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Localization of the achondroplasia gene to the distal 2.5 Mb of human chromosome 4p

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Achondroplasia has been mapped to 4p16.3 using 18 multigenerational families with achondroplasia and 10 short tandem repeat polymorphic markers from this region. No evidence of genetic heterogeneity was found. Analysis of a recombinant family localizes the achondroplasia locus to the 2.5 Mb region between D4S43 and the telomere. Multipoint linkage analysis favors placement telomeric of D4S412. The establishment of closely linked markers will facilitate positional cloning of the achondroplasia gene and permit prenatal diagnosis of homozygous achondroplasia for at risk couples.

INTRODUCTION

Achondroplasia (MIM 100800) is the most common form of disproportionate short stature and is transmitted as an autosomal dominant trait with virtually complete penetrance (1). The gene frequency has been estimated to be between 1/15,000 and 1/40,000 (2–4). More than 80% of recorded cases are sporadic, presumably due to new mutations; sporadic cases have been associated with increased paternal age (5). Achondroplasia is easily recognizable at birth and there is usually little confusion about the diagnosis. The classic physical features include rhizomelic (short limbed) dwarfism, megalencephaly, characteristic facies with frontal bossing and midface hypoplasia, exaggerated lumbar lordosis, limitation of elbow extension, genu varum and trident hand. The radiologic features include small cuboid shaped vertebral bodies with progressive narrowing of caudal interpedicular distance, lumbar lordosis, thoracolumbar kyphosis with occasional anterior beaking of the first and second lumbar vertebrae, small iliac wings with a narrow greater sciatic notch and short tubular bones with metaphyseal flare and cupping (1,2).

There are many medical complications associated with achondroplasia, especially in the homozygous state. Heterozygotes have normal intelligence but have delayed motor milestones. Communicating hydrocephalus and cervicomedullary compression, secondary to a small foramen magnum, occur in some individuals and may be associated with life threatening apnea (5). Caudal narrowing of the lumbar spinal canal leads to symptomatic spinal stenosis with age. Orthopedic problems are common (6). Mortality is particularly increased for people with achondroplasia from birth to 4 years and in the late fourth to fifth decades of life (7).

Homozygous achondroplasia, i.e. the condition resulting from inheritance of an achondroplasia allele from each of two affected parents, results in a much more severe phenotype with quantitatively different radiological changes (8). The condition is virtually always lethal in the first year of life and is characterized by a small thoracic cage with respiratory insufficiency and severe neurologic deficits which include hydrocephalus and brainstem compression (8,9). There is currently no reliable method for prenatal diagnosis of homozygous achondroplasia for couples at risk.

Several candidate genes for achondroplasia have been studied. Evidence for an association with type II collagen (10) was subsequently retracted and disproved (11,12). The chondroitin sulfate proteoglycan core protein and type X collagen were also excluded by linkage analysis (13,14). Linkage to neurofibromatosis type I was proposed on the basis of a child who had achondroplasia and NF-1 in conjunction with a translocation of chromosome 17 (15) but was subsequently disproved (16,17). Association with neuroblastoma on chromosome 1 has been reported (18) but no further studies in this region have been published.

We have used short tandem repeat markers throughout the human genome to identify a region genetically linked to achondroplasia. One hundred and twenty-five markers covering approximately 50% of the genome were excluded (CAF, RIOL, TWH, unpublished data). Linkage of the achondroplasia phenotype to markers on human chromosome 4p was subsequently established as the result of our collaborative effort with colleagues at the University of Connecticut (19). In this study, 10 markers on chromosome 4p were tested to establish their linkage relationships to achondroplasia in 18 multigenerational families.

RESULTS

Our results corroborate the mapping of the achondroplasia locus to the distal portion of chromosome 4p (19,20). Markers D4S127, D4S182, D4S412, D4S43, D4S115 and D4S227 had maximum LOD scores greater than 3 (Table 1). The highest two-point LOD score achieved was with D4S412 which reached a value of 6.44 at $\theta = 0.02$. No recombinants were seen with D4S227, D4S182, D4S114 and D4S115, with maximum LOD scores at $\theta = 0$ of

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Table 1. Achondroplasia LOD scores at recombination distances shown

