8-3-1989

Linkage Analysis of Reading Disability on Chromosome 6.

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Linkage Analysis of Reading Disability on Chromosome 6

A Thesis Presented to the
Department of Biology
and the
Faculty of the Graduate College

In Partial Fulfillment
of the Requirements for the Degree
Master of Arts
University of Nebraska at Omaha

by
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August 3, 1989

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THESIS ACCEPTANCE

Acceptance for the faculty of the Graduate College, University of Nebraska, in partial fulfillment of the requirements of the degree Master of Arts, University of Nebraska at Omaha.

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8/1/89

Date

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ABSTRACT

Reading disability (RD) is defined as difficulty learning to read and spell despite adequate intelligence and educational opportunity and without demonstrable physical, neurological, or emotional handicap. Investigators have suggested a genetic influence and postulated an autosomal dominant mode of inheritance. The strongest support for this hypothesis came from the observation of linkage between RD and a heteromorphism of the short arm/centromere of chromosome 15. Further analysis indicated the possibility of genetic heterogeneity with some families showing RD due to a gene not on chromosome 15.

This research is a report of the results of a linkage analysis of RD versus four genetic markers on chromosome 6. Two are restriction fragment length polymorphisms (D6S8, D6S9) and two are classical red cell and serum markers, Glyoxylase (GLO) and Properdin clotting factor (BF). No linkage between D6S9 and and the BF/ GLO linkage group was found. Evidence now indicates that D6S9 may not lie on chromosome 6. The distance between BF and GLO was estimated at 10% and the position of D6S8 was determined to be BF-D6S8-GLO or BF-GLO-D6S8. The final analysis assumed order BF-D6S8-GLO. A maximum LOD score of 1.486 was obtained for RD at about 21% from GLO towards the centromere side. This LOD score rose to 2.645 when one family showing linkage with chromosome 15 was omitted. The results suggest that a second gene for RD may lie on chromosome 6.

Introduction

Studies on the Causes of Reading Disability

The nature of reading disability (RD) has interested scientists since the early 1900's. Orton (1937) suggested that an apparent dysfunction in visual perception and visual memory caused reading disability. Other studies considered RD to be due to: a) a dysfunction of storing acoustic information in permanent memory; b) an inability to concentrate and pay attention; and c) an inability to relate stimuli perceived through a sensory system to stimuli perceived by another system (Vellutino, 1979). However, none of these theories have had strong support from experimental evidence.

Recent studies consider that RD is more a symptom of dysfunction occurring during the storage and retrieval of linguistic information rather than as a consequence of some defect in the visual system (Liberman, 1982). To prove the memory theory, several comparison tests between RD children and normal children were performed. These researches were based on the assumption that the storage of information in memory proceeds in three stages: 1) a sensory storage stage; 2) a short term memory stage; and 3) a long term memory stage. Sensory stimulation is held in the sensory storage system briefly. If the stimulus captures the subject's attention, it will then enter the short term memory system where the stimulus is transformed into a more abstract symbolic representation for long term memory storage. If the encoded form of the stimulus is well categorized it will store in long term memory, otherwise, it will be discarded from memory.

RD children were asked to copy words after a brief visual presentation, and then were asked to name the words. The results showed that RD children could correctly reproduce a stimulus word even when they were not able to name the word accurately (Vellutino, 1987). Also, visual recall of complex, word-like symbols lacking any linguistic association caused the same degree of difficulties in normal and RD children (Mann, 1986). Since the RD readers were able to hold a memory trace for as long as normal readers, it suggests that memory for visual symbols representing words is mediated by the linguistic properties of those words. Other experiments suggested that RD was the consequence of limited facility in using language to code other types of information such as the association of sounds with the words, segmentation of words into component sounds, and development of vocabulary (Brady, 1986). An example was described as the following: normal readers break printed words into individual phonemes to learn the sounds associated with given letters, combinations of letters and to learn the names of printed words as whole entities; RD readers fail to segment the printed words into individual phonemes and therefore cannot store the words with complete phonological codes. RD readers have difficulty retrieving correct phonological name codes from long term memory, and thus they cannot name the words correctly (Bradley and Bryant, 1985).

