laumen *et al.* 2010). The HOARSI gives scores from 1-4, which correspond to absent, mild, moderate and severe clinical signs respectively. Briefly, horse owners were contacted by phone and only horses with clinical signs that had persisted for at least 2 months were included in the study. All horses were 5 years or older, with at least a 12-month history of hay-feeding. A standardised questionnaire was used to gather information from the horse owners about the animals' history of chronic, regular or frequent coughing, increased breathing effort at rest, increased breathing effort after exercise, and nasal discharge. The HOARSI classification refers to the period when the horses were exhibiting the most severe clinical signs. We selected horses with HOARSI 1 as controls and horses with HOARSI 3 or 4 as RAO cases. We used an overlapping set of horses in a related study (Shakhsi-Niaei *et al.* 2012).

Mutation analysis and genotyping

We amplified all 62 *DNAH3* exons and their flanking regions in 4 horses (Table 1). The PCR products were directly sequenced on an ABI 3730 sequencer (LifeTechnologies, Carlsbad, CA, USA) and polymorphisms were identified using Sequencher software v4.9. (GeneCodes, Ann Arbor, MI, USA). The newly identified variants were submitted to the Single Nucleotide Polymorphism Database (dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/).

For the association study we determined the genotypes of 35 SNPs by using Golden Gate assays on a BeadXpress station (Illumina Inc., San Diego, CA, USA) followed by data analysis with the BeadStudio v3 software (Illumina Inc, San Diego, CA, USA). The genotypes of 4 additional SNPs in the region were available from equine SNP50 Beadchip data (Illumina Inc, San Diego, CA, USA). All genomic positions refer to the EquCab 2 assembly (http://www.ncbi.nlm.nih.gov/projects/mapview/). The equine cDNA and protein positions refer to the database accessions XM_001491853.3 and XP_001491903.2, respectively.

Table 1
Primer sequences. The positions correspond to chromsome 13 in the *Equus caballus* 2 genome assembly.

