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An Autosomal Recessive Nonsyndromic-Hearing-Loss Locus Identified by DNA Pooling Using Two Inbred Bedouin Kindreds

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Summary

Autosomal recessive nonsyndromic hearing loss (ARNSHL) is the most common form of severe inherited childhood deafness. We present the linkage analysis of two inbred Bedouin kindreds from Israel that are affected with ARNSHL. A rapid genomewide screen for markers linked to the disease was performed by using pooled DNA samples. This screen revealed evidence for linkage with markers D9S922 and D9S301 on chromosome 9q. Genotyping of individuals from both kindreds confirmed linkage to chromosome 9q and a maximum combined LOD score of 26.2 (recombination fraction [θ] .025) with marker D9S927. The disease locus was mapped to a 1.6-cM region of chromosome 9q13-q21, between markers D9S15 and D9S927. The disease segregates with a common haplotype in the two kindreds, at markers D9S927, D9S175, and D9S284 in the linked interval, supporting the hypothesis that both kindreds inherited the deafness gene from a common ancestor. Although this nonsyndromic-hearing-loss (NSHL) locus maps to the same cytogenetic interval as DFNB7, it does not overlap the currently defined DFNB7 interval and may represent (1) a novel form of NSHL in close proximity to DFNB7 or (2) a relocalization of the DFNB7 interval to a region telomeric to its reported location. This study further demonstrates that DNA pooling is an effective means of quickly identifying regions of linkage in inbred families with heterogeneous autosomal recessive disorders.

Introduction

Profound early-onset deafness is present in 4/10,000–11/10,000 children (Marazita et al. 1993). Although deafness may be caused by a number of environmental and disease related factors, ⩾50% of cases are inherited. Approximately 10%–20% of cases of inherited deafness are autosomal dominant, 2%–3% are X linked, and >75% are autosomal recessive (Reardon 1992). Mitochondrially inherited deafness has been documented, as well as mitochondrially linked susceptibility to antibiotic-induced deafness (Bu et al. 1993; Prezant et al. 1993). Nonsyndromic deafness, which occurs without other clearly associated signs or symptoms, accounts for ~70% of all genetically determined deafness (Moatti et al. 1990). Nineteen loci for nonsyndromic hearing loss (NSHL) have been reported—10 associated with dominant inheritance and 9 with recessive inheritance. Recessive loci include DFNB1, linked to chromosome 13q12 (Guilford et al. 1994*a*); DFNB2, linked to chromosome 11q13.5 (Guilford et al. 1994*b*); DFNB3, linked to chromosome 17p11.2-q12 (Friedman et al. 1995); DFNB4, linked to 7q31 (Baldwin et al. 1995); DFNB5, linked to 14q12 (Fukushima et al. 1995*a*); DFNB6, linked to 3p21-p14 (Fukushima et al. 1995*b*); DFNB7, linked to 9q13-q21 (Jain et al. 1995); DFNB8, linked to 21q22 (Veske et al. 1996); and DFNB9, linked to 2p22-p23 (Chaib et al. 1996). DFNB1 shares the same genetic interval as DFNA3, a dominant form of nonsyndromic deafness (Chaib et al. 1994; Scott et al. 1995). Although no specific genes have been identified for DFNB1 or DFNA3, one explanation for this shared interval is that the two forms are allelic.

In this paper we present the linkage analysis of two Bedouin kindreds from Israel that are affected with autosomal recessive nonsyndromic hearing loss. Our data indicate that hearing loss in these families maps to a locus on chromosome 9q13-q21, between markers D9S15 and D9S927. Although this NSHL locus maps to the same cytogenetic region as DFNB7, it does not overlap the currently defined DFNB7 interval and represents either a novel form of NSHL in close proximity to DFNB7 or a relocalization of the DFNB7 interval to a region telomeric to its originally described location (Jain et al. 1995).

