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Research paper

Mapping quantitative trait loci for hearing loss in Black Swiss mice

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Abstract

In common inbred mouse strains, hearing loss is a highly prevalent quantitative trait, which is mainly controlled by the *Cdh23*^{753A} variant and alleles at numerous other strain-specific loci. Here, we investigated the genetic basis of hearing loss in non-inbred strains. Mice of Swiss Webster, CF-1, NIH Swiss, ICR, and Black Swiss strains exhibited hearing profiles characteristic of progressive, sensorineural hearing impairment. In particular, CF-1, Black Swiss, and NIH Swiss mice showed early-onset hearing impairment, ICR and Swiss Webster mice expressed a delayed-onset hearing loss, and NMRI mice had normal hearing. By quantitative trait locus (QTL) mapping, two significant QTLs were identified underlying hearing loss in Black Swiss mice: one QTL mapped to chromosome (chr) 10 (named *ahl5,* LOD 8.9, peak association 35–42 cM) and a second QTL localized to chr 18 (*ahl6,* LOD 3.8, 38–44 cM). *Ahl5* and *ahl6* account for 61% and 32% of the variation in the backcross, respectively. Cadherin 23 (*Cdh23*) and protocadherin 15 (*Pcdh15*), mapping within the 95% confidence interval of *ahl5,* bear nucleotide polymorphisms in coding exons, but these appear to be unrelated to the hearing phenotype. Haplotype analyses across the *Cdh23* locus demonstrated the phylogenetic relationship between Black Swiss and common inbred strains.

Keywords: Outbred strains; Hearing loss; Quantitative trait loci; Cadherin 23; Protocadherin 15

1. Introduction

Sensorineural hearing loss in inbred strains, commonly referred to as age-related hearing loss (AHL), is a frequently occurring, quantitative trait affecting all major genealogical subgroups (Zheng et al., 1999). Typically, elevated hearing thresholds result from the progressive degeneration of sensory hair cells in the organ of Corti followed by the loss of cochlear ganglion neurons (Henry and Chole, 1980; Shnerson et al., 1981; Willott et al., 1998). A phenotypic survey of eighty representative inbred strains showed that approximately 50% of strains expressed hearing loss with varying kinetics and age of onset (Zheng et al., 1999). The BUB/BnJ and DBA/2J strains express early-onset hearing loss, and the C57BL/6J and BALB/cJ strains develop late-onset hearing loss. CBA/CaJ, C3HeB/FeJ and all wild-derived inbred strains however maintain robust life-long hearing. The genetics of AHL is strain-specific and consistent with a multifactorial, oligogenic inheritance model in which one or a few recessive and mostly hypomorphic alleles interrelate to control age of onset, progression and severity of the deficit (Noben-Trauth et al., 2003). The most prevalent hearing loss allele is the *Cdh23*^{753A} variant, which is present in approximately 80% of inbred strains with hearing loss. Strains transmitting the alternative *Cdh23*^{753G} variant, such as CBA/CaJ and wild-derived inbred strains, are likely to maintain life-long hearing. Late-onset hearing loss in C57BL/6J and BALB/cJ strains is best explained by homozygosity of the *Cdh23*^{753A} allele together with epigenetic, stochastic, or environmental factors. Less frequent and apparently strain-specific alleles, such as *mt-Tr*, *ahl2*, and *Mass1*^{Frings}, mediate epistatic and additive effects (Johnson and Zheng, 2002; Johnson

Abbreviations: QTL, quantitative trait locus; ahl, age-related hearing loss; cM, centiMorgan

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et al., 2001, 2005). The *ahl2* and *Mass1*^{Frings} alleles have profound impacts on AHL onset and progression such that, respectively, NOD/LtJ and BUB/BnJ (both of which bear the *Cdh23*^{753A} variant) develop severe hearing impairment by four to five weeks of age (Zheng et al., 1999). The contribution of these strain-specific loci to the phenotypic variance and their effect on the *Cdh23*^{753A} allele present in these strains is variable. *Ahl2* appears to be a weak hypomorph with a mild, if any, phenotypic effect but epistatically interacts with *Cdh23*^{753A} accelerating onset of hearing loss. In contrast, the *Mass1*^{Frings} allele has a pathogenic effect that is sufficient to induce a severe to profound hearing impairment in young BUB/BnJ mice in the absence of the *Cdh23*^{753A} allele. A progressive worsening of AHL, however, is observed when transmitted with the *Cdh23*^{753A} allele.

With the aim of extending this inheritance model and identifying new hypomorphic hearing loss alleles we investigated hearing function in outbred strains. The Swiss Webster, ICR, NMRI, N:NIH, and Black Swiss strains are descendants of Swiss mice brought to the Rockefeller Institution during the early 1920s (Beck et al., 2000). Black Swiss is a late derivative of the N:NIH Swiss strain and was developed by Carl Hansen at the Veterinary Resource Program at the National Institutes of Health (USA). N:NIH Swiss mice were outcrossed to C57BL/6N mice, and serially backcrossed (N10) to N:NIHS mice by selecting for the recessive agouti and wildtype albino alleles. Subsequent intercrossing resulted in the Black Swiss strain (a/a,$+^b/+^b$,$+^c/+^c$). The CF-1 strain originated in a laboratory in Missouri but its ancestry is unknown. Although these strains are currently maintained as outbred stocks they have also at various times been bred as inbred lines.

2. Materials and methods

2.1. Mice

BUB/BnJ, A/J, and CAST/Ei strains were obtained from The Jackson Laboratory (Bar Harbor, ME, USA); NMRI mice were obtained from Charles River, France; ICR, Swiss Webster, NIH Swiss and Black Swiss mice were purchased from Taconic (Germantown, NY, USA) and CF-1 mice were obtained from Charles River Laboratories (Wilmington, MA, USA). Animal studies were conducted following the guidelines of the National Institutes of Health approved by the ACUC NINDS/NIDCD.

2.2. Crosses

Reciprocal matings were established between the Black Swiss strain and the BUB/BnJ, A/J, CF-1 and NIH Swiss strains. The hearing ability of F1 hybrids was examined between eight and twelve weeks of age. To generate the [(Black Swiss × CAST/Ei) F1 × F1] intercross, a Black Swiss female mouse with a 75 decibel sound pressure level (dB SPL) hearing threshold for the 16 kHz stimulus at eight weeks of age was crossed with a CAST/Ei male and ten F1 hybrids were intercrossed and produced 147 F2 progeny. To generate the [(Black Swiss × CAST/Ei) × Black Swiss] backcross population, Black Swiss mice with hearing thresholds between 50 and 60 dB SPL (16 kHz stimulus) at eight weeks of age were crossed with CAST/Ei mice and five N1 hybrids were backcrossed to Black Swiss males and females with thresholds of 50–80 dB SPL at four to eight weeks of age to produce the N2 population ($n = 234$).