Locus	0.00	0.01	0.05	0.10	0.15	0.20	0.30	0.40	Z max.	θ
D4S394	−∞	−14.9	−2.04	−0.37	0.31	0.59	0.62	0.35	0.67	0.25
D4S431	−∞	−8.22	−0.24	0.69	0.99	1.02	0.73	0.30	1.03	0.18
D4S432	−∞	0.26	2.97	2.86	2.52	2.09	1.21	0.45	2.98	0.06
D4S127	−∞	3.46	4.49	4.09	3.57	3.01	1.85	0.79	4.54	0.03
D4S412	−∞	5.56	6.16	5.35	4.44	3.53	1.84	0.55	6.44	0.02
D4S182	3.99	3.98	3.63	3.21	2.75	2.29	1.39	0.58	3.99	0.00
D4S43	−∞	2.21	3.40	3.12	2.70	2.23	1.28	0.49	3.40	0.04
D4S114	2.31	2.31	2.04	1.76	1.47	1.19	0.66	0.22	2.31	0.00
D4S115	3.91	3.90	3.34	2.76	2.21	1.69	0.81	0.22	3.91	0.00
D4S227	4.83	4.81	4.20	3.58	2.98	2.40	1.33	0.49	4.83	0.00

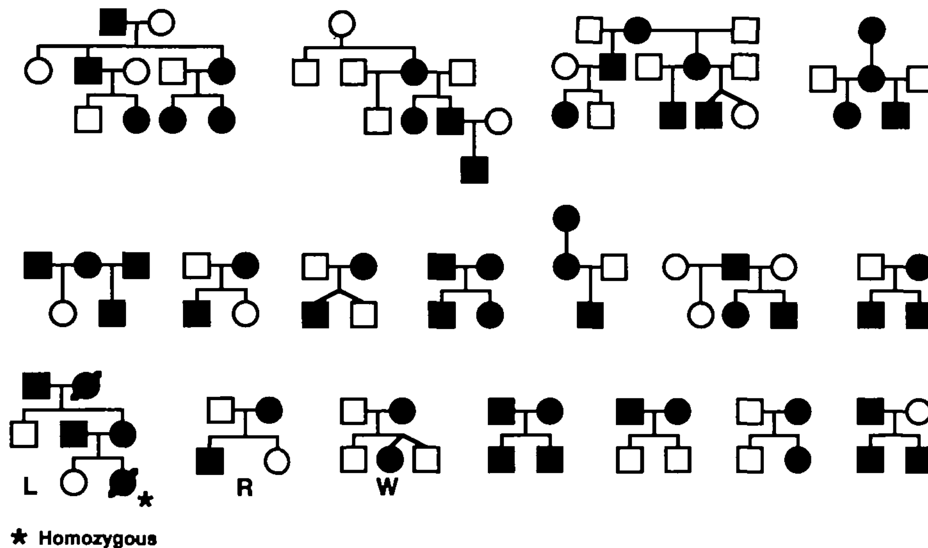

Figure 1. Families used for linkage analysis.

4.83, 3.99, 2.31 and 3.91 respectively. Using the HOMOG program (21) no evidence for genetic heterogeneity was found. However, the small size of most of the families (as illustrated in Figure 1) does not give much power to detect heterogeneity. The multipoint analysis (Figure 2) provides strong evidence for the placement of the achondroplasia locus telomeric to D4S431. The most likely position, with a maximum LOD score of 9.9, is telomeric to D4S115. This position is approximately 100 times more likely than placement between D4S127 and D4S431.

We have also performed haplotype analysis in an attempt to place the achondroplasia locus relative to physically mapped markers. Informative recombination events were identified in three families (Figure 3A–C). Family L had a recombination event between D4S43 and D4S115 which places the achondroplasia locus distal to D4S43 (Figure 3A). A second recombination event occurred in family L between D4S431 and D4S432 in individual II-2, whose daughter (III-2) was clinically homozygous for achondroplasia and died in the first year of life. This recombinant places the achondroplasia locus distal to D4S431. Families W and R, depicted in Figure 3B and 3C, show recombination between achondroplasia and D4S432, both of which indicate that the achondroplasia locus is distal to D4S432.