Modern theories of the etiology of RD suggest that an underlying biologic dysfunction is the cause (Finucci et al., 1976). The question as to whether this is mediated, partly at least, by genetic influences then arises. Genes may be expected to play some role in the development of these

faculties which underlie RD. Furthermore, early investigators have suggested a strong genetic influence, especially for a severe type of RD, often referred to as dyslexia (Hallgren, 1950). RD is probably not simply caused by a single gene. There may be several single gene types as well as multifactorial types, or combinations of both. However, at least one of the subtypes could be caused by one single gene. Finding a major gene associated with the symptoms of RD would contribute greatly to understanding this condition.

By separating out specific genetic forms of RD, the influence produced by non genetic reading disability may be more easily identified. The subdivision of RD into more than one type will benefit other researchers by allowing them to focus their studies on specific and presumably different groups of RD patients.

A linkage between reading disability and chromosome 15 heteromorphisms was reported, with the mode of the inheritance being autosomal dominant (Smith et al., 1983). A replication study is currently underway. If this finding is confirmed, the chromosome 15 location could account for one heritable subtype of RD. However, analysis also suggested genetic heterogeneity with some families showing RD due to a gene not on chromosome 15. No more than 20% of the families showed linkage with chromosome 15, thus, making corroboration of the finding difficult. Consequently, a search for a second gene involved in RD was began.

There is some evidence of a higher frequency of immune illness in a familial dyslexic population (Bruce et al., 1987). Since chromosome 6 contains a locus related to immune disorders, markers on this

chromosome were considered to be good candidates for linkage with RD. Confirming whether RD is linked to chromosome 6 markers is the main purpose of this research.

Chromosome 6 contains several clinically important markers. The one related to immune disorders is HLA complex. The HLA system is located on the short arm at 6p21-3 (Kidd et al., 1977). According to Olaison et al.(1987), the marker GLO is 7 cM from HLA toward the centromere side, D6S8 is on the telomere side of HLA, and BF was between HLA-A and HLA-B (See figure 1).

Linkage Analysis

Background

Mendel's second law is: alleles of two (or more) gene pairs "located on nonhomologous chromosome" will be assorted independently of one another into gametes. This is also called the law of random assortment. However, Mendel did not realize that this rule could not be applied in all cases. Nonrandom assortment results when the two genes are located close to one another on the same chromosome. The closer the two loci are, the more they tend to be inherited together. The phenomenon of nonrandom assortment is called linkage and provides the basis for creating the genetic map.

Figure 2 illustrates the concept of linkage. Since the genotypes of two grandparents are AABB and aabb, the phase of the first generation offspring must be Aa/Bb. This first generation can then be backcrossed with either of the homozygous parents and the resulting F2 offspring are AaBb, Aabb, aaBb and aabb which should occur in 1:1:1:1 proportions.