Patients and Methods

Patient Evaluation

Two Bedouin kindreds with congenital profound prelingual sensorineural deafness were ascertained through medical records in the Audiology Institute of the Soroka

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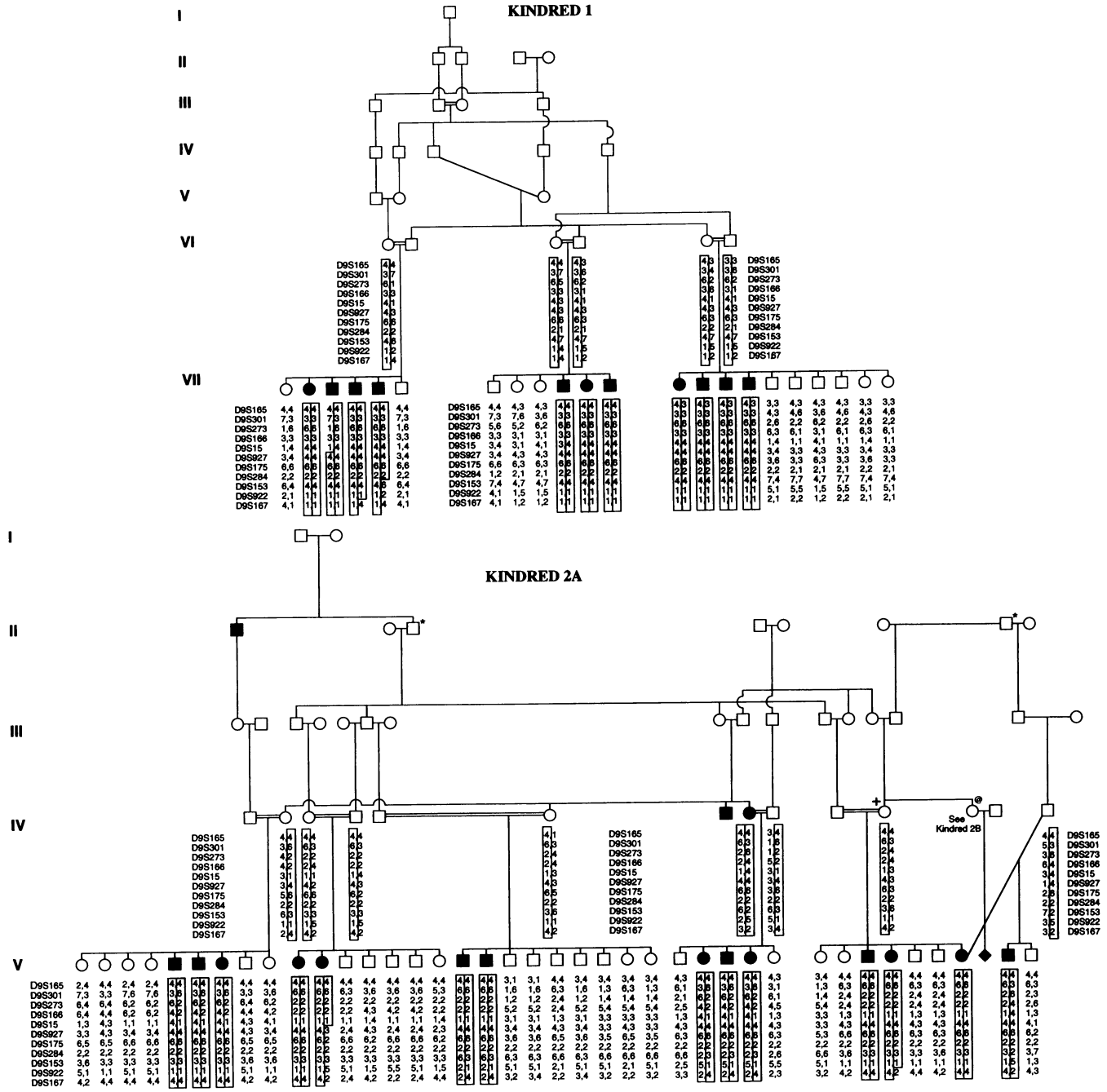

Medical Center and the "NIV" School for the Deaf in Beer-Sheva, Israel. Kindreds 1, 2A, and 2B each have different surnames, and all claim to have originated in Iraq approximately eight generations ago (~200 years ago). Kindreds 2A and 2B appear to be related (the founders of kindreds 2A and 2B were reported to be brothers).

