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A region on equine chromosome 13 is linked to recurrent airway obstruction in horses

U. JOST, J. KLUKOWSKA-RÖTZLER, G. DOLF†, J. E. SWINBURNE‡, A. RAMSEYER, M. BUGNO§, D. BURGER#, S. BLOTT‡ and V. GERBER*

Equine Clinic, Department of Veterinary Clinical Studies, and †Institute of Genetics, University of Berne, Vetsuisse-Faculty Berne, Länggass-strasse 124, CH-3012 Berne, Switzerland; ‡Animal Health Trust, Newmarket, Suffolk CB8 7UU, UK; §Department of Immunology and Cytogenetics, National Research Institute of Animal Production, Balice, Poland; and #National Stud, Avenches, Switzerland.

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For further information and explanation of terminology see www.evj.co.uk/glossary

Summary

Reasons for study: **Equine recurrent airway obstruction (RAO) is probably dependent on a complex interaction of genetic and environmental factors and shares many characteristic features with human asthma. Interleukin 4 receptor α chain (*IL4RA*) is a candidate gene because of its role in the development of human asthma, confirmation of this association is therefore required.**

Methods: **The equine BAC clone containing the *IL4RA* gene was localised to ECA13q13 by the FISH method. Microsatellite markers in this region were investigated for possible association and linkage with RAO in 2 large Warmblood halfsib families. Based on a history of clinical signs (coughing, nasal discharge, abnormal breathing and poor performance), horses were classified in a horse owner assessed respiratory signs index (HOARSI 1–4: from healthy, mild, moderate to severe signs). Four microsatellite markers (*AHT133*, *LEX041*, *VHL47*, *ASB037*) were analysed in the offspring of *Sire 1* (48 unaffected HOARSI 1 vs. 59 affected HOARSI 2–4) and *Sire 2* (35 HOARSI 1 vs. 50 HOARSI 2–4), age ≥7 years.**

Results: **For both sires haplotypes could be established in the order *AHT133-LEX047-VHL47-ASB37*. The distances in this order were estimated to be 2.9, 0.9 and 2.3 centiMorgans, respectively. Haplotype association with mild to severe clinical signs of chronic lower airway disease (HOARSI 2–4) was significant in the offspring of *Sire 1* (P = 0.026) but not significant for the offspring of *Sire 2* (P = 0.32). Linkage analysis showed the ECA13q13 region containing *IL4RA* to be linked to equine chronic lower airway disease in one family (P<0.01), but not in the second family.**

Conclusions: **This supports a genetic background for equine RAO and indicates that *IL4RA* is a candidate gene with possible locus heterogeneity for this disease.**

Potential relevance: **Identification of major genes for RAO may provide a basis for breeding and individual prevention for this important disease.**

Introduction

Equine chronic lower airway disease, in its severe form known as recurrent airway obstruction (RAO) or heaves, is probably a polygenetic disorder (Marti and Harwood 2002) and its evolution is associated with genetic and environmental factors (Marti *et al*. 1991; Ramseyer *et al*. 2007). RAO shares many characteristic features with human asthma (Robinson 2001).

In man, many genes associated with various forms of asthma have been mapped (Malerba and Pignatti 2005). Studies on the human interleukin 4 receptor α chain (*IL4RA)* gene have revealed numerous polymorphisms, some of which are associated with phenotypes related to asthma and atopy (Ober *et al*. 2000a; Youn *et al*. 2000). In preliminary analysis investigating 10 microsatellite markers in the neighbourhood of 10 functional candidate genes in a limited sample of 10 affected and 10 unaffected paternal halfsibs, evidence was found for an association of a marker near *IL4RA* with the RAO-phenotype (U. Jost, unpublished data). Interleukin 4 probably plays an important role in the development of human asthma as it induces isotopic switch to immunoglobulin E (IgE) (Coffman *et al*. 1993) and differentiation of T-helper type 2 (Th2) lymphocytes (Hu-Li *et al*. 1987). Interleukin 4 receptor alpha, a 140 kDa high affinity binding chain, composes the interleukin 4 receptor together with the common γ chain (Idzerda *et al*. 1990; Russell *et al*. 1993) and also serves as the alpha chain of the interleukin 13 receptor (Chomerat and Bancherau 1998). The coding gene for *IL4RA* has been localised to the short arm of chromosome 16 (HSA16p12.1; Pritchard *et al*. 1991) in man, and in horses to chromosome ECA13 by synteny mapping in a somatic cell hybrid panel (Solberg *et al*. 2004).