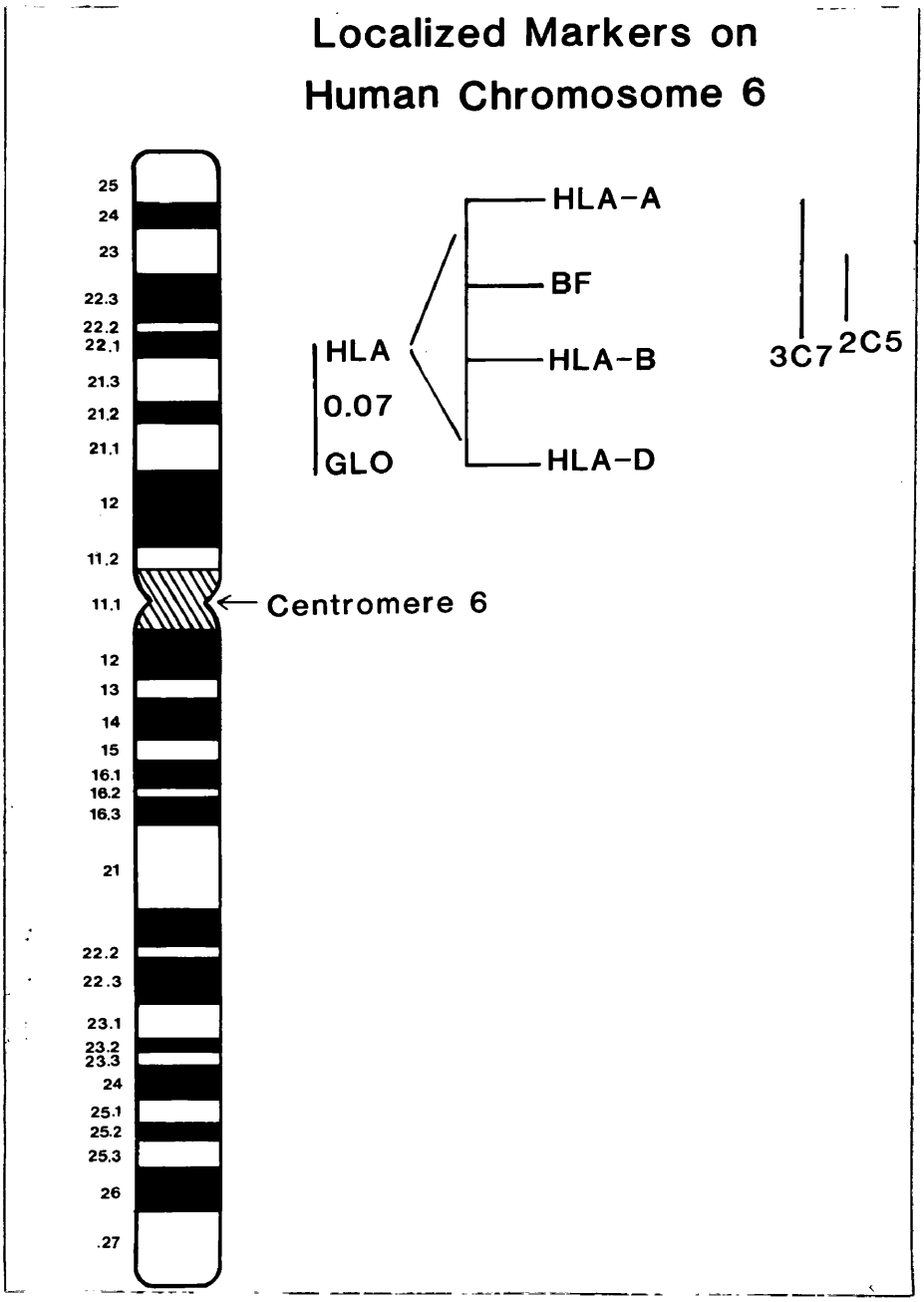


Figure 1. Genetic markers on chromosome 6.
Physical and genetic map of chromosome 6 shows locations of markers used in this study. The recombination distance between GLO and BF is estimated as 7 centiMorgan based on male recombination rates (Olaisen et al., HGM9, 1987). This study uses 10% recombination as a sex average. 3C7 is now known not on to be chromosome 6 but belongs to chromosome 11. The distance between HLA-A and HLA-D is no greater than 2 centiMorgan. 3C7 = D6S8, 2C5 = D6S8 (HGM9, 1987).

Any deviation from these proportions is indicative of linkage. The offspring, Aa/bb, and aa/Bb, are called recombinants or crossovers and the frequency of crossover offspring is the recombination fraction, θ. The expected proportions for a recombination of 10% are shown in the next to last row of Figure 2 and given as a function of θ in the last row.

Recombination is due to the reciprocal exchange of genetic material between homologous chromosomes and occurs more often when two linked genes are far apart. As the distance between two loci increases, recombination increases also, up to but never over 50%. At this point, two genes are so far apart on the same chromosome that linkage cannot be distinguished from independent assortment. In unlinked conditions, the transmission of an allele derived from one given parent has no relation to

P	AA BB	X	aa bb	
F1 (Backcross)		Aa Bb	X	aa bb
F2	Aa Bb	Aa bb	Aa bb	aa bb
No linkage	0.25	0.25	0.25	0.25
Linkage at $\theta = 0.1$	0.05	0.45	0.45	0.05
Linkage at $\theta = \theta$	$\theta/2$	$(1-\theta)/2$	$(1-\theta)/2$	$\theta/2$

θ = recombination fraction

Figure 2. The results of a double backcross show either random assortment or linkage.

the transmission of alleles for the other locus, free recombination should be observed between two unlinked genes.