	Start	Stop	Exon	Primer	Forward Sequence (5'>3')	Reverse Sequence (5'>3')	T_m	Product, bp
1	26.419.946	26.420.804	1	DNAH3_P1	TGCATTTAGGCCTCTCCAA	CCTTCACACCCACTTCCAA	59.43	859
2	26.424.250	26.425.322	2+3	DNAH3_P2+3	TGGGGATGAGAAGGGTGTT	ACAGCCAGGACCAGAATCC	55.16	1 073
3	26.427.888	26.428.853	4+5	DNAH3_P4+5	TCCCCTTAGTGTGCTCCTG	GCTGCCATTCTGCAACCTA	59.39	966
4	26.431.430	26.432.444	6	DNAH3_P6	TCTGGTGTCATGGGGAAAA	TGACCTACTGCGTGCACAA	59.96	1 015
5	26.432.363	26.433.609	7	DNAH3_P7	AGCTCTTTGATGGTGATGGA	GCAGGGCTACTCCCACAGT	59.14	1 247
6	26.436.353	26.437.412	8	DNAH3_P8	AGCCATCAGGGGTAGGTCT	GGATTTCTCTGGCTTTGGA	58.17	1 060
7	26.438.081	26.438.589	9	DNAH3_P9	ATGAGCCATCTGCCTTGTC	AACGACTTCTCAGCTTCTGC	58.09	509
8	26.439.899	26.440.415	10	DNAH3_P10	AGCCATACCCAGCCCTTAT	AGATGCACGGCCATATTTT	58.27	517
9	26.440.671	26.441.693	11	DNAH3_P11	GCCCAAGCTCTTTCCAGT	TCATGCTGACGTTTTTCCA	58.09	1 023
10	26.442.941	26.443.713	12	DNAH3_P12	GGAAGTGGGGAGAGGAAGT	TGCTGCCTTTTCTTATCATGT	57.81	773
11	26.445.159	26.446.002	13+14	DNAH3_P13+14	TTCAGTGTATGTTTTGCCACA	ATTGCCTCAGGATGCTTTC	57.75	844
12	26.450.454	26.451.432	15	DNAH3_P15	CCCATCAAGGATTTTCAGG	GGTAGAGGGCACAGCTCAT	57.87	979
13	26.451.267	26.452.213	16	DNAH3_P16	GCTCTCCACAAGCCAAGTT	CAGTGAAGCAGGCTGAAAA	57.85	947
14	26.460.129	26.460.774	17	DNAH3_P17	AATGTGACTGGGCTTTCGT	GCTGACTTGCCTCAAAGGT	58.08	646
15	26.460.362	26.461.431	18	DNAH3_P18	TTGGTTCTGGTCCTTGGTT	TCCCCACTTTCCATCCTTA	57.94	1 070
16	26.470.588	26.471.110	19	DNAH3_P19	GCTCAGGGAATTACAGCTACA	CTGGCTGGCACAATACTCA	57.76	523
17	26.474.688	26.475.249	20	DNAH3_P20	CAAGTGCTGGTGGGAGAC	CACCCAAAGTGAAGGTTCC	57.79	562
18	26.478.447	26.478.714	21	DNAH3_P21	TGTCTCCTCCCGTTGAGTT	GGGACCCTTTCTCTGTGAG	57.69	268
19	26.485.549	26.485.919	22	DNAH3_P22	GGGTGGAGATCTGAGCTGT	TGCCCTTACTTGGATGGTT	57.86	371
20	26.486.494	26.486.969	23	DNAH3_P23	ATCAGTGGGGCTGTGATG	CCTCTAGGGAGGTGGGATT	57.93	476
21	26.487.920	26.488.405	24	DNAH3_P24	TGGCTCAGTTGGTTTCAGA	GTCTCTTGGGGAATGGAGA	57.71	486
22	26.491.118	26.491.979	25	DNAH3_P25	CAACCTCTATGCCCCAGAC	CGGCAGGAGTGGAAGTAAT	57.92	862
23	26.492.393	26.492.909	26	DNAH3_P26	TGTCATTTGCCTCCTGAGA	GCCCCAAGATTTACAAGGA	57.70	517
24	26.494.125	26.494.769	27	DNAH3_P27	TAAGCCCAGACGTGTCAAG	ATTGGAGGAGGAGGAGAGG	57.79	645
25	26.497.108	26.498.098	28	DNAH3_P28	GCTAATGCCTGGAGGTCAG	GGGTGTGTGAGGAGGAAAG	58.22	991
26	26.499.486	26.499.957	29	DNAH3_P29	AGTGAGAGGTGGCCTTCTG	AGTAAGAGCCCTGCTTGGA	58.01	506
27	26.501.026	26.501.613	30+31	DNAH3_P30+31	CCAGTTTCTTTCCCGTGTC	CCTGTCATTGGGGAGCTG	59.16	588
28	26.501.601	26.502.619	32	DNAH3_P32	CCCCAATGACAGGGTCTTA	TGTTGGGAAAGTGGTTGTG	57.67	1 019
29	26.503.380	26.504.117	33	DNAH3_P33	AGCCCTCCTTTCCTTGATT	ACTCTTGGTGCGTGTGATG	57.99	738
30	26.505.289	26.506.431	34+35	DNAH3_P34+35	CAAATGATAGGGCTTCTCCA	TTCCTGACTCCAGCAAACA	57.43	1 143
31	26.507.364	26.507.813	36	DNAH3_P36	TAGTTGGGTGCAGAGCAAA	AGAATCCAGGGACAGCAAA	58.10	450

Table 1 continued
Primer sequences. The positions correspond to chromsome 13 in the *Equus caballus* 2 genome assembly.