2.3. Auditory-evoked brainstem response (ABR) analyses

ABR testing was performed as described previously (Zheng et al., 1999). Briefly, anesthetized mice were subjected to computer-controlled acoustic stimuli, delivered through high-frequency transducers (Intelligent Hearing Systems, Miami, FL, USA). Sub dermal needle electrodes were placed at the vertex of the cranium and responses were recorded, amplified, averaged and displayed on a monitor. Hearing thresholds were determined by decreasing the stimulus intensity in 5–10 dB SPL increments until the weakest observable response was obtained. The experimental error range was ±5 dB SPL. To determine hearing thresholds in various outbred strains, we recorded responses following broadband click and pure tone single frequency stimuli of 8, 16 and 32 kHz. Mice of the normal hearing strains CBA/CaJ and C3HeB/FeJ were used to establish the phenotypic base line of normal hearing. At the 16 kHz stimulus, mice of these strains exhibited mean (±one standard deviation) thresholds of 22 ± 9 and 24 ± 9 dB SPL, respectively. Mice are most sensitive to acoustic stimuli around the 16 kHz frequency band. Unless otherwise indicated in the text, hearing thresholds, LOD scores, and phenotypic variances are given in relation to the 16 kHz stimulus.

2.4. DNA extraction and genotyping

DNA was extracted from tail clips using DNaesy® (Qiagen, Valencia, CA). Microsatellite markers polymorphic between C57BL/6J and CAST/Ei were chosen from the Center for Inherited Disease Research website (www.cidr.jhmi.edu/mouse/mouse_strp.html) (Witmer et al., 2003) and purchased from Applied Biosystems, Invitrogen or IDT. Allele-specific products were amplified in the presence of 10–50 ng genomic DNA, 200μM dNTP, 0.1 μM of each primer, and the AmpliTaq™ PCR system (Applied Biosystems) in a 10 μl reaction using a GeneAmp 9700 PCR system (Applied Biosystems) under the following conditions: 1 min at 95 °C for initial denaturation, 45 s at 94 °C, 1 min at 55 °C, 1 min 72 °C for 50 cycles, followed by a 10 min extension step at 72 °C. PCR products were diluted 1:10 in 10 μl formamide including 0.1 μl of GeneScan™ 500ROX™ Standard (Applied Biosystems) and electrophoresed on a 3730 DNA Analyzer (Applied Biosystems). Electropherograms were analyzed with ABI Prism™ GeneMapper™ v3.5 software (Applied Biosystems).

2.5. QTL analysis

Hearing thresholds were obtained for four stimuli (click, 8, 16 and 32 kHz). The average age of N2 backcross mice was 13 ± 4 weeks with a range of 6–19 weeks and the mean age of F2 progeny was 37 ± 5 weeks with a range of 20–46 weeks. For the selective genome-wide linkage scan, twenty-two N2 progeny from each extreme (<2.5th and >90th percentile) of the phenotypic spectrum (threshold range: 10–15 dB SPL and 70–100 dB SPL) and twenty-one F2 progeny from each tail (<7.5th and >86th percentile) of the phenotypic distribution (threshold range: 10–15 dB SPL and 70–100 dB SPL) were selected and typed at 131 microsatellite marker loci. The least-squares linear regression method of MapManager QTXb20 was used to detect and localize marker association with trait values (Manly et al., 2001). Empirical thresholds to estimate genome-wide significance of likelihood-ratio-statistic (LRS) scores were computed by performing 1000 permutations (Churchill and Doerge, 1994). Suggestive, significant, and highly significant linkage levels correspond to a genome-wide p value of 0.63, 0.05 and 0.001, respectively (Lander and Kruglyak, 1995). For each significant QTL detected in the selective scan, the rest of the segregating population was typed with markers from the linked chromosome. Interval mapping was performed to localize the QTL and composite interval mapping was conducted to search for additional linked and unlinked QTLs. Bootstrap testing was used to compute the 95% confidence interval of the QTL peak association. Pair-wise marker regression-analyses were performed at $p = 10^{-5}$ and the significance level for the interaction was computed by permutation testing. LRS scores were converted to LOD scores by dividing the LRS value by the factor 2ln(10). Regression coefficients underlying the LRS statistic were

computed assuming a normal distribution of trait values. Even though hearing threshold distributions in the F2 and N2 crosses were not normally distributed, but skewed to the right, the statistic is robust enough to produce correct regression coefficients (Manly and Olson, 1999). Centi-Morgan position of markers and genes were retrieved from the Mouse Genome Informatics website at www.informatics.jax.org.

2.6. DNA sequencing

Cdh23 and *Pcdh15* coding exons were sequenced using genomic DNA and cDNA generated from cochlea and brain mRNA. PCR was performed in a 15 μl reaction containing 50–100 ng genomic DNA, 200 μM dNTP, 0.2 μM of each forward and reverse primers, and Advantage cDNA Polymerase Mix (Clontech Laboratories, Inc, Palo Alto, CA). Amplification was carried out in a thermal cycler denaturing at 95 °C, 1 min and cycling 35 times at 94 °C, 30 s, 68 °C, 2 min followed by a final extension of 68 °C for 2 min. Reactions were treated with 1U shrimp alkaline phosphatase (Roche) and 1U Exocuclease (New England BioLabs) at 37 °C for 60 min and inactivated at 80 °C for 10 min. Sequencing of PCR products was performed using BigDye™ Terminator v 3.1 and electrophoresed on a 3730 × 1 capillary sequencer (Applied Biosystems). Sequence chromatograms were analyzed using Sequencher v 4.5. Primer sequences are available upon request.

2.7. Statistical analyses

Descriptive statistics, ANOVA, Bonferroni's multiple comparison tests, D'Agostino and Pearson normality test, Fisher's exact test, and linear regression-analyses were computed using Prism™ 4.0b software (GraphPad, Inc.). Cluster analyses and dendrograms were generated using the JMP™ statistical software package (JMP, INC).

3. Results

3.1. Hearing thresholds in outbred strains

We tested groups of 10–20 mice at different ages from six outbred strains and identified three phenotypic classes according to their 16 kHz tone threshold profiles: normal hearing, early-onset, and delayed-onset hearing loss. Mice of the NMRI strain had a mean threshold of 18 ± 5 dB SPL at ten weeks of age and showed normal responses to all four stimuli over the following forty-two week test period (Fig. 1, Table 1). Swiss Webster mice had normal hearing at ten weeks of age (17 ± 9 dB SPL), but had developed a delayed-onset hearing loss by around seventeen weeks of age with mean thresholds of 47 ± 34 dB SPL. Mice of ICR, NIH Swiss, and CF-1 strains expressed an early-onset hear-

Table 1
Time-dependent hearing thresholds in outbred strains

Strain	Age	Click	8 kHz	16 kHz	32 kHz	*N*
ICR	5	47 ± 12	41 ± 8	26 ± 12	57 ± 7	10
	8	45 ± 14	35 ± 20	32 ± 26	56 ± 18	20
	14	57 ± 22	52 ± 30	57 ± 36	73 ± 26	20
	24	81 ± 17	81 ± 22	85 ± 22	94 ± 16	10
	32	81 ± 16	71 ± 29	93 ± 4	95 ± 8	8
Swiss Webstar	6	42 ± 7	28 ± 7	17 ± 6	49 ± 9	10
	10	34 ± 5	30 ± 8	17 ± 6	44 ± 9	10
	17	46 ± 19	34 ± 21	47 ± 34	73 ± 20	10
	26	60 ± 26	54 ± 26	51 ± 36	69 ± 22	20
Black Swiss	4	63 ± 9	43 ± 15	59 ± 16	67 ± 16	37
	14	71 ± 13	56 ± 19	70 ± 15	78 ± 15	35
	27	79 ± 12	76 ± 25	85 ± 17	88 ± 16	27
	60	89 ± 10	91 ± 11	97 ± 4	99 ± 2	7
CF-1	6	55 ± 23	46 ± 22	49 ± 27	67 ± 19	10
	12	87 ± 21	81 ± 24	81 ± 31	91 ± 18	10
	26	87 ± 24	81 ± 24	92 ± 6	100	9
NMRI	10	34 ± 12	32 ± 5	18 ± 5	52 ± 19	10
	28	41 ± 6	33 ± 9	24 ± 6	54 ± 8	10
	52	47 ± 9	31 ± 8	26 ± 11	50 ± 9	10
NIH Swiss	5	45 ± 17	33 ± 16	41 ± 31	61 ± 21	10
	13	51 ± 22	42 ± 31	47 ± 40	67 ± 28	10
	31	68 ± 25	55 ± 31	60 ± 38	89 ± 10	9
	52	73 ± 28	65 ± 33	68 ± 36	84 ± 22	8
CBA/CaJ	12–32	37 ± 7	33 ± 11	22 ± 9	51 ± 8	11
C3HeB/FeJ	11	36 ± 2	34 ± 6	24 ± 6	49 ± 6	14