DISCUSSION

Since not all the markers studied have been placed on both the physical and genetic maps it is not possible to completely reconcile the results of the multipoint linkage analysis and the haplotype analysis of recombinant chromosomes. Two of the markers used in this study (D4S432 and D4S412) have not yet been placed on the physical map of 4p. Although the haplotypes illustrated in Figure 3 suggest that D4S432 lies distal to D4S431 and centromeric to D4S127, the linkage distance is unknown and the data generated in these families is insufficient to establish that distance. D4S43 has not been placed on the genetic map and therefore could not be used in the multipoint analysis. Although the multipoint analysis does not formally exclude a position centromeric of D4S127, the haplotype analysis in Family L strongly suggests that the locus lies distal to D4S43 (Figure 4).

Assignment of the achondroplasia locus to distal chromosome 4p will facilitate positional cloning of the achondroplasia gene. Of the several known expressed sequeces in the region, FGFR3, encoding a fibroblast growth factor receptor, is the most likely candidate gene for achondroplasia. FGFR3 is distal to D4S43, within 500 kb on the physical map (22). The murine homolog

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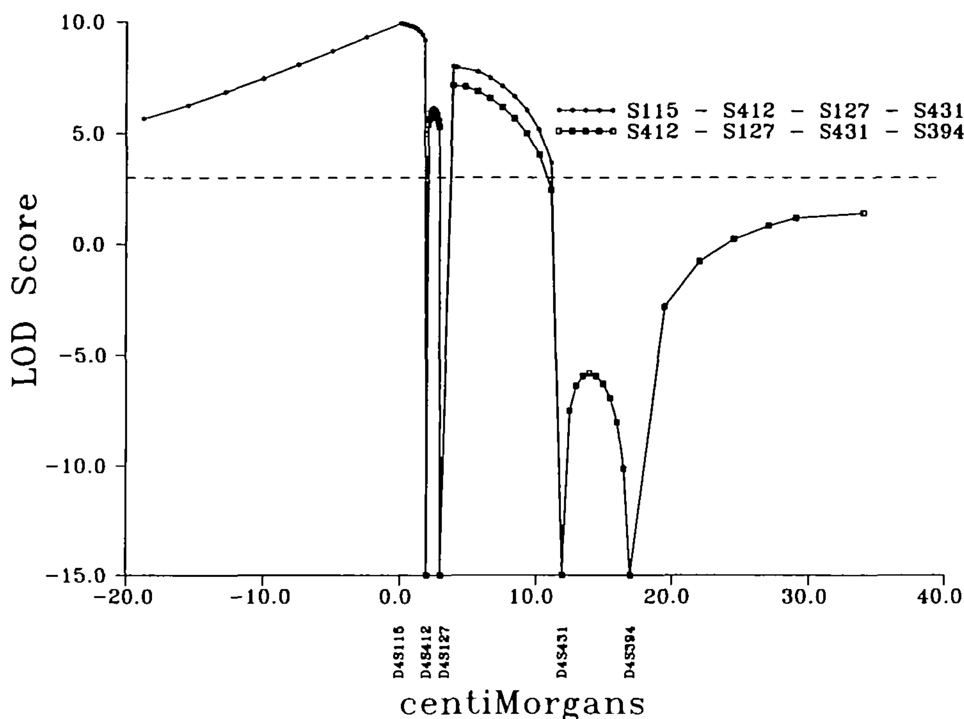

Figure 2. Multipoint linkage analysis of the achondroplasia locus to genetic markers on chromosome 4p. Analysis was performed as described in Materials and Methods.

is expressed during endochondral ossification and in the cartilage rudiments of developing bone, as well as in the developing nervous system, cochlea, and ocular lens (23). In addition to FGFR3, ZNF141 (a zinc finger gene; 24) and HDA1-1 (unknown function; 25) are found in the candidate region and are possible candidate genes.Three other genes have been mapped to the candidate region, α-L-iduronidase (26), myosin light chain 5 (27) and rod photoreceptor cGMP phosphodiesterase β-subunit (28). Although all three of these have known functions that are unlikely to be related to achondroplasia they have yet to be formally excluded.

The candidate region for achondroplasia, as defined by the recombinant events in Family L, includes the 2.2 Mb smallest region of deletion overlap for the Wolf–Hirschhorn (4p-) syndrome (24). This 2.2 Mb region begins 80 kb telomeric to D4S43 and extends to within 200 kb of the telomere. It is likely, therefore, that the achondroplasia locus is deleted in patients with Wolf–Hirschhorn syndrome. Since there is no overlap between the Wolf–Hirshhorn and achondroplasia phenotypes, achondroplasia is probably the result of a dominant-negative mechanism, and not merely the consequence of haploinsufficiency for the gene product.