Linkage analysis tests whether the trait in question is transmitted in random fashion with another inherited trait and thus whether alleles at both loci conform with Mendel's second random assortment law.

The detailed genetic maps have been developed for a number of lower organisms using the principle of linkage (Mertens, 1972). When dealing with other animals, recombination fractions can be estimated directly from counts of offspring in arranged matings and a simple Chi-square test can be used to test deviations away from random assortment. Unfortunately, linkage study is much more complex in humans. The following problems greatly limit the linkage research in humans:
1) matings cannot be controlled in human studies and key individuals are often missing; 2) sample size is relatively small; 3) the human generation is much longer (Gardner et al., 1985).

In order to allow for these problems, Morton (1955) derived the sequential probability ratio (or LOD score) tests in linkage analysis. His test provided a highly efficient method for the extraction of information on linkage from pedigree data. This test allows pooling of complex results across families and takes advantage of the powerful sequential analysis approach to minimize sampling requirements.

Figure 3 shows the derivation of LOD scores for two generation families data and illustrates the problem faced when parental phase is unknown. Assume two loci, A and B. Given a father with genotype Aa and Bb, phase would be either AB/ab, or Ab/aB. In either case, if the two loci

show independent segregation, the four possible gamates AB, ab, Ab, and aB will appear in offspring with the expected ratio 1:1:1:1. If two loci are linked, the probability of each child depends upon the parental phase. If the father is AB/ab, the likelihood of an AB or ab gamete (non crossover) is $(1-\theta)/2$ and the likelihood of a Ab or aB gamete (crossover) is $\theta/2$, where θ is the recombination frequency. A single child sibship is useless for linkage if parental phase is not known since the sum of both likelihood is $\theta/2 + (1-\theta)/2 = 1/2$, a constant. With two or more children, some information about linkage can be obtained. For example, the likelihood for two children given parental phase AB/ab will be $(1-\theta)^2/8$ and the probability of both children given a parental phase of Ab/aB is $(\theta)^2/4$. The likelihood of the sibship assuming both parental phases are equally likely, is $[(1-\theta)^2 + \theta^2]/4$. If there is no linkage, $\theta = 1/2$ and the likelihoods is 1/16. The likelihood ratio (odds) of linkage versus non linkage between the two loci A and B, computed for this family, is $2[(1-\theta)^2 + \theta^2]$. The log of the odds (which is termed the LOD score) is Log $2\ [(1-\theta)^2 + \theta^2]$. The reason for transforming the likelihood ratio into a LOD score is so that the scores can be easily combined by simple addition across families.

Linkage analysis using family studies is generally performed by investigation a battery of known marker genes to see if one of the markers is close to the gene for the trait. The odds of linkage versus no linkage between two loci is computed for each member in each informative pedigree at the following levels of recombination: 0.0, 0.1, 0.2, 0.3, and 0.4. Since the number of offspring in a single family is rarely enough to give a statistically significant result, LOD scores must be added together from different

families. The recombination fraction that gives the maximum LOD is taken as the best estimate of the distance between the genes.

One must be very careful in making the decision to accept linkage. A stringent rule now being used is that the hypothesis of linkage is accepted when the maximum LOD score is 3 (1000:1 odds in favor of linkage) or greater; a LOD score of -2 (100:1 odds against linkage) rejects linkage at that level of recombination, and a LOD score between -2 and 3 indicates that more data is necessary.

Usefulness of Linkage Analysis

Linkage analysis can be used to localize and map genes. By correlating the genetic patterns of a marker and a gene of particular interest, the disease-causing gene can be localized on a certain chromosome. Collective markers can then be used to construct a human chromosome map (White and Lalouel, 1988). Genetic markers have a linear order on a chromosome and the order of these markers can be inferred from the observed recombination values. The genetic distance between the markers can be measured with the detection of linkage and the estimation of recombination fractions between the loci. Physical location of an unlocalized gene may be deduced from linkage analysis and known locations of linked markers. Therefore, genetic mapping is based on both the physical distribution of loci on chromosomes and the distribution of cross-overs in all intervals considered.