Age, weeks; kHz, kiloHertz; *N*, number of animals tested; values are mean ± SD.

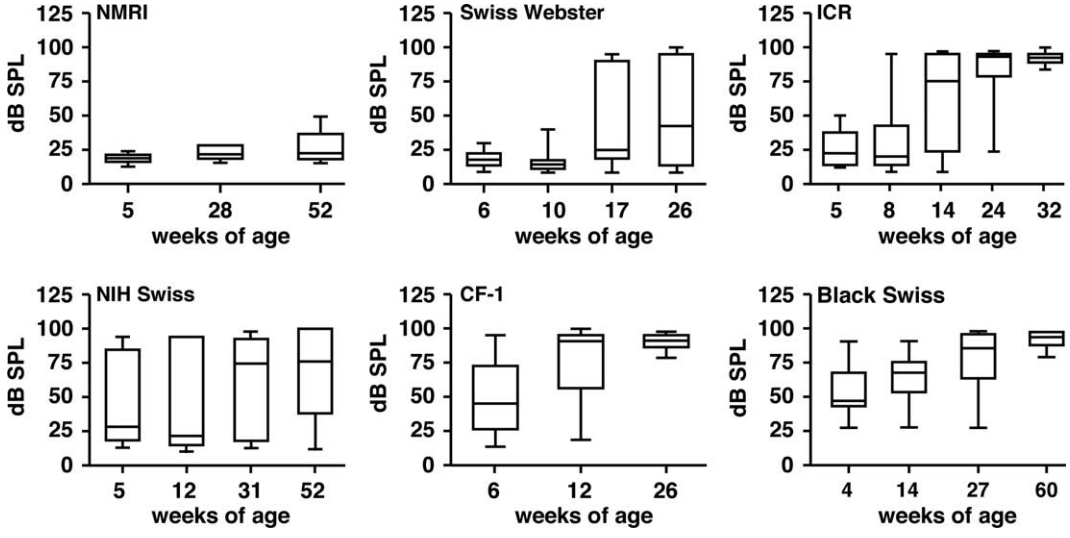

Fig. 1. ABR thresholds in outbred strains. Whisker plots show median, 25th and 75th percentiles of data; bars show the minimum and maximum values. Empirically determined ABR thresholds were obtained following a 16 kHz stimulus. dB SPL, sound pressure levels in decibel.

ing loss that had become apparent around four weeks after birth with mean thresholds of 26 ± 12, 41 ± 31, and 49 ± 27 dB SPL, respectively (Fig. 1, Table 1). In CF-1 mice, thresholds at the six-week time point showed a wide scatter ranging from normal hearing to deafness. At twelve weeks of age, 64% of the mice had lost hearing and by twenty-six weeks of age all mice had become deaf. NIH Swiss mice showed a similar progression profile, but the threshold mean at fifty-two weeks of age did not reach the level of a complete hearing loss. Hearing loss in ICR mice followed a sigmoid progression curve with most of the hearing impairment occurring between eight and twenty-four weeks of age.

Black Swiss mice expressed an early-onset hearing impairment showing a moderate to severe hearing loss by four weeks of age with a mean threshold of 59 ± 16 dB SPL (range: 35–95 dB SPL). Thresholds were also significantly elevated at the click (63 ± 9 dB SPL; $p < 0.001$) and the 32 kHz stimulus (67 ± 16 dB SPL; $p < 0.001$) compared with normal hearing C3HeB/FeJ mice, but appeared almost normal at the 8 kHz stimulus (43 ± 15 dB SPL; $p > 0.05$; ANOVA followed by Bonferroni post tests) (Fig. 1, Table 1). In eight-week old mice, thresholds were significantly elevated at all four stimuli; hearing loss commenced in the high frequency range and progressively affected the lower frequencies (Fig. 2(A)). The thresholds of thirty-nine mice at eight weeks of age were normally distributed ($p > 0.05$ under the null hypothesis; D'Agostino and Pearson test) and ranged from 30 to 100 dB SPL (Fig. 2(B)). Hearing loss progressed slowly with an average threshold increase of 1 dB SPL per week (Fig. 2(C)). At sixty weeks of age all mice tested had thresholds higher than 80 dB SPL with a mean of 97 ± 4 dB SPL.

Since mice of inbred strains bearing the *Cdh23*^{753A} allele are at high risk developing hearing loss, we asked whether hearing thresholds in the outbred panel correlated with segregation of the *Cdh23*$^{753G>A}$ dimorphism. All ICR mice ($n = 20$) tested were homozygous for the *Cdh23*^{753A} allele. Mice of Swiss Webster ($n = 20$), CF1 ($n = 38$) and NIH Swiss ($n = 21$) strains segregated both alleles without significant correlation between allele type and hearing function ($p > 0.05$, Fisher's exact test). All NMRI ($n = 10$) and Black Swiss ($n = 20$) mice were homozygous for the *Cdh23*^{753G} allele.

3.2. Complementation tests

Because of the relatively high frequency of hearing loss in inbred and outbred strains, we tested whether some of the early-onset hearing loss strains share alleles with Black Swiss mice. We outcrossed Black Swiss to BUB/BnJ, A/J, CF-1, and NIH Swiss and analyzed hearing function of F1 hybrids at various ages. Hybrids from Black Swiss × BUB/BnJ and Black Swiss × A/J crosses complemented the Black Swiss phenotype at all tested frequencies (Table 2). In contrast, Black Swiss × CF-1 and Black Swiss × NIH Swiss hybrids responded with thresholds at the 32 kHz stimulus that were significantly ($p < 0.05$) higher than those of normal hearing C3HeB/FeJ mice.