	Start	Stop	Exon	Primer	Forward Sequence (5'>3')	Reverse Sequence (5'>3')	T_m	Product, bp
32	26.509.133	26.509.688	37	DNAH3_P37	TGTTGGCTGGTCTTTGAGA	AGCAACAAGGCTTTTAACCA	57.72	556
33	26.512.163	26.512.417	38	DNAH3_P38	TCAGACCATCTCTGTCTCCAC	GAGCTGTCCCTCCTGCTTA	57.94	255
34	26.514.273	26.514.847	39	DNAH3_P39	CTGGTCCTGGAGTTGGTGT	GTGAGGCAATGGAGAAAGG	58.36	575
35	26.516.444	26.516.835	40	DNAH3_P40	CCTATGGGCCGTACAATTC	TCATGGGAGGAAAAACAGG	58.19	392
36	26.519.054	26.519.787	41	DNAH3_P41	GGCCTCTCACAACAAAACC	CAAACGGCTTAGGGACATT	57.90	734
37	26.531.911	26.532.668	42	DNAH3_P42	CTGACAACATGGAGTTTCACC	AATGGGCAGGAGTTGTAGG	57.82	758
38	26.534.404	26.535.138	43	DNAH3_P43	TGCCAAGACATAGCCTCCT	ACACCACAATCCCCTTCTG	58.37	735
39	26.536.286	26.536.965	44	DNAH3_P44	TGCCAATCTGTGCATCTTT	TGGAGGCTTTCTGTTCCTC	57.85	680
40	26.541.797	26.542.677	45+46+47	DNAH3_P45+46+47	GGATAACTAGGCCCAAGAGG	AACGGAACCCGAAATCTG	58.13	881
41	26.543.437	26.544.439	48	DNAH3_P48	GAATGTGCTCAGGGGAAAC	TGGAGAGGGTTCAGAGGAA	58.17	1 003
42	26.545.331	26.545.711	49	DNAH3_P49	CACTTCCCAGGCACTCAC	TGGAATATTTGGTTGGGGTA	57.64	381
43	26.547.068	26.547.557	50	DNAH3_P50	CACCTCCCCAGTTCTTCTC	GTCTCATTTGGTGCCCTCT	57.40	490
44	26.551.417	26.551.713	51	DNAH3_P51	TCATTTCTGCTCTTGCCAAT	GTTCCCTCCCTTTGCTCTC	58.63	297
45	26.556.761	26.557.283	52	DNAH3_P52	CCAGTTAGAGCCACCCTTG	ACATCCTGGTGCCATTCTT	58.11	523
46	26.560.227	26.561.280	53	DNAH3_P53.1	GGCTTTACGTCTCGACTGG	TGCTGCTTGAATGTGGACT	58.18	1 054
47	26.559.366	26.560.614	53	DNAH3_P53.2	GCTCCTGTTGCCTTCTCC	TCTGCTCGCTTCTGGTTC	58.06	1 249
48	26.560.466	26.561.379	53	DNAH3_P53.3	TCTGCCTGTGAGGGTCTGT	GGGAGGTAATGTGGGTTCC	59.22	914
49	26.561.162	26.562.138	53	DNAH3_P53.4	TATGTGAGGACGCTGGAAA	ATCAGGCCTTCGAGTTTTG	57.82	977
50	26.561.940	26.562.481	53	DNAH3_P53.5	AGCTGCTTTTCTCCCTCCT	TCCACCCATTCTGGACTTT	58.06	542
51	26.566.835	26.567.681	54	DNAH3_P54	TCAGAGCAAAAGAGGAATCAA	TCACCTCCCTCCAACAACT	57.87	847
52	26.570.239	26.570.543	55	DNAH3_P55	GGGTCTGTTGTTCCTCCAG	GATCTCTCCCCCTGCAAC	58.05	305
53	26.571.932	26.572.318	56	DNAH3_P56	ATTCATGGGGCTGAAAGAG	CCAGCACAGTAACCAGCAT	57.46	387
54	26.575.939	26.576.594	57	DNAH3_P57	GTAGAGCTCCTTGGCTGGA	CCCCTTTTTCATCCTTCCT	58.07	656
55	26.576.497	26.577.249	58	DNAH3_P58	TTCAGAAGGTGCCAGAAGG	TGGAAAACTGCATGGAAAA	58.30	753
56	26.578.965	26.579.435	59	DNAH3_P59	TTCACTGCTTGAAGTTTACCC	TACGGTTAGCCTGTGCTTG	57.27	471
57	26.579.711	26.580.619	60	DNAH3_P60	ATCTGGAGCAGCAAGAACC	TCCCCTAACTCTCGGAAGA	57.67	909
58	26.581.215	26.582.035	61	DNAH3_P61	CAGCTTGTACCTGGGCTTT	TTGGTATGATGGTGGCAGA	58.19	821
59	26.582.915	26.583.900	62	DNAH3_P62	GCATCTGACTCTAACCCCACT	ACCCAAACCTCTTTCAACCT	57.94	986