The etiology of achondroplasia remains unknown. The name achondroplasia is a misnomer because cartilage is formed. Early descriptions of severe disturbances of endochondral ossification were misleading as many of the cases were probably misdiagnosed cases of other lethal forms of dwarfism (2). Rimoin *et al* reported minor quantitative changes in the width of endochondral ossification zones (29) but others have reported more extensive quantitative and qualitative disruption of ossification at other growth plates (30,31). More recently Briner *et al* (32) performed a detailed investigation of all major ossification centers in an infant with heterozygous achondroplasia and demonstrated a wide variability of changes between ossification centers. Growth plates of tubular bones were most severely affected and showed clusters of large, disorganized and vacuolized cells, focal vacuolizations of intercellular matrix, replacement of endochondral ossification by fibrous bands and precocious calcification with focal membranous ossification. Vertebral bodies and iliac bones showed minor narrowing of ossification zones. Mackler and Shepard have presented evidence that there is a deficiency of cytochrome a_3 in the growth plates of humans with achondroplasia as well as in rabbits with a phenotypically similar form of dwarfism (33).

In concordance with other studies (19,20), we found no evidence for genetic heterogeneity. The distinctive phenotype of achondroplasia therefore most likely results from defects in a single gene at 4p16.3. The data of Le Merrer *et al* (20) are consistent with the previous hypothesis that hypochondroplasia, a skeletal dysplasia which resembles achondroplasia but with a less pronounced phenotype, is allelic with achondroplasia (34). However, more hypochondroplasia families will have to be studied to give a significant LOD score.

The recognition of markers tightly linked to the achondroplasia locus will have immediate clinical implications. In some families at risk for homozygous infants with achondroplasia, the linkage method will allow prenatal diagnosis and offer reproductive options not previously available. The availability of prenatal diagnosis in these families is important because infants with homozygous achondroplasia invariably die within the first year of life. Pregnancy is particularly arduous for women with achondroplasia, as it exacerbates symptomatic spinal stenosis and further compromises an already limited respiratory system. Moreover, deliveries are necessarily by Caesarian section because