The goals of the present study were to determine the chromosomal localisation of *IL4RA* by fluorescence *in situ* hybridisation (FISH) and to evaluate associations and linkage of 4 microsatellite markers in the ECA13q13 region harbouring *IL4RA* with a horse owner assessed respiratory signs index and with individual signs of chronic lower airway disease (coughing and nasal discharge) in 2, previously described (Ramseyer *et al*. 2007), high RAO-prevalence Warmblood horse families.

*Author to whom correspondence should be addressed.
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Materials and methods

Horses

Two unrelated RAO-affected Warmblood sires were identified. Both stallions showed obvious clinical signs of respiratory distress (nostril flare, increased abdominal lift or increased respiratory rate) and airway obstruction when stabled in stalls with straw bedding and fed hay; and remission of these signs when stabled in a barn complex especially adapted to the requirements of RAO patients (bedding of dust-free shavings; haylage feeding). Offspring of the affected sires were classified according to a horse owner assessed respiratory signs index (HOARSI 1–4; healthy, mild, moderate and severe clinical signs) based on a history of clinical signs (coughing, nasal discharge, abnormal breathing and poor performance) as described in detail by Ramseyer *et al*. (2007). Cough frequency (absent, occasional, regular, frequent) and nasal discharge (absent or present) were also recorded as separate traits. The classification refers to the period when the horses were exhibiting their most severe disease manifestation. Only horses with clinical signs that had persisted for at least 2 months were included in the study, and all individuals were age ≥7 years with at least a 12 months history of hay-feeding. Clinical examination, comprehensively complemented by TBS and BALF cytology and scoring of mucus accumulation in the trachea as described (Gerber *et al*. 2004), showed that HOARSI 3 and 4 are consistent with the RAO phenotype (69 horses in the 2 families examined; V. Gerber, unpublished data). Blood samples (EDTA 10 ml) from 107 offspring of *Sire 1* and 85 offspring of *Sire 2* were collected for this investigation.

BAC characterisation

An equine genomic BAC clone (CH241-23J08) harbouring *IL4RA* was grown overnight in 500 ml LB medium with 25 mg/ml chloramphenicol at 37°C with shaking. DNA was extracted and purified by alkaline lysis according to the manufacturer's protocol[1]. PCR was performed on BAC DNA with primers AF (AAGCTCCTGCCCTGTTTACTG) and CR (GGACCGCAGCAACCAGAG) (Solberg *et al*. 2004), for exon 12 of *IL4RA* with Multiplex PCR KIT[1] according to the manufacturer's protocol. Amplifications of 1.5 kb PCR product were carried out in a total volume of 50 μl containing 50 ng DNA, 2 μmol/l of each primer, 25 μl of 2 x Qiagen Multiplex PCR Master Mix[1] and 10 μl Q-Solution in a Perkin-Elmer 9700 thermocycler. PCR conditions were as follows: 20 min at 95°C, 35 cycles of 30 s at 95°C, 90 s at 61°C and 60 s at 72°C, and a final extension of 30 min at 60°C. The amplicon was sequenced on 8% denaturing polyacrylamide gels using a LI-COR DNA sequencer model 4200[2].

Fluorescence in situ *hybridisation (FISH)*

Chromosome preparations were obtained after lymphocyte culture and stained applying G-banding technique prior to FISH (Wang and Fedoroff 1972). Chromosomes were identified according to the international domestic horse chromosome banding standard (Bowling *et al*. 1997). Sheared BAC DNA (100 ng) containing *IL4RA* was labelled with biotin-16-dUTP by nick translation and hybridised to the G-banded horse metaphase chromosome preparations. After denaturation, an overnight hybridisation at 37°C was carried out. Signals were amplified and detected using avidin-FITC and anti-avidin. Chromosome staining was performed with propidium iodide. Slides were analysed using an Axiophot fluorescence microscope[3] equipped with a digital CCD camera, driven by the computer aided software Lucia[4].