Deaf individuals in each kindred were clinically examined, and, when possible, results of pure-tone audiograms and auditory brainstem–evoked response (ABR) tests were obtained. Appropriate informed consent was obtained from study subjects.

Pooling of DNA

DNA was prepared from blood samples by a standard nonorganic protocol. DNA concentrations were determined by spectrophotometer readings at OD_{260}. Samples were diluted to 20 ng/μl and were amplified by PCR. To insure equal amplification between individual samples, DNA pools were made by combining equal quantities of DNA from each individual. Affected DNA pools were made from DNA from all available affected individuals in each kindred (11 individuals from kindred 1 and 15 individuals from kindred 2A). Unaffected pools were made from a similar number of unaffected siblings and

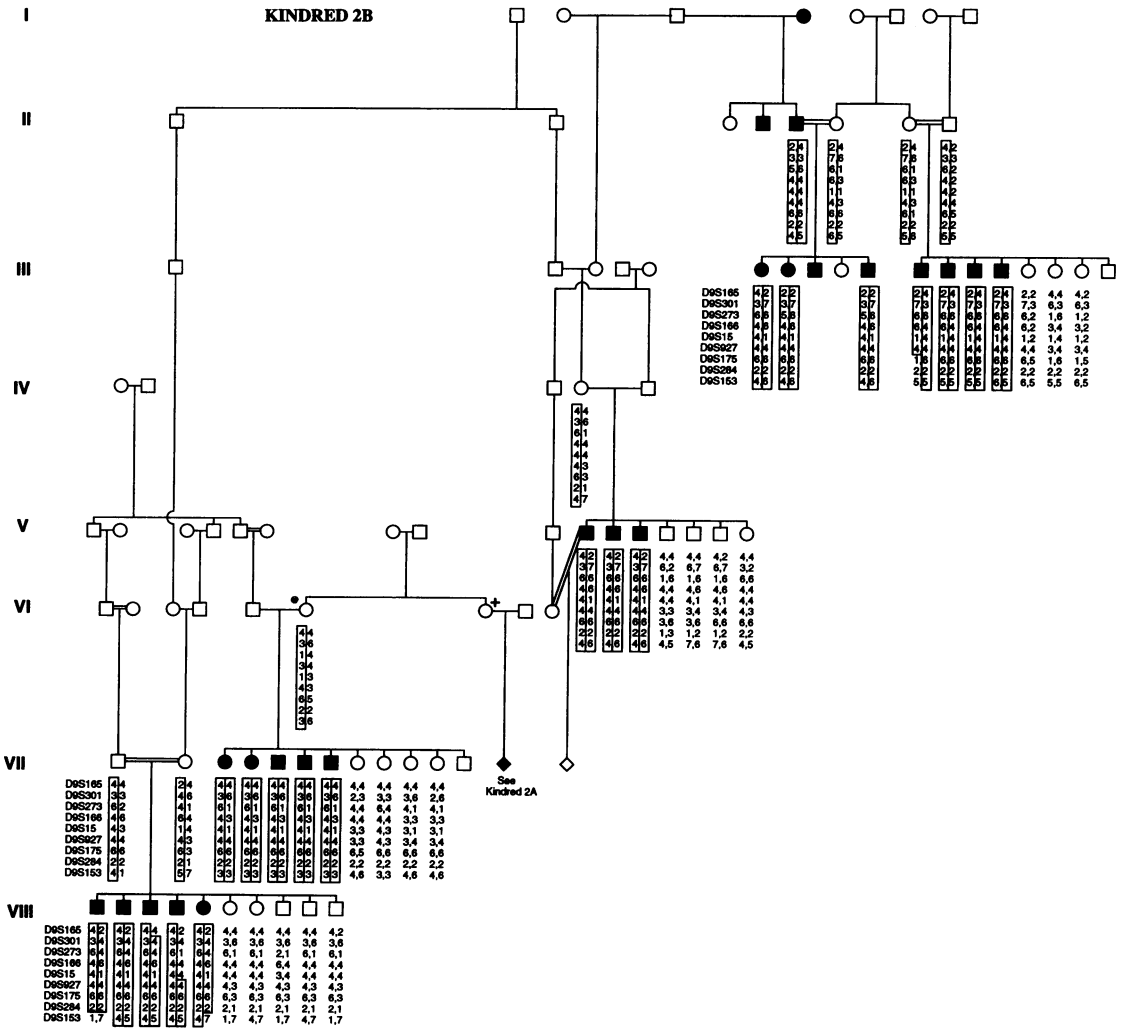

Figure 1 Pedigree of Bedouin kindreds 1, 2A, and 2B. Affected individuals are represented by blackened symbols, and unaffected individuals are represented by unblackened symbols. Genotypic data for markers D9S165, D9S301, D9S273, D9S166, D9S15, D9S927, D9S175, D9S284, D9S153, D9S922, and D9S167 are shown. Boxes indicate the haplotype of the disease chromosome. Asterisks (*) indicates a single individual who is shown twice in order to simplify the pedigree drawing for kindred 2A. The symbols + and @ indicate individuals who appear both in kindred 2A and in kindred 2B. In kindred 1, individuals VII-3 and VII-5 define the disease interval for kindred 1 to the region between D9S15 and D9S153. A spontaneous mutation or a double-crossover event is seen in individual VII-10 with marker D9S15. In kindred 2A, individual V-11 defines the disease interval for kindred 2A as centromeric to D9S927. Because of the complexity of the pedigree, LOD scores for this kindred were calculated with a break between individual III-9 and his parents II-4 and II-5. Likely spontaneous mutations are found in V-13 with marker D9S166 and in V-16 with marker D9S301. In kindred 2B, individuals III-11 and VIII-4 define the disease interval for kindred 2B as the region between D9S15 and D9S175.

parents (10 siblings and 1 parent in kindred 1 and 15 siblings from generation V in kindred 2A).

PCR Amplification