Protein analysis

In order to analyse the evolutionary conservation of the non-synonymous variants, we prepared multi-species alignments of the *DNAH3* protein sequences using the ClustalW2 software (European Bioinformatics Institute, Cambridge, UK; http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Association analysis

We removed individuals and markers with less than 90 % call rate with the Plink software (Purcell *et al.* 2007). We also excluded markers with a minor allele frequency of less than 0.05 and markers that strongly deviated from Hardy-Weinberg equilibrium ($P<0.001$). Subsequently, we performed an allelic association study using chi-square tests. We also performed haplotypic association analyses using sliding windows of 3 and 7 markers, respectively. We corrected for multiple testing by applying the Bonferroni correction.

Results and discussion

Mutation analysis of the DNAH3 gene

We sequenced 34 156 nucleotides containing all 62 exons with 12 255 nucleotides of coding sequence of the *DNAH3* gene in two RAO affected and two control horses. These four horses were half-sibling offspring of the European Warmblood family, in which the QTL on ECA 13 was originally detected. We identified a total of 53 new polymorphisms including 7 non-synonymous variants in addition to 4 previously known SNPs in the region (Table 2). Protein sequence alignment showed that 4 of these 7 non-synonymous variants led to amino acid exchanges in highly conserved positions (p.Gln1227Arg, p.Met2357Val, p.Gln2665Arg, p.Ser3033Cys; Figure 1). These variants are located either in or very close to functionally annotated domains of the *DNAH3* protein (Table 3). It has to be cautioned that the 5'-end of the predicted horse *DNAH3* transcript (XM_001491853.3) corresponding to the first exon is very different from the human ortholog. Thus there may be an error in the equine genome assembly or the annotation of the equine *DNAH3* gene. Such a hypothetical error might alter the positions of the identified variants by about 30 amino acids. The human-horse relationships are given in Table 3.

Association analysis of DNAH3 variants with RAO

For the association study we genotyped 39 SNPs including all 13 exonic variants in a cohort of 464 animals. We removed one marker due to low minor allele frequency and 42 horses due to low call rates, so that 422 horses (230 cases & 192 controls) and 38 markers remained for the final allelic association analysis (Table 2). All of the tested markers were in Hardy-Weinberg equilibrium and had similar allele distributions between cases and controls with raw *P*-values greater than 5 %. As 38 markers were tested, a Bonferroni-corrected significance threshold would have been $0.05 / 38 = 0.0013$. We also did not detect a significant haplotype association.