Genotyping

Genomic DNA was obtained from peripheral blood using a High Pure PCR Template Preparation Kit[5]. Equine genomic DNA was amplified using the primers presented in Table 1. PCR was carried out in a total volume of 12 μl containing 20 ng DNA, 2.5 pmol of each primer, 0.25 mmol/l of each dNTP, 1 x PCR buffer containing 1.5 mmol/l $MgCl_2$ and 0.35 units *Taq* polymerase[1] in a Perkin-Elmer 9700 thermocycler. PCR was performed using a touchdown program (Don *et al*. 1991). After initial denaturation for 5 min at 94°C, the annealing temperature was lowered 1°C at every 30 s cycle in touch-down range indicated in Table 1, followed by an additional 8 cycles of 30 s at the lowest annealing temperature. In each cycle the extension lasted 30 s at 72°C, while the final extension lasted 15 min at 72°C. Allele sizes were determined on 8% denaturing polyacrylamide gels using a LI-COR DNA sequencer model 4200[6] and were analysed using GeneImagIR 4.05 software.

Haplotyping

In order to test associations with HOARSI and individual clinical signs, paternal haplotypes were constructed by genotyping

TABLE 1: Equine PCR primer conditions and DNA markers used in this study

Locus	Accession number	Repeat type	Alleles (n)*	PCR product size (bp) *	Primer sequences 5'→3'	Annealing temperature *	References
VHL47	X86449	$(CA)_{13}$	4	122–140	F - GTTTGCTGTGGTTACCAGGCAGA R - GCAAATTGAATATTTGAAGTTGAGAC	TD 63–55	van Haeringen *et al.* (1998)
ASB37	AF004767	$(CA)_{20}$	6	122–136	F - CCTGCAACTTTTTCCCAGCC R - GGCAGATGTTAGCTCATGGC	TD 68–60	Lindgren *et al.* (1998)
LEX041	AF075643	$(TG)_{12}$	7	143–161	F - TATTTTCTGAATGCTTCTGTGC R - CTCTACACCCAATGCCTGAT	TD 63–55	Coogle *et al.* (1997)
AHT133	DQ381466	$(GT)_{17}$	8	188–210	F - CCAGTCAGCTCCTGTGAGGC R - TCCCTGGACACCCCACTGTG	TD 63–55	J.E. Swinburne, unpublished data

* Data from present study; TD = touchdown.

the 4 microsatellite markers *AHT133*, *LEX041*, *VHL047* and *ASB037*. *LEX041* and *ASB037* were reported to be syntenic with the *IL4RA* gene on equine chromosome 13 (Solberg *et al*. 2004). *VHL047* has been located distal to *LEX041* and *ASB037* by linkage analyses (Penedo *et al*. 2005). *AHT133* is a microsatellite developed from the BAC clone (CH241-23J08) harbouring *IL4RA*. The haplotypes of the sires were reconstructed based on their offspring where the paternal alleles could be identified unambiguously. Paternal haplotypes were assigned to the remaining offspring so that recombinational events were kept at a minimum. The nonpaternal alleles in the offspring were considered to constitute the maternal haplotype.

The order of the 4 microsatellites was established by linkage analysis using CRI-MAP (Green *et al*. 1990). Using χ^2 tests and Fisher's exact test, haplotype frequencies, except those with recombination, in offspring of *Sire 1* and *Sire 2* were compared grouped by HOARSI, coughing frequency and absence/presence of nasal discharge.

Linkage mapping

To determine whether the microsatellite markers on ECA13 were linked to chronic lower airway disease, halfsib regression interval mapping was performed using the QTL Express software (Knott *et al*. 1996; Seaton *et al*. 2002). Three phenotypic traits were included in the analysis: HOARSI, frequency of coughing, and nasal discharge. Chromosome-wise significance thresholds for the resulting F statistic of the traits were obtained using 1000 random permutations of the data in QTL Express.

Results

Physical mapping

The equine genomic clone containing the *IL4RA* gene was assigned to ECA13q13 (Fig 1), which is in accordance with the horse/human comparative map (HSA16p12.1) (Chowdhary *et al*. 2003).

Genetic mapping

The 2 sires with the dams and their offspring formed a single halfsib family (because 4 of the dams had offspring by both stallions) comprising a total of 388 animals. As the genotypes of the dams were unknown, only the male recombination rates were estimated. The order *AHT133 - LEX041 - VHL047 - ASB037* was 12 times more likely than the next best order. The distances between the microsatellites in this order were estimated at 2.9 cM - 0.9 cM - 2.3 cM.