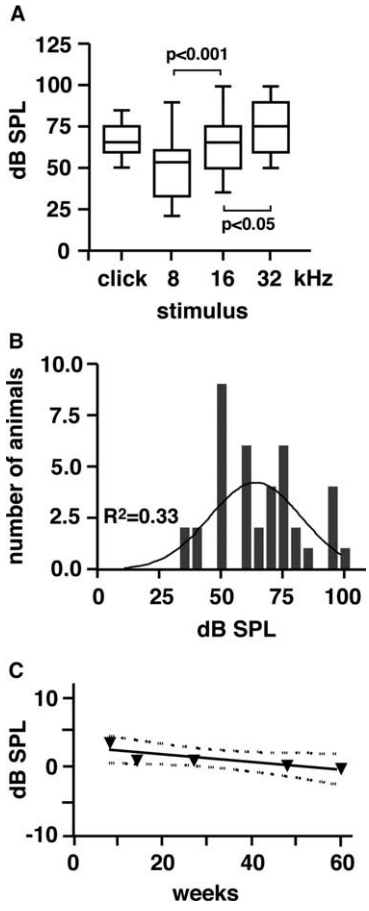

Fig. 2. Hearing loss profile in the Black Swiss strain: (A) hearing thresholds at low and mid-range frequencies in Black Swiss mice. Whisker plots showing median, 25th and 75th percentiles of data, obtained at the indicated stimulus frequencies from a cohort of thirty-nine eight-week-old Black Swiss mice. The bars show the minimum and maximum threshold values. Statistical significant differences of mean thresholds (ANOVA followed by Bonferroni's multiple comparisons tests) are indicated; (B) histogram of 16 kHz stimulus thresholds from eight-week old Black Swiss mice. The best-fit Gaussian distribution curve is superimposed and R^2 value indicating the goodness-of-fit is shown; (C) linear regression model of hearing loss progression. Weekly rates of threshold increases as a function of weeks of age are shown as triangles. The best-fit line (solid) of the mean threshold increases is shown with the 95% confidence intervals (dotted line).

3.3. Hearing threshold distributions in backcross and intercross

Hybrids between Black Swiss and CAST/Ei showed normal responses to the 16 kHz stimulus (15 ± 4 dB SPL; $n = 28$) and to all other stimuli, suggesting recessive inheritance in the outcross. In the F2 and N2 populations, hearing thresholds followed a near normal distribution; the majority of thresholds were clustered in the normal hearing spectrum and the rest were widely scattered in the 35–100 dB SPL range (Fig. 3(A) and (B)). The distributions, however, were skewed to the right with a skewness

Table 2
ABR thresholds in F1 hybrids

Strain/cross	Click	8 kHz	16 kHz	32 kHz	Age	N
Black Swiss (BLKSW)	61 ± 11	44 ± 17	58 ± 16	70 ± 19	8	5
CAST/Ei	32 ± 3	26 ± 8	15±7	45 ± 13	7	4
[BLKSW × CAST/Ei]Fl	34 ± 6	25 ± 7	15 ± 5	44 ± 8	5–10	28
A/J	82 ± 6	85 ± 9	91 ± 7	100	3	5
[BLKSW × A/J]F1	41 ± 10	36 ± 10	23 ± 15	55 ± 12	6–8	25
	55 ± 20	36 ± 16	27 ± 15	51±19	48	10
BUB/BnJ	78 ± 12	81±12	87 ± 12	96 ± 6	4	7
[BLKSW × BUB/BnJ]F1	39 ± 7	33 ± 10	19 ± 9	48 ± 7	6–8	23
	40 ± 6	27 ± 5	23 ± 6	42 ± 7	46	17
CF-1	83 ± 18	80 ± 28	95 ± 7	90 ± 14	13–34	2
[BLKSW × CF-1]F1	41 ± 8	37 ± 10	23 ± 9	58 ± 14	9	27
	52 ± 5	40 ± 6	36 ± 7	71 ± 16*	33	11
NIH Swiss	67 ± 24	62 ± 41	93 ± 3	100	13	3
[BLKSW × NIH Swiss] F1	43 ± 13	35 ± 14	35 ± 22	65 ± 18*	9–10	37

ANOVA followed by Bonferroni's multiple comparison test. *N*, number of animals tested; kKz, kiloHertz; values are mean ± SD.
* $p < 0.05$.

coefficient of 1.9 for the intercross population and of 1.6 for the backcross population. Logarithmic (ln) transformation decreased the skewness coefficient to 1.0 for the intercross and to 0.6 for the backcross population (Fig. 3(A) and (B)), but transformed and non-transformed data sets, deviated significantly from a normal distribution ($p < 0.001$ under the null hypothesis; D'Agostino and Pearson test).

To ascertain whether the age contributed to the variation in the crosses, ABR thresholds were grouped by age and means were compared by ANOVA. There was no significant difference in threshold means across the age groups in both F2 and N2 populations ($p > 0.05$). Linear regression-analyses however revealed a small effect of age on ABR thresholds in the backcross ($r^2 = 0.04$, $p = 0.002$), but not in the intercross ($r^2 = 0.001$, $p = 0.7$).

To approximate the number of hearing loss alleles segregating in the crosses, the pathological threshold was estimated. Presumed that hearing thresholds in normal mice followed a normal distribution, we examined the dB SPL ranges in the F2 and N2 crosses, which best fit a Gaussian curve. In both crosses, thresholds in the 10–30 dB SPL range were normally distributed ($p > 0.05$; D'Agostino and Pearson test). ABR analyses of 430 normal hearing mice from sixteen different inbred strains identified a mean threshold of 18 ± 4.2 dB SPL at the 16 kHz stimulus (Zheng et al., 1999). All Black Swiss mice tested had thresholds higher than 30 dB SPL (Fig. 2(B)). These data suggested that the 30 dB SPL threshold represented a reasonable approximation of the pathological cut-off value. Hence, the ratio between normal and hearing impaired mice in the F2 and N2 crosses was 3.5:1 and

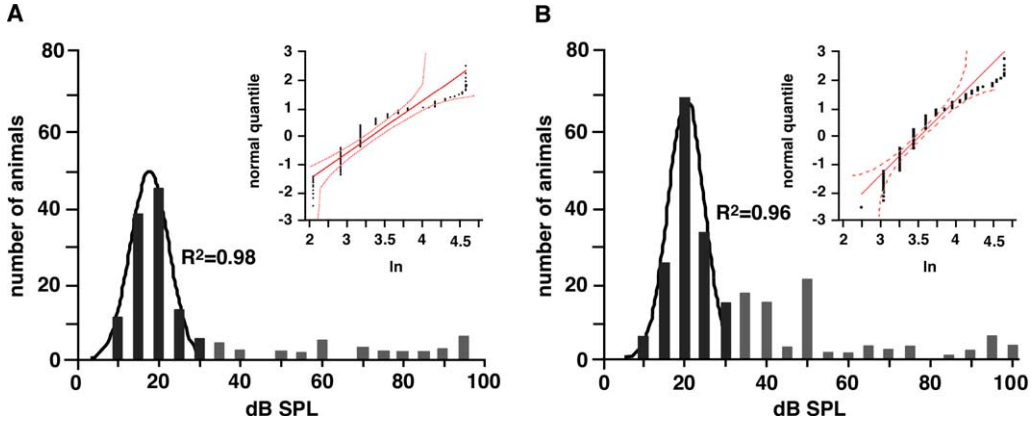

Fig. 3. Hearing threshold distribution in Black Swiss × CAST crosses. Hearing threshold histograms of (A) the [(Black Swiss × CAST/Ei) F1 × F1] F2 and (B) the [(Black Swiss × CAST/Ei) × Black Swiss] N2 population. Thresholds obtained following presentation of a 16 kHz stimulus are shown in decibel sound pressure levels (dB SPL). Numbers of animals are given on the left. The bell-shaped curve indicates the Gaussian distribution of thresholds between 10 and 25 dB SPL and the R^2 value indicating the goodness-of-fit is given. Black and grey columns represent animals with normal hearing and animals with pathological thresholds, respectively. Insets show normal quantile plots of ln transformed threshold data (black squares) and the best fit regression curve (red straight line) including the 95% confidence interval (red dotted line).