Table 2
Polymorphisms of the equine *DNAH3* gene and association data

SNP	*DNAH3* Position	ECA 13 Position	Function	Allele 1	Frequency Allele 1 Cases (n = 230)	Frequency Allele 1 Controls (n =192)	Allele 2	Chi-square	*P*-value	Odds Ratio
c.-263C>T	5'-UTR	26 420 431	-	C	0.400	0.466	T	3.706	0.054	0.764
c.-206T>A	5'-UTR	26 420 488	-	T	0.446	0.396	A	2.169	0.141	1.230
c.135+46C>G	intron 2	26 424 538	-		-	-		-	-	-
c.135+238T>C	intron 2	26 424 730	-	C	0.469	0.486	T	0.212	0.645	0.936
c.135+336C>T	intron 2	26 424 828	-		-	-		-	-	-
c.135+388T>C	intron 2	26 424 880	-		-	-		-	-	-
c.243T>C	exon 3	26 425 126	p.=	T	0.074	0.091	C	0.828	0.363	0.796
c.431+11A>G	intron 4	26 428 077	-	G	0.465	0.479	A	0.167	0.683	0.945
c.431+169C>A	intron 4	26 428 235	-		-	-		-	-	-
BIEC2-226005	intron 5	26 429 354	-	C	0.454	0.466	T	0.129	0.720	0.951
BIEC2-226006	intron 5	26 429 577	-	T	0.454	0.466	G	0.129	0.720	0.951
c.992+3309A>T	intron 7	26 436 824	-	A	0.450	0.477	T	0.594	0.441	0.899
c.1033A>G	exon 8	26 436 881	p.Lys345Glu	G	0.352	0.397	A	1.787	0.181	0.825
c.1494+58A>G	intron 10	26 440 284	-		-	-		-	-	-
c.14942+936C>T	intron 10	26 441 163	-	T	0.448	0.474	C	0.569	0.451	0.900
c.1531G>A	exon 11	26 441 265	p.Val511Ile	A	0.450	0.477	G	0.603	0.438	0.898
c.1824+121C>T	intron 13	26 445 527	-	C	0.441	0.448	T	0.037	0.847	0.974
c.2424+558A>G	intron 17	26 460 868	-	G	0.091	0.096	A	0.063	0.802	0.942
c.2781+168T>C	intron 19	26 471 046	-		-	-		-	-	-
c.2988+12T>G	intron 21	26 478 616	-	T	0.067	0.073	G	0.098	0.754	0.919
c.3285+49T>C	intron 23	26 486 786	-	T	0.194	0.181	C	0.226	0.635	1.088
c.3624+52G>A	intron 25	26 491 543	-		-	-		-	-	-
c.3624+85C>T	intron 25	26 491 576	-		-	-		-	-	-
c.3624+124T>C	intron 25	26 491 615	-		-	-		-	-	-
c.3624+272G>A	intron 25	26 491 763	-		-	-		-	-	-
c.3624+306T>C	intron 25	26 491 797	-		-	-		-	-	-
c.3624+1110G>A	intron 25	26 492 602	-	A	0.363	0.375	G	0.127	0.721	0.950
c.3680A>G	exon 26	26 492 718	p.Gln1227Arg	A	0.312	0.307	G	0.024	0.877	1.023
c.4142+60T>C	intron 29	26 499 841	-	T	0.480	0.453	C	0.627	0.429	1.116
c.4272A>G	exon 31	26 501 439	p.=	A	0.435	0.430	G	0.022	0.882	1.021
c.4634+314A>G	intron 33	26 503 913	-		-	-		-	-	-
c.4815C>T	exon 34	26 505 595	p.=	C	0.117	0.079	T	3.297	0.069	1.542
c.4854+525A>T	intron 34	26 506 159	-	T	0.117	0.083	A	2.653	0.103	1.463
c.4989+1139T>C	intron 35	26 507 511	-	T	0.298	0.302	C	0.018	0.893	0.980
c.5101+1509C>T	intron 36	26 509 216	-	T	0.137	0.104	C	2.100	0.147	1.365
c.5160T>C	exon 37	26 509 281	p.=	T	0.117	0.081	C	3.020	0.082	1.506
c.5343+22A>G	intron 37	26 509 486	-	-	-	-	-	-	-	-
c.5444+1973T>C	intron 38	26 514 321	-	-	-	-	-	-	-	-
c.5444+1983T>C	intron 38	26 514 331	-	-	-	-	-	-	-	-
c.5550+17T>C	intron 38	26 514 536	-	T	0.132	0.115	C	0.514	0.473	1.164
c.5550+141C>T	intron 38	26 514 660	-		-	-		-	-	-
BIEC2-226016	intron 39	26 516 372	-	T	0.309	0.308	G	0.001	0.977	1.004
BIEC2-226017	intron 39	26 516 538	-	T	0.309	0.308	C	0.001	0.977	1.004
c.5644G>A	exon 40	26 516 727	p.Ala1882Thr	G	0.306	0.310	A	0.017	0.895	0.980
c.5926+53T>A	intron 41	26 519 441	-	T	0.493	0.489	A	0.014	0.906	1.017
c.5926+270T>C	intron 41	26 519 658	-		-	-		-	-	-
c.6030+79G>C	intron 42	26 532 238	-	G	0.011	0.008	C	-	-	-