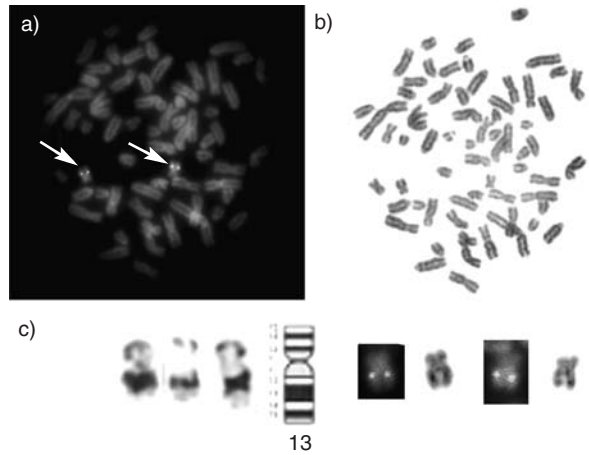

Fig 1: Chromosomal assignment of the equine BAC CH241-23J08 containing IL4RA *by FISH (a) double signals (indicated by arrows) visible on both chromosomes ECA13q13 (b) GTG-banded horse chromosome (c) comparison of standard G-banded chromosome 13 (left), standard idiogram of ECA13 (centre), and G-banded ECA13 showing fluorescent signal (right)*

Haplotype reconstruction

Paternal haplotypes of *Sire 1* could be identified unambiguously in 57 of the 107 offspring. Haplotypes were 1-4-5-5 and 4-1-1-4. In another 44 offspring the established paternal haplotypes could be assigned without resorting to recombination. In 6 offspring recombinational events must have taken place. In the 107 offspring of *Sire 1*, 57 different maternal haplotypes could be established. Paternal haplotypes of *Sire 2* could be identified unambiguously in 19 of the 85 offspring. Haplotypes turned out to be 3-4-5-1 and 1-2-4-4. In another 63 offspring the established paternal haplotypes could be assigned without resorting to recombination. In 3 offspring, recombinational events must have taken place. In the 85 offspring of *Sire 2*, 53 different maternal haplotypes could be established.

Association analysis

The 48 unaffected (HOARSI 1) offspring of *Sire 1* received 4-1-1-4 in 26 instances and 1-4-5-5 in 20 instances. In 2

TABLE 2: Haplotype distribution and P values in offspring categories of *Sires 1* and *2*

Offspring groups	*Sire 1*			*Sire 2*		
Haplotype	1-4-5-5[#]	4-1-1-4*	P value	1-2-4-4[δ]	3-4-5-1[β]	P value
HOARSI 2	9	9		4	13	
HOARSI 3	13	5		6	9	
HOARSI 4	15	4	0.148 (2-sided)	4	4	0.379 (2-sided)
Unaffected group (HOARSI 1)	20	26		14	14	
Affected group (HOARSI 2+3+4)	37	18	0.026 (Fisher's)	14	26	0.320 (Fisher's)

[#]Haplotype 1-4-5-5 (188 bp (*AHT133*)- 153 bp (*LEX041*)- 140 bp (*VHL47*)- 136 bp (*ASB37*); *Haplotype 4-1-1-4 (200 bp (*AHT133*)- 143 bp (*LEX041*)- 124 bp (*VHL47*)- 132 bp (*ASB37*); [δ]Haplotype 1-2-4-4 →188 bp (*AHT133*)- 149 bp (*LEX041*)- 138 bp (*VHL47*)- 132 bp (*ASB37*); [β]Haplotype 3-4-5-1 →196 bp (*AHT133*)- 153 bp (*LEX041*)- 140 bp (*VHL47*)- 122 bp (*ASB37*).

unaffected offspring of *Sire 1* recombinational events must have taken place. There was no difference in the haplotype distribution between HOARSI 2, HOARSI 3 and HOARSI 4 group of *Sire 1* (P = 0.148, Table 2). Therefore, the 3 groups formed the affected (HOARSI 2–4) group in the further analyses. The 59 affected offspring of *Sire 1* received 4-1-1-4 in 18 instances and 1-4-5-5 in 37 instances. In 4 affected offspring recombinational events must have taken place. The comparison of the haplotype frequencies showed a significant difference between affected (HOARSI 2–4) and unaffected (HOARSI 1) offspring of *Sire 1* (P = 0.0261, Table 2). In *Sire 2* the haplotypes seemed to segregate at random and showed no associations with the affection status (Table 2). There was no difference in the haplotype distribution between offspring of *Sire 1* with frequent, regular and occasional coughing (P = 0.417). Therefore, they formed the coughing group in the further analysis. The 51 coughing offspring of *Sire 1* received 4-1-1-4 in 14 instances and 1-4-5-5 in 34 instances. In 3 coughing offspring of *Sire 1* recombinational events must have taken place. The 56 noncoughing offspring of *Sire 1* received 4-1-1-4 in 30 instances and 1-4-5-5 in 23 instances. In 3 noncoughing offspring, recombinational events must have taken place. The comparison of the haplotype frequencies showed a significant difference between coughing and noncoughing offspring of *Sire 1* (P = 0.008, Table 3). In *Sire 2* there was no haplotype association with coughing frequency (P = 0.46, Table 3). Comparison of haplotype frequencies in offspring of *Sire 1* with and without nasal discharge showed no significant difference (P = 0.0722, Table 3). In the family of *Sire 2* there was no association of haplotype with nasal discharge (P = 0.57, Table 3).