1.7:1, respectively, and indicated the segregation of one to two loci.

3.4. Genome-wide linkage scans

To identify the quantitative trait loci underlying hearing loss in Black Swiss mice, a genome-wide scan was performed. Selective genotyping of the N2 population ($n = 44$) followed by marker regression analyses using the 16 kHz trait revealed a QTL on chromosome (chr) 10 with a highly significant LOD score of 8.9 at *D10Mit20* (genome-wide significance threshold at $p = 0.001$: 4.8) (Table 3). This QTL, named *ahl5*, had a strong effect and accounted for 61% of the variance in the backcross. A second QTL was identified on chr 18 with a significant LOD score of 3.8 at *D18Mit103* (genome-wide significance threshold at $p = 0.05$: 3.2). This QTL was named *ahl6* and explained 32% of the threshold variation. For the click stimulus similarly significant LOD scores were obtained (Table 3). The 8 kHz stimulus produced a highly significant LOD score with *D10Mit20*, but yielded only suggestive linkage with *D18Mit144*. No linkage however was found with the 32 kHz trait. A selective genome-wide scan of F2 progeny ($n = 42$) detected one QTL with highly significant linkage at *D10Mit139* with a LOD of 7 (genome-wide significance threshold at $p = 0.05$: 4.4), but failed to find linkage at chr 18 at all stimuli (Table 3). The chr 10 QTL in the F2 cross had a strong effect (54%) and replicated the *ahl5* QTL detected in the backcross.

3.5. Chromosome-wide QTL interval mapping

To localize the QTLs identified in the backcross, the rest of the N2 progeny ($n = 190$) were genotyped with markers from chr 10 and chr 18. Interval mapping followed by bootstrap tests placed the 95% confidence interval (CI) of the *ahl5* QTL peak between *D10Mit20* and *D10Mit207* (35–42 cM position) (Fig. 4, Table 3). The *ahl6* QTL peak on chr 18 was defined by the *D18Mit208–D18Mit103* inter-

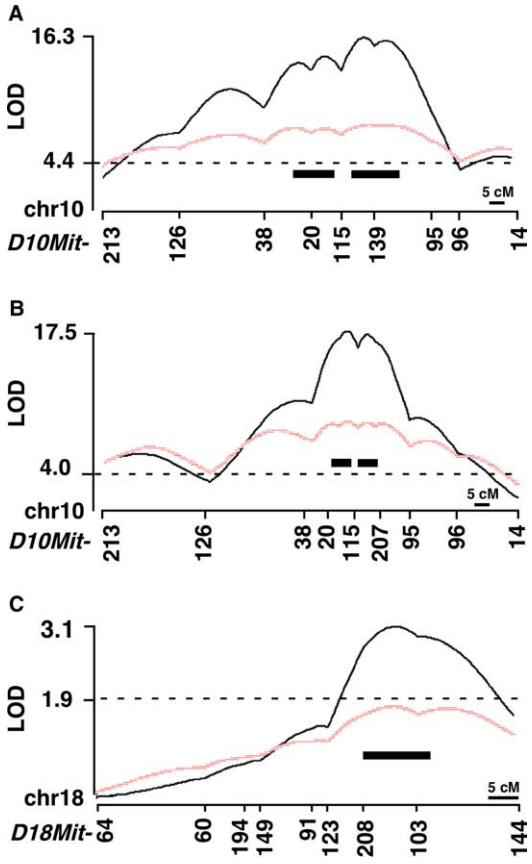

Fig. 4. QTL interval mapping. Interval mapping plots of the *ahl5* QTL in the (A) intercross and (B) backcross populations and of (C) *ahl6* in the backcross population are shown. Each black line represents the LOD profile of the 16 kHz trait as a function of map position. Dashed lines and corresponding LOD values indicate the permutation-derived chromosome-wide significance level for highly significant ($p = 0.001$; LOD 4.0) and significant ($p = 0.05$; LOD 1.9) linkage. Chromosome-wide peak LOD scores for each QTL are shown on the left. Solid bars indicate the bootstrap based 95% confidence intervals for each QTL. The red line shows the positive regression coefficient. Each horizontal axis represents the genetic map of the indicated chromosome (chr) using Kosambi mapping functions and the microsatellite marker loci (*D–Mit—)*. A scale bar indicating the genetic length in cM is given.

Table 3
Mapping statistics of hearing loss QTLs in Black Swiss × CAST/Ei crosses

Cross	Trait	Marker	LOD	var[a]	QTL	95% CI[b]	QTL peak[c]
Backcross	Click	*D10Mit20*	7.1	53	*ahl5*	*D10Mit20-207*	35–42
	8 kHz	*D10Mit20*	6.2	48	*ahl5*	*D10Mit20-207*	35–42
	16 kHz	*D10Mit20*	8.9	61	*ahl5*	*D10Mit20-207*	35–42
	Click	*D18Mit144*	3.2	29	*ahl6*	*D18Mit208-103*	38–44
	8 kHz	*D18Mit144*	2.8	26	*ahl6*	*D18Mit208-103*	38–44
	16 kHz	*D18Mit103*	3.8	32	*ahl6*	*D18Mit208-103*	38–44
Intercross	Click	*D10Mit139*	8.1	59	*ahl5*	*D10Mit20-95*	35–51
	8 kHz	*D10Mit126*	5.5	45	*ahl5*	*D10Mit38-95*	27–51
	16 kHz	*D10Mit139*	7	54	*ahl5*	*D10Mit38-95*	27–51

[a] Percent of genome-wide phenotypic variation explained by QTL.
[b] Bootstrap-derived 95% confidence interval of chromosome-wide QTL peak interval.
[c] Position of QTL peak location in cM.

val (38–44 cM position). Interval mapping using the entire F2 population ($n = 147$) genotyped with chr 10 markers localized the *ahl5* QTL peak between *D10Mit38* and *D10Mit95* (95% CI, 27–51 cM) (Fig. 4, Table 3).

3.6. QTL effects

To determine the singular and collective effects of the QTLs in the N2 and F2 crosses, we compared the threshold means as a function of the genotype for each QTL. The mean threshold for the 16 kHz stimulus of *ahl6* single homozygotes (+/*ahl5* *ahl6*/*ahl6*) was in the normal hearing range and did not differ ($p > 0.05$, ANOVA followed by Bonferroni's test) from that of animals heterozygous at both QTL intervals (+/*ahl5* +/*ahl6*) (Fig. 5(A)). The mean threshold of *ahl5* single homozygotes (*ahl5*/*ahl5* +/*ahl6*) was higher than that of *ahl6* single homozygotes ($p < 0.001$). N2 progeny that were homozygous at both QTL intervals had a mean threshold that was significantly higher than that of the single homozygotes (*ahl5*/*ahl5* +/*ahl6* and+/*ahl5* *ahl6*/*ahl6*) ($p < 0.001$). To estimate the contribution of *ahl6* to the *ahl5* effect, ABR thresholds were grouped (above and below the mean threshold of *ahl5*/*ahl5* +/*ahl6*) and the risk factor was computed. *Ahl5*/*ahl6* double homozygotes (*ahl5*/*ahl5* *ahl6*/*ahl6*) were 1.8 more likely to develop thresholds higher than 40 dB SPL than *ahl5* single homozygotes ($p = 0.017$; Fisher's test). There was no significant difference in age between the four genotypic classes (ANOVA, $p = 0.65$), but among double homozygotes thresholds tended to be increased with age ($r^2 = 0.06$, $p = 0.04$). In *ahl5*/*ahl6* double homozygotes the mean threshold was significantly lower than that of the parental Black Swiss mice of a comparable age group (12 weeks of age). F2 progeny homozygous at *ahl5* produced a wide scatter of thresholds with a mean that was different ($p < 0.01$) from heterozygotes and F2 mice homozygous for the CAST/Ei-derived allele at the *ahl5* QTL interval (Fig. 5(B)).