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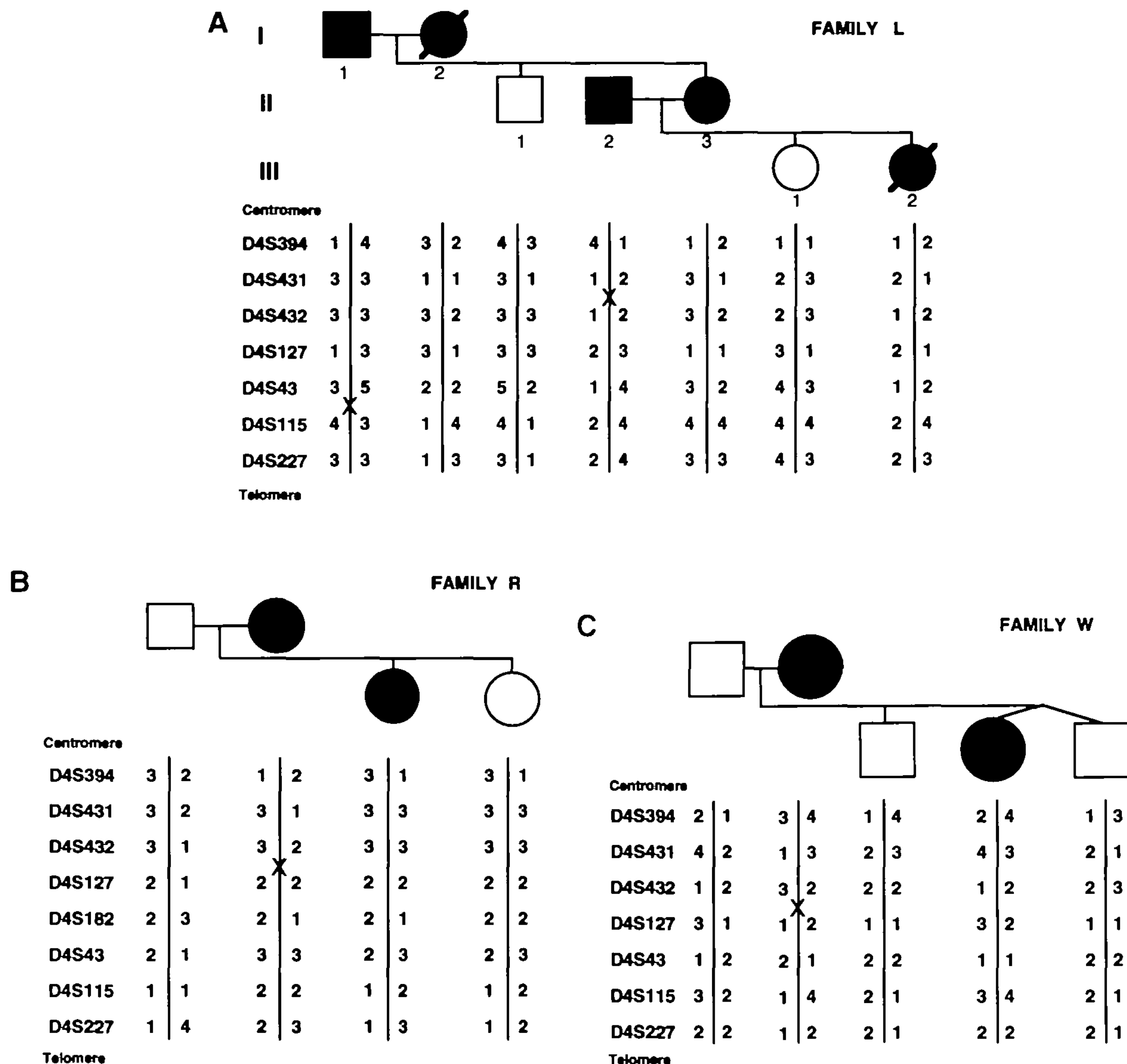

Figure 3. Recombinant chromosomes. Haplotypes are shown for markers D4S394, D4S431, D4S432, D4S127, D4S182, D4S43, D4S115 and D4S227. The L and W families were both uniformly homozygous at the D4S182 locus and therefore this marker was not depicted in these families. A depicts family L which has had at least two recombination events, as shown. The genotype of individual I-2 was reconstructed from the other genotypes. The event between D4S43 and D4S115 places the achondroplasia locus distal to D4S43. B and C depict families R and W which both demonstrate a crossover between D4S432 and D4S127, and place the achondroplasia locus distal to D4S432.

of the small pelvic outlet. For all of these reasons, not to mention the emotional trial of carrying and delivering an infant with a fatal phenotype, the linkage method for prenatal diagnosis of homozygous achondroplasia will be extremely valuable. Until the achondroplasia gene itself is identified and direct detection of mutations is possible, linkage can only be applied predictably in those families where both parents come from multiplex pedigrees, or in situations where a homozygous or unaffected infant has been born previously and tissue or DNA is available from the previous infant for testing.

MATERIALS AND METHODS

Eighteen multiplex families with achondroplasia were identified through the Medical Genetics Clinics at the Johns Hopkins Hospital and University of Texas Medical Center at Houston and through the membership of the Little People of America Inc (Figure 1). All affected individuals have heterozygous achondroplasia except for individual III-2 in Family L, who had homozygous achondroplasia based on the severity of her clinical and radiographic phenotype and markedly small foramen magnum. Informed consent for venipuncture and DNA studies was obtained and DNA was isolated from blood leukocytes as previously described (35).

The polymerase chain reaction (PCR) was used to amplify 10 short tandem repeat markers on 4p (Table 2). All PCR reactions, except D4S115, were performed in 10 μl volumes in 96 well microtiter dishes using Perkin Elmer Amplitaq polymerase and dNTPs under conditions recommended by the manufacturer. Markers D4S182 and D4S431 required an increased $MgCl_2$ concentration of 2.5 mM for optimal amplification. Unlabelled PCR reactions were run for each marker to optimize conditions. Radiolabelled PCR reactions used 50 ng of DNA from each patient, 7 pmol of unlabelled primer and 1 pmol of primer end labelled with ^{32}P-γ-ATP. PCR reactions were carried out for 30 cycles with denaturing, annealing and extension times of 30 seconds. The products were electrophoresed on 6% polyacrylamide DNA sequencing gels (Sequagel) and autoradiographed. PCR for D4S115 was performed as described (36) except the $MgCl_2$ concentration was reduced to 5.5 mM.