Linkage mapping

Evidence for a quantitative trait locus (QTL) linked with HOARSI was found at 26 cM (F value 5.35, P<0.05), coinciding with the marker *ASB037*. The peak for a QTL for coughing frequency occurred at 22 cM (F value 5.42, P<0.01), between markers *AHT133* and *LEX041*, and no significant effect was found for nasal discharge. Segregation of the QTL for HOARSI was only found for *Sire 1* (within-family *t* value 3.12) and coughing (*t* value 3.2)

Discussion

This study demonstrated association and linkage of the tested microsatellite markers in the ECA13q13 region with clinical signs of chronic lower airway disease, in particular with coughing, in the family of *Sire 1* but not in that of *Sire 2*. The recombinational events in the 6 offspring of *Sire 1* did not allow us to narrow down the region harbouring the putative major gene as its location relative to this linkage group could not yet be established.

The association of paternal microsatellite marker haplotypes, as well as the linkage analysis, in the family of *Sire 1* support the hypothesis of a genetic background for equine RAO. To the authors' knowledge, the familial predisposition for equine chronic lower airway disease was first described by Schaeper (1939), who reported the prevalence of RAO in 14 of 27 offspring out of the affected stallion 'Egmont' and described the progeny of several affected mares, mated to unaffected stallions and producing affected foals, which themselves produced affected descendants. However, that study did not include a control group, e.g. offspring, from an unaffected stallion. Koch (1957) examined a 7-year-old mare with clinical signs of heaves. Dam and foal (age 6 months) of this mare were also heaves-affected. Gerber (1989) reported the family history of 'Julia', an affected mare, who gave birth to 5 affected foals, out of 2 healthy stallions. However, mated to an affected stallion she gave birth to a colt that remained unaffected.

Marti *et al*. (1991) showed that the risk for developing moderate to severe equine chronic lower airway disease was significantly increased in offspring with one and, even more, with 2 affected parents. The inheritance mode did not appear to follow a simple Mendelian-pattern. Also, no association with major histocompatibility complex (MHC) associated equine leucocyte antigens was found in these groups. In a sample of the Swiss Warmblood population, the prevalence and clinical manifestation of moderate to severe equine chronic lower airway disease (HOARSI 3 and 4) was increased in offspring of 2 affected sires in comparison with a control group and a group of maternal halfsiblings (Ramseyer *et al*. 2007). HOARSI is a composite score based on owner-observed coughing frequency, presence or absence of nasal discharge, increased breathing effort and poor performance. Therefore, coughing and nasal discharge are naturally correlated with HOARSI. They were not equally associated with the ECA13q13 region harbouring *IL4RA*, however. The significant difference in haplotype distribution and linkage analysis between coughing and noncoughing offspring of *Sire 1* in the present study is in agreement with findings of Ramseyer *et al*. (2007), which suggested coughing to be the most markedly heritable characteristic. In contrast to coughing, the comparison of haplotype distribution and linkage analysis between offspring of *Sire 1*, with and without nasal discharge, showed no significant difference. A simple explanation

TABLE 3: Haplotype distribution and P values for coughing and nasal discharge in the offspring of *Sires 1* and *2*

Offspring groups	*Sire 1*			*Sire 2*		
Haplotype	1-4-5-5[#]	4-1-1-4*	P value	1-2-4-4[δ]	3-4-5-1[β]	P value
Occasional coughing	11	7		5	11	
Regular coughing	13	5		7	12	
Frequent coughing	10	2	0.417 (2-sided)	2	1	0.510 (2-sided)
Not coughing	23	30		14	16	
Coughing (occasional+regular+frequent)	34	14	0.008 (Fisher's)	14	24	0.46 (Fisher's)
No nasal discharge	27	29		22	24	
Nasal discharge	30	15	0.072 (Fisher's)	24	16	0.570 (Fisher's)