Genome-wide marker regression-analyses in the backcross populations did not reveal any significant interaction of QTL pairs. Chromosome-wide analyses of markers at chr 10, 13 and 18 performed with all N2 mice, yielded only suggestive evidence for an interaction between *ahl5* (*D10Mit20*) and *D13Mit19* with a LOD score of 3.9. An interaction between *ahl6* (*D18Mit103*) and *D13Mit19* produced a LOD score of 2.7.

3.7. Candidate gene evaluation in the ahl5 interval

The 95% CI of the *ahl5* QTL interval in the intercross (*D10Mit38–D10Mit95*) encompassed at its proximal and medial region two genes previously shown to cause deafness in waltzer (*Cdh23*^{v}, position 30 cM) and Ames waltzer (*Pcdh15*^{av}, position 40 cM) mouse mutants (Alagramam et al., 2001; Di Palma et al., 2001b). Cadherin 23 (*Cdh23*) and protocadherin 15 (*Pcdh15*) encode proteins that are essential for the structure of the mechanosensitive stereocilia hair bundle (Alagramam et al., 2000; Holme and Steel, 2002). The nucleotide sequences of all thirty-four *Pcdh15* coding exons were determined in Black Swiss, CBA/CaJ and CAST/Ei (Table 4). Twenty variants between the musculus and castaneous subspecies were found, four of which were synonymous changes and were located within exon 35 encoding the intracellular portion of protocadherin 15. No variants were found among the Black Swiss, CBA/CaJ and C57BL/6J strains.

We also determined the *Cdh23* coding sequence in Black Swiss mice and mice of representative strains and subspecies. Eight synonymous nucleotide differences between Black Swiss and CBA/CaJ were detected (Table 5), but

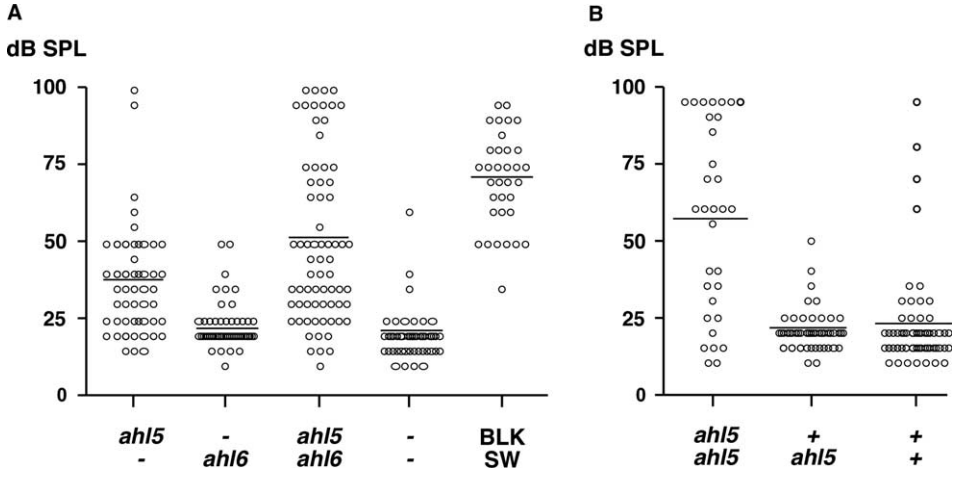

Fig. 5. Individual and combined QTL effects: (A) shown are the means (lines) and distribution of thresholds (circles) measured with presentation of the 16 kHz stimulus of N2 progeny as a function of the genotypes at *ahl5* (typed at *D10Mit207*) and *ahl6* (typed at *D18Mit103*). *ahl5* and *ahl6* denotes homozygosity of Black Swiss alleles at the marker locus and hyphens (-) indicate a heterozygous genotype. BLKSW, Black Swiss; (B) shown are the means (lines) and distribution of thresholds (circles) of F2 progeny as a function of the genotype at *ahl5* (typed at *D10Mit139*). Plus sign (+) indicates the CAST/Ei-derived allele. Vertical axes represent the hearing thresholds in decibel sound pressure level (dB SPL).

Table 4
Strain-specific SNPs in *Pcdh15*

Exon	Nucleotide[a]	C57BL/6J	CBA/CaJ	BLKSW	CAST/Ei	aa Change
1	102	C	C	C	T	
	352	T	T	T	C	
	353	G	G	G	A	
4	171	C	C	C	T	
5	189	T	T	T	C	
9	816	T	T	T	C	
12	1288	T	T	T	G	
17	1953	T	T	T	C	
20	2406	C	C	C	T	
	2517	C	C	C	T	
21	2592	A	A	A	G	
	2673	G	G	G	C	
	2751	C	C	C	G	
25	3204	T	T	T	C	
35	4698	G	G	G	A	
	4823/24	AT	AT	AT	GG	Asnl611Gly
	4841	C	C	C	A	Ala1614Asp
	4849	G	G	G	A	Glu1617Lys
	5082	T	T	T	C	
	5140	A	A	A	G	lle1714Val
	5160	T	T	T	A	
	5463	G	G	G	A	
	5589	C	C	C	T	

BLKSW, Black Swiss; aa, amino acid.
[a] GenBank accession number: AF281899.

none of these variants was unique to Black Swiss; they also were present in the closely related PERC/Ei strain and/or more distantly related wild-derived strains (CAST/Ei, MOLF/Ei, CZECH/Ei), all of which have normal hearing. Using these eighty-eight single-nucleotide polymorphisms (SNPs), spread out over a genomic distance of 350 kb (~30 cM position), the phylogenetic origin of the genomic segment that is part of the *ahl5* QTL interval was determined. Cluster analyses showed that across the *Cdh23* locus on chr 10 Black Swiss had the closest relationship to M.m. *domesticus* strains, and was genetically clearly distinct from common laboratory inbred strains (Fig. 6).

4. Discussion

4.1. Hearing loss in non-inbred strains

This study was initiated to investigate the genetic basis of polygenic hearing loss in non-inbred strains. The six strains examined were chosen based upon their frequent use (Morris et al., 2005) and their common descent from Swiss albino founders (Beck et al., 2000). The perhaps most interesting finding was, that each strain showed a distinct and characteristic hearing profile, which was unexpected given the close genealogical relationship. The CF-1 strain, with an uncertain ancestry, showed a severe early-onset hearing impairment that rapidly progressed to a complete hearing loss by twenty-six weeks of age. The apparent biphasic progression suggests segregation of early- and late-onset hearing loss allele(s) in CF-1 mice. Among the Swiss albino-derived strains, Black Swiss mice had the most severe phenotype, with all animals responding with pathological thresholds ($\geqslant$30 dB SPL at 16 kHz stimulus) by four weeks of age. Hearing loss in Black Swiss mice was robust, increased steadily over a one-year time period, but was characterized by a slower progression than that of CF-1 mice. The hearing loss in NIH Swiss mice was generally less severe than in Black Swiss mice. We observed the slowest progression of hearing loss in the Swiss Webster strain. Similarly distinct progression profiles have been observed among inbred strains, with BUB/BnJ, NOD/LtJ and DBA/2J (similar to CF-1) showing very early-onset, A/J (similar to ICR) and C57L/J, C57BR/cdJ and C57BLKS/J (similar to NIH Swiss and Swiss Webster) showing a delayed-onset hearing loss (Zheng et al., 1999).