Short tandem-repeat–polymorphisms (STRPs) screening set version 6, developed from markers generated by the Cooperative Human Linkage Center (Sheffield et al. 1995*b*), available from Research Genetics, were used in this study. PCR was performed with 40 ng pooled DNA in a 8.4-ml PCR reaction mixture containing 1.25 μl PCR buffer (100 mM TRIS-HCl pH 8.8), 500 mM KCl, 15 mM $MgCl_2$, and 0.01% [w/v] gelatin), 200 μM each of dATP, dCTP, dGTP, and dTTP, 2.5 pmol each of forward and reverse primer, and 0.25 U *Taq* polymerase. Samples were subjected to 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. PCR products were analyzed on 6% denaturing polyacrylamide gels (7.7 M urea). Visualization of the DNA was accomplished by the silver-staining protocol of Bassam et al. (1991). Genotyping of individual samples was performed in an identical manner, except that 40 ng DNA from each individual was used as the PCR template.

Statistical Analysis

Linkage analysis for all markers was performed by the linkage program MENDEL on a Sun workstation

(Lange et al. 1988). Linkage was tested by the LOD-score method, and a LOD score ≥3.0 was used as the criterion for significance. For kindred 1, linkage analysis was conducted with all of the individuals and their respective relationships from generation IV to VII. Individuals and relationship information from generations I–III were not used to calculate LOD scores. Kindred 2A was analyzed with all individual and relationships intact, except for a single break between individual III-9 and his parents, individuals II-4 and II-5 in figure 1 (kindred 2A). Because of the complexity of kindred 2A, it was sometimes necessary to reduce the number of alleles when markers with four or more alleles were analyzed. This allele reduction was accomplished by assigning the same number to two alleles that did not segregate with the disease. Kindred 2B was analyzed as a single kindred with all individuals and relationships intact.

Results

Clinical Findings

Hearing evaluation of affected individuals in both kindreds revealed profound prelingual neurosensory hearing loss. Representative pure-tone audiograms of several deaf individuals from both kindreds showed profound bilateral hearing loss of 90–110 decibels in all speech frequencies (500–4,000 Hz). ABR examinations were also compatible with profound hearing impairment. All hearing-impaired individuals had an otherwise normal phenotype with normal developmental milestones and the absence of external ear abnormalities or other medical problems. All were of normal intelligence.