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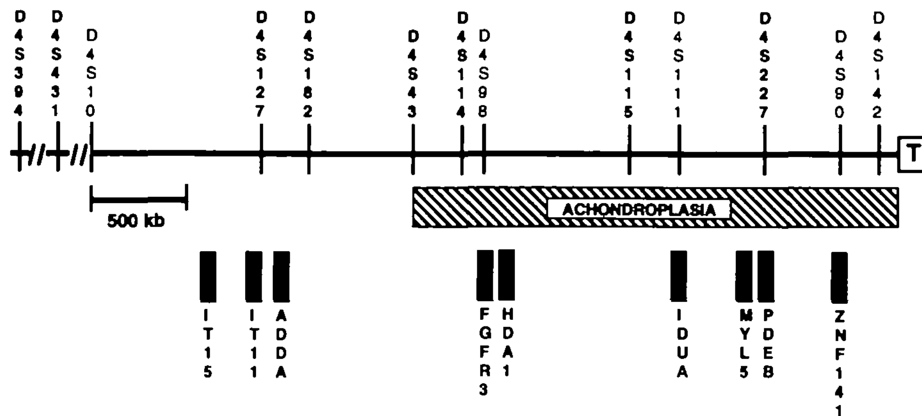

Figure 4. Physical map of 4p16.3. Approximate positions of genes and anonymous markers were derived from references 24,25, 44–49. Markers used in this study are shown in bold. Gene positions are indicated by the black boxes below the map and the achondroplasia candidate region is indicated by the hatched box. IDUA is α-L-iduoronidase, MYL5 is myosin light chain 5 and PDEB is phosphodiesterase β-subunit. Map is drawn approximately to scale distal of D4S10; the physical distances between D4S394, D4S431 and D4S10 are not known.

Table 2. Polymorphic markers used for linkage analysis

	Sequence	Anneal. Temp.	Ref
D4S394	5'-CCCTTGAGCATCCTGACTTC-3' 5'-GAGTGAGCCCCTGTACTCCA-3'	60°C	39
D4S431	5'-AGGCATACTAGGCCGTATT-3' 5'-TTCCCATCAGCGTCTTC-3'	52°C	39
D4S432	5'-ACTCTGAAGGCTGAGATGGG-3' 5'-CTGAACCGCAGATCCCC-3'	56°C	39
D4S127	5'-CCTCTGTTTGCAATCCATTT-3' 5'-GTCCCTTGCATGCCCTGGCT-3'	54°C	40
D4S182	5'-GGATCCAATCCAAAGGAAAGTTCC-3' 5'-TTTTTCTCCCCCCATGACACCATG-3'	55°C	41
D4S43	5'-CTTCCTTTTCTCTCTGGATGC-3' 5'-ACATCACGCTTATCTTTGGGG-3'	56°C	42
D4S412	5'-ACTACCGCCAGGCACT-3' 5'-CTAAGATATGAAAACCTAAGGGA-3'	55°C	39
D4S114	5'-TATGGCTCACCTCTCATCTCTGTG-3' 5'-CTGTCATAAGAGGGCCCAGTACTC-3'	65°C	(D.Tagle, pers. comm.)
D4S115	5'-GGTGATGTAGAGGAGGCACCT-3' 5'-GGTGATGACTTCTCATGAGC-3'	60°C	36
D4S227	5'-GATCAGGTATCTACTTTCTTATGTAC-3' 5'-GTCCACCCACGGGAACTGATGTTTC-3'	55°C	43

Genotypes were read and confirmed by at least two of us. Two point LOD scores and maximum likelihood estimates were generated using the MLINK and ILINK subroutines of the LINKAGE package 5.1 (37). The multipoint analysis was performed using a modified version of LINKMAP (38). Because of computational difficulties with using many loci, two overlapping multipoint maps were generated, the first considering the interval between D4S115 and D4S431, the second for the interval between D4S412 and D4S394. Distances between markers were from published sources (19).

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