Table 2 continued
Polymorphisms of the equine *DNAH3* gene and association data

SNP	*DNAH3* Position	ECA 13 Position	Function	Allele 1	Frequency Allele 1 Cases (n = 230)	Frequency Allele 1 Controls (n =192)	Allele 2	Chi-square	*P*-value	Odds Ratio
c.6120T>C	exon 43	26 534 479	p.=	T	0.298	0.310	C	0.149	0.700	0.943
c.6505+25T>G	intron 44	26 536 729	-	G	0.454	0.442	T	0.126	0.723	1.051
c.6786+39A>G	intron 46	26 542 273	-		-	-		-	-	-
c.7069A>G	exon 48	26 543 688	p.Met2357Val	G	0.135	0.115	A	0.729	0.393	1.197
c.7994A>G	exon 51	26 551 513	p.Gln2665Arg	G	0.100	0.096	A	0.031	0.859	1.042
c.8362+2699G>A	intron 52	26 559 821	-	G	0.093	0.083	A	0.266	0.606	1.134
c.9098C>G	exon 53	26 561 025	p.Ser3033Cys	G	0.092	0.079	C	0.430	0.512	1.178
c.10245G>A	exon 53	26 562 172	p.=	G	0.093	0.083	A	0.266	0.606	1.134
c.11247+235A>G	intron 57	26 576 428	-	A	0.300	0.304	G	0.017	0.898	0.981
c.11558+725T>C	intron 59	26 579 915	-	T	0.222	0.208	C	0.222	0.637	1.083

```
Species          p.K345E  p.V511I  p.Q1227R p.A1882T p.M2357V p.Q2665R p.S3033C

Horse wildtype   ELLKGKL  FTAVDQQ  ASMQKVI  IHLAFMM  TVVMEYY  AVMQVEL  DSFSIDN
Horse mutated    ...E...  ...I...  ...R...  ...T...  ...V...  ...R...  ...C...
Human            .L.....  ...AAAD  ...RE..  .....S.  .....H.  ....R..  .......
Mouse            D.....   SFIFLYL  ...RQ..  .....S.  .......  .......  ....V..
Dog              ...G...  ...I---  ...RE..  V....S.  .......  .......  .......
Platypus         ...AST.  -------  E.IRH..  V...VS.  .T.....  V...K..  ....S..
Chicken          .ICG...  -------  ..VRQ.L  IH.TYSL  .A...S.  .E..K..  .......
Fish             S..SAD.  -------  M.IRA..  ....YSL  .E..DF.  ....Q..  .L..T..
```

Figure 1
Multiple species alignment of the *DNAH3* protein sequence at the sites of non-synonymous variants in the horse. Four of the seven variants lead to amino acid exchanges in highly conserved positions.

Table 3
Protein domain assignment of non-synonymous variants

Variants	Corresponding human position	Domain	Remark	Region
p.Lys345Glu	375	Stem		1..1390
p.Gln1227Arg	1 259	Stem		1..1390
p.Ala1882Thr	1 914	--	Between the AAA 2 and AAA 3 domains	1904..2035
p.Met2357Val	2 389	--	Between the AAA 3 and AAA 4 domains	2285..2394
p.Gln2665Arg	2 697	Stalk		2661..2960
p.Ser3033Cys	3 065	AAA 5		3045..3275

The numbering refers to the equine protein accession XP_001491903.2. The predicted equine protein differs at its N-terminus from the human protein, possibly due to an annotation error in the horse genome reference sequence. The predicted equine protein shows high homology to its human ortholog starting from amino acid 11 in horse and amino acid 41 in human. The domain positions correspond to the human *DNAH3* protein from the UniProtKB/Swiss-Prot accession Q8TD57.

In previous association studies for RAO on ECA 13, we found the strongest association for the marker BIEC2-224511 (Shakhsi-Niaei *et al.* 2012). This marker has a raw *P*-value of 0.015 in the 422 horses of this study and thus shows an almost four-fold stronger association than the best-associated marker from the *DNAH3* gene. BIEC2-224511 is located at position 24 309 405, approximately 2 Mb away from the *DNAH3* gene. The best associated marker in the *DNAH3* gene (c.-263C>T) is at the left boundary of the *DNAH3* gene and thus also physically closest to BIEC2-224511 of all tested markers (Table 2).

The lack of association of the tested *DNAH3* markers indicates that variants at the *DNAH3* gene are most likely not responsible for the RAO QTL in European Warmblood horses.

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