Linkage Mapping Using Pooled DNA Samples

Because of the highly inbred nature of the kindreds in this study, rapid screening for markers linked to the disease was possible by use of pooled DNA samples (Sheffield et al. 1994, 1995*a*; Carmi et al. 1995; Nystuen et al. 1996). In this method, STRPs amplified from pools of DNA from affected and unaffected individuals are electrophoresed on denaturing acrylamide gels and are visualized by silver staining. The relative intensity of each resulting allelic band on the polyacrylamide gel is approximately proportional to the frequency of that allele in the pooled DNA samples. If a disease gene has been inherited by the affected individuals from a common ancestor, the banding pattern of an STRP that is linked to the disease phenotype will show a shift in intensity, toward a predominant allele in the affected pool, compared with the unaffected pool. In the case of a recessive gene inherited from a common ancestor, individuals will share a single homozygous allele at the disease locus (individuals in the affected pool will be homozygous for both the disease gene and the linked STRP allele).

A genomewide screen using affected and unaffected pools from each of the two Bedouin kindreds (kindreds 1 and 2A) was conducted. For kindred 1, 96 STRPs were screened, and three markers showed a reduced number of alleles in the affected DNA pool compared with the unaffected pool. Two of these markers, D9S922 and D9S301, were located within the same region of chromosome 9q. For kindred 2A, 69 STRPs were screened, and 2 showed a reduction in the number of alleles in the affected DNA pool compared with the unaffected pool. One of the markers was D9S301. On the basis of the DNA-pooling results, individual members of kindreds 1 and 2A were genotyped with D9S922, D9S301, and nine neighboring chromosome 9 markers.

Linkage was tested by the LOD-score method, and a score of ≥3.0 was used as the criterion for significance. Linkage was found in kindred 1 by using marker D9S301, with a LOD score of 4.98 at $\theta = .025$, and by using marker D9S922, with a LOD score of 4.96 at $\theta = .025$. Linkage was found in kindred 2A at D9S301, with a LOD score of 5.79 at $\theta = .05$. Evaluation of marker D9S922 yielded a LOD score below the level of significance (LOD score of 1.83 at $\theta = .10$). With linkage established for kindred 2A, linkage analysis was also performed for additional family members composing kindred 2B. Initial linkage for kindred 2B was found with marker D9S175 (a marker between D9S301 and D9S922). The LOD score at this marker was 5.06 at $\theta = .025$. LOD scores were determined for a number of other markers in this region, as summarized in table 1. Marker D9S927 had the highest LOD scores of the markers tested (kindred 1, maximum LOD score 7.15 at $\theta = .00$; kindred 2A, maximum LOD score 9.42 at $\theta = .05$; and kindred 2B, maximum LOD score 10.12 at $\theta = .00$).

Fine Mapping

Linkage of deafness in kindreds 1 and 2 to chromosome 9q raised the question of whether the disorder was caused by a previously reported ARNSHL locus, DFNB7, which maps to chromosome 9q13-q21 (Jain et al. 1995). In order to answer this question and to refine the genetic interval, additional genotyping followed by recombination analysis was performed.

Recombination analysis was performed independently for each of the kindreds in figure 1. Regions of crossover were defined by comparing data from each affected individual with data from his or her siblings and parents, rather than by attempting to analyze each pedigree as a whole. This conservative approach minimizes the likelihood of drawing inaccurate conclusions if affected individuals inherit the disease gene from more than one ancestor.

In kindred 1, affected individual VII-3 had a recombination event between D9S15 and D9S927. Affected individual VII-5 of kindred 1 showed a recombination event

Table 1

Two-Point LOD Scores between the ARNSHL Locus of Kindreds 1 and 2 and Associated Markers on Chromosome 9