[#]Haplotype 1-4-5-5 (188 bp (*AHT133*)- 153 bp (*LEX041*)- 140 bp (*VHL47*)- 136 bp (*ASB37*); *Haplotype 4-1-1-4 (200 bp (*AHT133*)- 143 bp (*LEX041*)- 124 bp (*VHL47*)- 132 bp (*ASB37*); [δ]Haplotype 1-2-4-4 →188 bp (*AHT133*)- 149 bp (*LEX041*)- 138 bp (*VHL47*)- 132 bp (*ASB37*); [β]Haplotype 3-4-5-1 →196 bp (*AHT133*)- 153 bp (*LEX041*)- 140 bp (*VHL47*)- 122 bp (*ASB37*).

for these results is that coughing as a very distinct clinical sign is more readily assessed than nasal discharge.

The investigated microsatellite markers are located near the *IL4RA* gene. Watson *et al*. (2003) reported the characterisation of 11 single nucleotide polymorphisms of exon 12 in the horse IL4RA gene and an elevated haplotype frequency in RAO-affected Quarter horses. The results of the present study support the hypothesis that the region of ECA 13 harbouring *IL4RA* plays a role in the development of lower airway disease in horses.

The *IL4RA* ligands IL4 and IL13 are key Th2-type cytokines. In animal models, *IL4RA* was shown to be essential for the development of an atopic phenotype: mice lacking the *IL4RA* gene show deficiencies in the production of Th2 lymphocytes and reduced IgE responses (Kopf *et al*. 1993; Noben-Trauth *et al*. 1997). However, human asthma and equine chronic lower airway disease are complex natural diseases resulting from an interaction of genetic background and environmental factors. In human asthma, candidate gene approaches and association analyses were performed in many studies. However, results have been contradictory, with associations noted in one population but not in another (Whittaker 2003). *IL4RA* has been reported to be associated with asthma susceptibility in German, Hutterites and Dutch families (Deichmann *et al*. 1998; Ober *et al*. 2000b; Howard *et al*. 2002). In contrast, results of a study of a Mexican family did not support an association (Mujica-Lopez *et al*. 2002).

Furthermore, the possible involvement of *IL4RA* in equine RAO is interesting in the light of the predominant Th2-type cytokine response in heaves affected horses reported in some studies (Lavoie *et al*. 2001; Cordeau *et al*. 2003; Horohov *et al*. 2005). However, results of other studies are contradictory, suggesting that RAO is a Th1-type disorder (Ainsworth *et al*. 2003). It is, therefore, all the more intriguing that the paternal haplotype association and the linkage was found only in the halfsib family of *Sire 1*, but not of *Sire 2*. This could suggest locus heterogeneity for this disease. Should this hold true, the established haplotype may point to a mutation in *IL4RA* as one of the 2 or more genes that modify susceptibility to equine chronic lower airway disease. In this case, it would be expected to find an association with a different candidate gene, presumably located elsewhere, in the offspring of *Sire 2*. Therefore, the present results could help explain the contradictory findings regarding Th1 vs. Th2 predominance in equine RAO. An alternative, less exciting explanation for the different results in the 2 families would be that recombination has broken down the association between the paternal haplotype and disease locus. Certainly, further studies on extended family material using markers directly in the *IL4RA* gene will be needed to test these hypotheses. Using data from more than 2 families would strengthen the conclusion at the population level. However, the present family material with so many available offspring by affected sires is unique, and, to the authors' knowledge this is the first study showing association and linkage of genetic markers with equine RAO.

In conclusion, the results provide further evidence for a genetic basis of equine RAO and support the hypothesis that the ECA13q13 region harbouring *IL4RA* plays a role in regulating susceptibility to equine chronic lower airway disease in some high-prevalence horse families. However, the functional variant in the region harbouring *IL4RA* is still unknown. Current knowledge suggests susceptibility to equine chronic lower airway disease to be a complex trait. Should *IL4RA* play an essential role, its effects would probably be modified by interaction with other genes and environmental factors. Further studies should investigate linkage and association of markers directly on candidate genes, in particular *IL4RA*, in the ECA13q13 region with equine RAO.

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Manufacturers' addresses

[1]Qiagen AG, Basel, Switzerland.
[2]LI-COR, Bad Homburg, Germany.
[3]Zeiss, Jena, Germany.
[4]Laboratory Imaging, Prague, Czech Republic.
[5]Roche, Basel, Switzerland.
[6]QTL, http://qtl.cap.ed.ac.uk.

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