Although each of the five examined strains exhibited a unique temporal progression, it appeared that in all strains hearing loss progressed from higher to lower frequencies. Analyses of mean thresholds of mice with clearly pathological thresholds ($\geqslant$50 dB SPL at 16 kHz) showed that impaired responses were most pronounced to the 32 kHz stimulus followed by those of the 16 and 8 kHz stimuli, which is characteristic of a sensorineural-type hearing impairment. This leads us to the proposition that, similar to inbred strains, hearing loss alleles in outbred strains act in a cell-autonomous manner in the neuroepithelium.

Despite these phenotypic similarities, the complementation tests support the notion that unique alleles are acting in outbred mice. F1 hybrid progeny of Black Swiss mice crossed with mice of the early-onset hearing loss strains A/J and BUB/BnJ had normal hearing. Thus, given the profound hearing loss in A/J and BUB/BnJ mice, Black Swiss mice would not be expected to bear the same deafness allele(s) acting in A/J or BUB/BnJ. The complementation data from F1 hybrids between outbred strains are less clear-cut. Crossing hearing-impaired NIH Swiss mice with hearing impaired Black Swiss mice produced F1 hybrids with normal hearing in the low-frequency range, but thresholds were elevated in the higher-frequency spectrum. This result is consistent with partially shared alleles, which would be expected due to the common ancestry of NIH Swiss and Black Swiss strains. This non-complementation in the higher-frequency range also was observed in CF-1 $\times$ Black Swiss hybrids. Although, this result is not inconsistent with partial, incomplete allelism, the strong alleles segregating in the CF-1 population suggests that the effect at the 32 kHz stimulus in the hybrids is more likely due to non-allelic non-complementation of one or several dominant alleles.

The breeding history of the outbred stains used in this study was not always readily accessible. However, cluster analyses of SNP distribution patterns across the *Cdh23* locus in different inbred strains showed a marked genetic difference between inbred strains and the Black Swiss strain. Interestingly, the genome of Black Swiss mice appeared less heterogeneous than expected. Genotype results of eighty-eight SNPs in *Cdh23* (Table 5) and seventy-five publicly available anonymous SNP markers

Table 5
Strain-specific SNPs in *Cdh23*

Exon	Nucleotide[a]	C57BL/6J	CBA/CaJ	BUB/BnJ	BLKSW	PERC/EI	MOLF/EI	V/Le	CZECHIL/EI	CAST/EI
1	14	T	T	T	T	C	C	C	C	C
2	72	G	G	G	G	G	G	G	G	A
	129	T	T	T	T	T	T	T	T	C
4	318	T	T	T	T	C	C	C	C	C
5	393	A	A	A	A	A	G	G	G	G
6	495	C	C	C	C	T	T	T	T	T
	471	C	C	C	C	T	C	C	C	C
	540	C	C	C	C	T	T	T	T	T
	561	C	C	C	C	G	C	C	C	C
	570	T	T	T	T	C	T	T	T	T
	618	T	T	T	T	C	T	T	T	T
7	654	A	A	A	A	G	G	G	G	G
	685	A	A	A	A	G	G	G	G	G
	729	T	T	T	T	C	C	C	C	C
	753	A	G	A	G	G	G	G	G	G
10	948	C	C	C	C	C	T	T	T	T
	1089	C	C	C	C	T	C	C	C	C
12	1154	G	G	G	G	G	A	A	G	A
	1263	T	T	T	T	T	C	C	C	C
15	1530	G	G	G	G	G	A	A	A	G
	1566	C	C	C	C	T	C	C	C	C
	1569	T	T	T	T	T	C	C	C	C
17	1587	C	C	C	C	C	C	C	C	T
	1906	C	C	C	C	C	C	T	C	C
18	2022	G	G	G	G	G	A	A	A	G
19	2082	C	C	C	C	C	T	T	T	C
	2169	T	T	T	T	C	C	C	C	C
20	2190	G	G	G	G	G	A	A	A	G
22	2478	G	G	G	G	G	A	A	A	A
23	2616	C	C	C	C	C	T	T	T	T
	2672	G	G	G	G	A	A	A	A	A
24	2754	C	C	C	C	C	T	T	T	T
	2835	T	T	T	T	T	C	C	C	C
25	2976	T	T	T	T	T	G	G	G	G
	2994	C	C	C	C	C	T	T	T	C
	3012	G	G	G	G	G	G	G	G	A
	3021	C	C	C	C	C	T	T	T	T
	3039	C	C	C	C	C	C	C	C	T
	3064	C	C	C	C	C	T	T	T	T
26	3168	C	C	C	C	C	T	T	C	C
27	3270	C	C	C	C	C	C	C	C	T
28	3409	G	G	G	G	G	G	G	G	A
29	3456	G	G	G	G	C	C	C	C	C
30	3591	T	T	T	T	T	T	T	T	C
	3618	T	T	T	T	C	T	T	T	C
	3707	A	A	A	A	A	A	A	A	G
31	3720	C	C	G	C	C	C	C	C	T
	3801	G	G	G	G	G	A	A	G	G
32	4137	A	A	A	A	A	A	A	A	G
	4146	C	C	C	C	C	C	C	C	T
	4158	A	A	A	A	G	A	A	A	A
	4161	G	G	G	G	G	A	A	A	G
	4179	T	T	T	T	T	C	C	C	C
34	4250	C	C	C	C	C	C	C	C	T
	4272	A	A	A	A	A	G	G	G	G
35	4576	T	T	T	T	G	G	G	G	G
37	4659	C	C	C	T	C	T	T	C	T
38	4879	C	C	C	C	C	T	T	nd	T
39	5154	C	C	C	C	C	T	T	C	T
41	5416	C	C	C	T	C	C	C	T	T
	5437	G	G	G	G	G	G	A	A	G
42	5541	T	T	T	T	T	T	T	C	C
	5563	A	A	A	A	A	G	G	G	G

Table 5 (*continued*)

Exon	Nucleotide[a]	C57BL/6J	CBA/CaJ	BUB/BnJ	BLKSW	PERC/EI	MOLF/EI	V/Le	CZECHIL/EI	CAST/EI
46	6073	A	A	A	A	A	G	G	G	G
	6076	A	A	A	A	A	G	G	G	G
	6123	G	G	G	G	G	A	A	A	A
47	6263	C	C	C	C	C	G	G	G	G
	6366	A	A	A	A	A	G	G	G	G
	6472	G	G	G	G	G	G	G	A	G
	6650	A	A	A	A	A	C	C	A	C
	6666	G	G	G	G	G	G	A	G	G
	6672	G	G	G	G	G	A	A	A	A
48	6809	A	A	A	A	A	G	G	G	G
49	7038	C	C	C	C	C	G	G	G	G
51	7281	G	G	G	G	G	A	A	nd	A
	7287	G	G	G	G	G	A	A	nd	A
	7311	T	T	T	T	T	C	C	nd	C
53	7569	A	A	A	A	A	T	T	T	T
	7611	T	T	T	T	T	C	C	C	C
54	7740	T	T	T	T	T	C	C	C	C
	7824	A	A	A	A	A	G	G	G	G
	7833	T	T	T	T	T	C	C	C	C
	7850	G	G	G	G	G	C	C	G	C
55	7856	G	G	G	A	G	A	A	A	A
	7941	A	A	G	A	G	G	G	G	T
56	8106	T	T	T	C	nd	C	C	C	C
	8134	T	T	T	C	nd	C	C	C	C
57	8247	T	T	T	T	T	C	C	C	C
60	8890	C	C	C	C	C	nd	C	G	C
62	9093	C	C	C	C	C	C	C	T	C
65	9375	C	C	C	C	C	T	T	T	T
66	9432	G	G	G	A	G	A	A	A	A
	9462	G	G	G	A	G	A	A	A	A

nd, not determined; BLKSW, Black Swiss.