Kindred and Locus	LOD Score at θ = .0	.10	.20	.30	.40	Maximum Lod Score	Maximum θ
1:							
D9S273	.92	4.55	3.42	2.12	.79	4.98	.025
D9S166	4.19	3.29	2.30	1.24	.33	3.97	.025
D9S15	−∞	3.75	2.95	1.86	.70	3.84	.075
D9S927	7.15	5.67	4.13	2.53	.95	7.15	.000
D9S175	4.15	3.32	2.43	1.49	.54	4.15	.000
D9S153	.98	4.54	3.36	2.05	.75	5.02	.025
2A:							
D9S273	4.60	5.60	4.07	2.36	.82	6.24	.025
D9S166	−∞	5.56	4.19	2.44	.88	5.89	.075
D9S15	−∞	5.07	3.86	2.27	.79	5.21	.075
D9S927	6.05	8.38	6.00	3.55	1.31	9.42	.050
D9S175	−∞	5.82	4.36	2.64	.99	6.23	.050
D9S153	−∞	1.44	1.40	.85	.26	1.53	.150
2B:							
D9S273	2.05	7.37	5.61	3.46	1.26	7.89	.050
D9S166	−∞	4.02	2.92	1.57	.52	4.11	.075
D9S15	−∞	5.26	3.98	2.38	.81	5.56	.050
D9S927	10.12	8.06	5.88	3.63	1.42	10.12	.000
D9S175	1.00	4.63	3.47	2.14	.84	5.06	.025
D9S284	−∞	2.40	1.92	1.21	.48	2.42	.075
D9S153	−∞	.98	1.69	1.26	.47	1.69	.200

between D9S284 and D9S153. These recombination events mark the centromeric (D9S15) and telomeric (D9S153) boundaries of the disease interval for kindred 1 (see fig. 1, kindred 1). This interval does not overlap the DFNB7 interval reported by Jain et al. (1995), which maps between D9S50 (centromeric) and D9S15 (telomeric).

In kindred 2A, affected individual V-11 had a recombination event between D9S15 and D9S927. This recombination marks the telomeric boundary of the disease gene (D9S927) of kindred 2A. No centromeric recombination events were observed with the STRPs genotyped in this kindred. We conclude from these data that the disease locus in kindred 2A maps centromeric to D9S927 (see fig. 1, kindred 2A). However, it is not possible to establish whether the disease in this kindred is located at the DFNB7 locus or in the novel interval defined by kindred 1.

To clarify the placement of the disease locus, a group of related individuals in kindred 2B were genotyped. Affected individual VIII-4 of kindred 2B had a recombination event between D9S15 and D9S927. Affected individual III-11 of the same kindred had a recombination event between D9S927 and D9S175. These recombinations define the centromeric (D9S15) and telomeric (D9S175) boundaries of the disease interval in kindred 2B. With the combined data from kindreds 2A and 2B, we can exclude the reported DFNB7 locus and define a novel disease locus for kindred 2. This locus is in a 1.6-cM interval of chromosome 9q13-q21, bounded by D9S15 and D9S927, as shown in figure 2, and is included in the disease interval defined by kindred 1.

To determine whether kindreds 1 and 2 were likely to be related, we compared the haplotypes of these kindreds with markers in the linked interval. The disease segregates with a common haplotype with markers D9S927, D9S175, and D9S284 located in the linked interval.

Discussion

We present the linkage analysis of two Bedouin kindreds from Israel that are affected with ARNSHL. The disease locus in these kindreds is within a 1.6-cM region of chromosome 9q13-q21, defined by D9S15 and D9S927.

The use of highly inbred families and a genomewide search scheme, which uses DNA pooled from affected and unaffected individuals as a PCR template, has proved to be effective in mapping disease loci (Sheffield et al. 1994, 1995*a*; Carmi et al. 1995; Nystuen et al. 1996). In this study, a rapid genomewide screening for markers linked to the disease revealed initial evidence for linkage with markers D9S922 and D9S301 on chromosome 9q. Linkage to this region was confirmed through genotyping of these and other STRPs on chro-

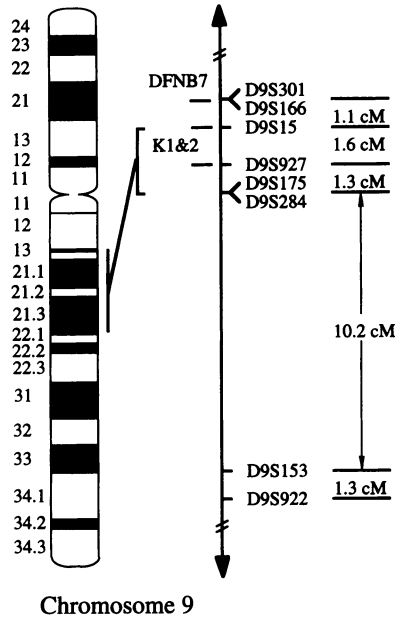

Figure 2 Schematic representation of chromosome 9, showing the relative location of the disease interval defined by kindreds 1 and 2, DFNB7, and polymorphic markers. The genetic map is based on data from the Cooperative Human Linkage Center (http://www.chlc.org) (Sheffield et al. 1995*b*).

mosome 9q in both kindreds. This study further demonstrates that DNA pooling is an effective means of quickly identifying regions of linkage in inbred families with recessive disease.

Evidence for linkage to chromosome 9q is compelling. A combined LOD score of 26.20 (θ = .025) was found with marker D9S927. The disease interval was fine-mapped to a 1.6-cM region of chromosome 9q13-q21 by using observed recombination events. Haplotype data supported the narrowest disease interval defined by observed recombination but did not result in further narrowing of the interval.

Both kindreds are from the same region of Israel and share a common ethnic background that strongly encourages consanguineous marriage. It is possible that affected individuals in both kindreds inherited the deafness gene from a common ancestor. The fact that the disease segregates with a common haplotype for markers D9S927, D9S175, and D9S284 in the linked interval supports this hypothesis.

Since both the disease locus of kindreds 1 and 2 and DFNB7 have both been localized to a small region of chromosome 9q, it is important to determine whether these loci overlap. The DFNB7 interval has been reported by Jain et al. (1995) to be located between D9S50 and D9S15, with D9S15 defining the most telomeric border. The disease interval defined by kindreds 1 and 2 maps between D9S15 and D9S927, with D9S15 defining the centromeric border, on the basis of observed recombinations found in each of the study kindreds. These recombination events place the disease loci of each of these kindreds outside the reported DFNB7 interval.

It should also be noted that the DFNB7 locus had been defined with the assumption that marker D9S166 is located telomeric to D9S15, whereas our work assumes that D9S166 is located centromeric to D9S15. Despite the ambiguity of the placement of D9S166 in relation to D9S15, the region between these two markers has been excluded from the disease interval defined by this study and has been reported to be excluded from the DFNB7 interval. Since the region between D9S166 and D9S15 has been mutually excluded from both regions, it follows that these two loci do not share a common border but are separated by the distance between D9S166 and D9S15, ~1.1 cM (see fig. 2).

Although it appears that the disease locus defined by kindreds 1 and 2 represents a novel ARNSHL locus, it is also possible that this locus represents a relocalization of the DFNB7 interval. The DFNB7 locus is presently defined by Jain et al. using an inbred family with six affected individuals. All affected individuals in the DFNB7 family are homozygous at marker D9S301, and ancestral recombinations are seen in some affected individuals with the flanking markers D9S50 and D9S15. It is possible that a second region of homozygosity exists in the DFNB7 family, in the disease interval defined by kindreds 1 and 2. Genotyping of newly available markers and the identification of additional families with linkage to this region may establish whether the disease locus defined by kindreds 1 and 2 represents a novel ARNSHL locus or a relocalization of the DFNB7 interval to a region telomeric to its originally described location.

During our investigation, we encountered what appear to be spontaneous mutations of STRP markers in three of our study subjects. Some spontaneous mutations are easily identified by the appearance of a new allele not found in either parent (see both kindred 2A individual V-13 with marker D9S166 and kindred 2A individual V-16 with marker D9S301). Other apparent mutations could also be interpreted as unusual double crossovers (see kindred 1 individual VII-10 with marker D9S15). Although the spontaneous mutations documented in this study do not affect our conclusions, it is important to note that spontaneous mutations can be a source of error, and caution must be used when more than one interpretation is possible.

The disease locus identified by kindreds 1 and 2 is the second form of ARNSHL to be reported in the Bedouin population in Israel. The first form of ARNSHL documented in the Bedouin population was localized to the DFNB1 locus in a large, highly inbred family similar to those used in this study (Scott et al. 1995).

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