[a] GenBank accession number: AF308939.

from the ≈26 Mb *D10Mit20–D10Mit95* interval (unpublished observations) did not detect heterozygosity at any of these marker loci. The Black Swiss genome harbors probably a large number of fixed regions.

4.2. Two QTLs control hearing loss in Black Swiss mice

For the QTL mapping study we chose the Black Swiss strain because of its early-onset robust phenotype and

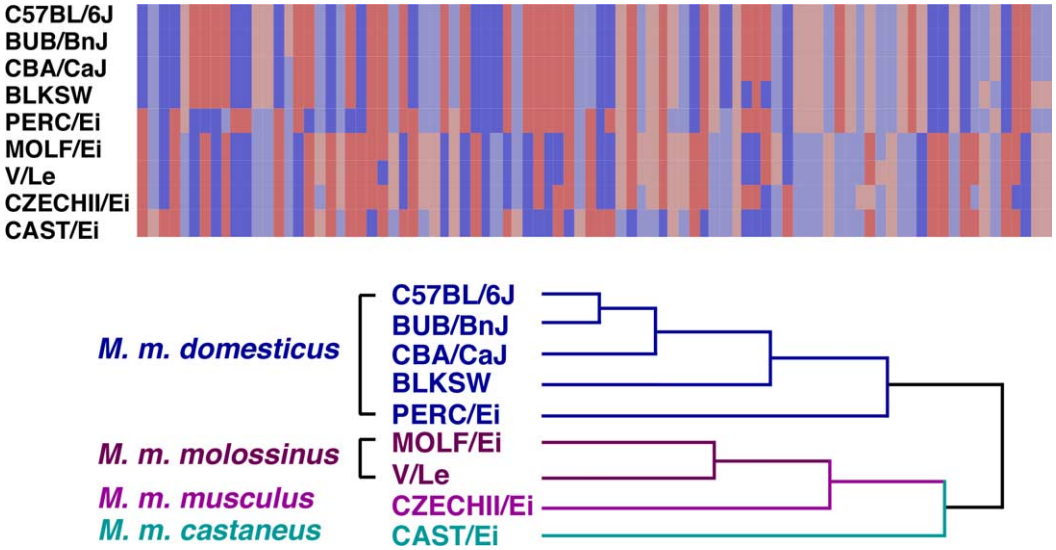

Fig. 6. SNP distribution pattern and genealogical dendrogram. Upper panel shows the distribution pattern of eighty-eight SNPs among nine strains. Each coloured column represents one di-allelic locus ordered from 5′ to 3′ of *Cdh23*. Blue, T; light blue, G; red, C; light red, A. Eleven SNPs were non-synonymous and were described elsewhere (Di Palma et al., 2001a). The lower panel shows the corresponding dendrogram summarizing the phylogenetic relationships across the *Cdh23* locus among the strains as indicated by hierarchical clustering. BLKSW, Black Swiss. M.m., Mus musculus.

absence of the *Cdh23*^{753A} allele. The hearing threshold distributions in both the N2 and F2 populations indicated the presence of a small number of QTLs, which was subsequently confirmed by the linkage scan. The *ahl5* QTL was particularly strong showing almost Mendelian segregation in the intercross. Interestingly, the *ahl* QTL mapped in inbred strains had a similarly strong effect in the C57BL/6J × CAST/Ei backcross (Johnson et al., 1997). *Ahl5* was identified in two crosses, providing strong support for its significance. *Ahl6* however was only detected in the backcross with genome-wide significant LOD scores at the click and 16 kHz stimuli. In the intercross, the effect of *ahl6* may be below the detection limit ($n = 147$) (Darvasi, 1998; Flint et al., 2005) or might be masked by the abundance of CAST/Ei alleles. The number and strength of QTLs in Black Swiss appears to be similar to that observed in inbred strains (Johnson and Zheng, 2002; Johnson et al., 2000, 1997; Nemoto et al., 2004).

The bootstrap-determined 95% CI of the *ahl5* QTL in the backcross was approximately 7 cM (between *D10Mit20* and *D10Mit207*) and in the intercross <24 cM (between *D10Mit38* and *D10Mit95*). Both regions included the *Pcdh15* gene and the interval in the F2 cross also included the *Cdh23* gene. Given their role in mouse, fish and human deafness we sequenced both genes for coding variants, but found none so far that could explain the hearing loss in Black Swiss. *Pcdh15* remains an intriguing candidate, as the QTL peak in the backcross comprised the upstream region of the *Pcdh15* transcription start site. Recently, polymorphisms in the regulatory region of *Tnfsf4* were associated with the atherosclerosis QTL *Ath1* (Wang et al., 2005). Given the large-effect size of *ahl5*, this QTL should be amenable to positional cloning combining genetic mapping, fine-structure haplotype determination and micro-array gene expression analyses.

To test whether the QTLs interact, we compared genotype-dependent threshold means and performed genome-wide regression-analyses but obtained ambiguous results. The regression-analyses did not support an interaction between *ahl5* and *ahl6* and produced only suggestive LOD scores for an interaction between *ahl6* and a suggestive QTL on chr 13. Threshold means sorted by genotype however showed that progeny homozygous at both QTLs were more likely to express thresholds that are higher than those of single homozygotes. This was independent of the age of the genotypic populations suggesting that *ahl6* had an accelerating effect on the *ahl5*-dependent hearing impairment. The combined effect of *ahl5* and *ahl6* did not reach the average hearing loss level observed in the parental Black Swiss strain indicating the existence of additional small-effect QTLs, such as the suggestive QTL on chr 13.

Hearing loss in Black Swiss mice also was noted in a study mapping the monogenic juvenile audiogenic seizure locus *Jams1* (Misawa et al., 2002). *Jams1* was localized to a 1.6 cM region on chr 10 (43.5 cM), which mapped within the 95% confidence interval of *ahl5*. It was noted that hearing loss and seizure susceptibility did not correlate in N2 and F2 mice (Misawa et al., 2002). However, the same allele could underlie both phenotypes as it was shown for the *Mass1*^{Frings} allele, which has been identified as the causative factor in both audiogenic seizures in the Frings strain and hearing loss in the BUB/BnJ strain (Johnson et al., 2005; Skradski et al., 2001).

In summary, five mouse strains each with a distinct hearing loss profile indicative of multifactorial, sensorineural hearing impairment and two new hearing loss QTLs were identified providing the framework to further investigate the genetic architecture and pathology of hearing loss in non-inbred strains.

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