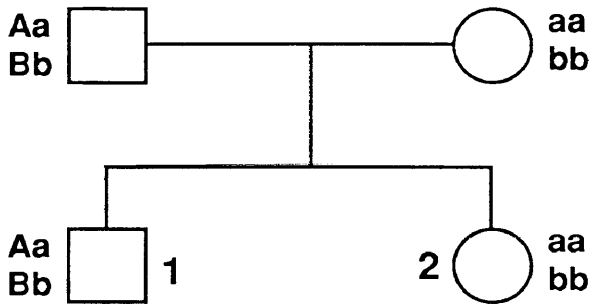
If a disorder is found to be linked to a known locus, this can be taken as proof that the disorder is due to a gene or tightly linked cluster of genes on the same chromosome (Morton, 1955). Therefore, linkage analysis can

serve as a tool to establish the involvement of a major gene in the etiology of a trait.

The presence of a linked marker gene can be very useful in genetic counseling, particularly in disorders with a late age of onset (Nei, 1977). For example, the onset of Huntington's chorea is gradual, usually starting in the fourth or fifth decade. Most gene carriers may have children before they realize they have this tragic disease. Since restriction fragment length polymorphism studies makes possible the diagnosis of this disease before symptoms occur, gene carriers will realize that they may pass the gene to their children (Gusella et al., 1983).

Linkage relationships might also be used for early detection in RD studies. Young, pre-reading children at risk for RD could be identified as gene carriers through linkage analysis, perhaps early training/education could be employed to lessen the associated problems later in school life. Unfortunately, since no predictive test is yet available, one can only speculate as to its practical utility.

Another advantage of linkage analysis is its ability to detect heterogeneity. This can be seen when some families show linkage of the trait in question to a given marker and other families may show linkage to yet another marker. Genetic heterogeneity is detected by comparing the LOD scores from individual families with the LOD scores form the entire sample (Ott, 1983). If genetic heterogeneity is proved, more specific study on subtypes of RD will be in order.



Father's Phase	$\frac{A}{B} \mid\mid \frac{a}{b}$	$\frac{A}{b} \mid\mid \frac{a}{B}$
Likelihood of Phase	1/2	1/2
Child #1	$(1-\theta)/2$	$(\theta)/2$
Child #2	$(1-\theta)/2$	$(\theta)/2$
Product	$1/2 \cdot (1-\theta)/2 \cdot (1-\theta)/2$	$1/2 \cdot (\theta)/2 \cdot (\theta)/2$
	$= (1-\theta)^2/8$	$= (\theta)^2/8$

The likelihood for linkage = $[(1-\theta)^2 + \theta^2]/8$

The likelihood for no linkage ($\theta = 1/2$) = 1/16

The likelihood of linkage/no linkage = $2[(1-\theta)^2 + \theta^2]$

The log of the likelihood ratio = Log $2[(1-\theta)^2 + \theta^2]$

Figure 3. Linkage analysis in a two generation family and derivation of the likelihood function for two children.

Applications of DNA polymorphisms

The genetic basis for RD was indicated in certain families where RD was found to be linked with a chromosomal heterophmorphism (Smith et al,.1983). Recent research has focused on corroboration of these findings by further investigation of DNA polymorphisms on chromosome 15. Data now indicate that only a few families are linked to chromosome 15 (Smith, in press, 1989) and attention is being directed to the study of DNA polymorphisms on other chromosomes.

Such DNA polymorphisms are detected using restriction enzymes. Restriction endonucleases can recognize a specific palindromic sequence of DNA that is a variable number of bases in length. Different restriction enzymes detect different sequences. Wherever the enzyme detects its particular recognition sequence of bases, it will cleave the DNA at or near that position. If two different chromosomal homologs differ in their DNA sequence within a recognition site for a restriction enzyme, one will be cleaved by the enzyme while the other not. This will cause a difference in the length of the DNA fragments that are produced by the cleavage of the restriction enzyme. Two types of polymorphism are detectable. One depends on the presence or absence of a restriction site and is usually due to a base pair substitution. This type of polymorphism typically has only two alleles and thus has limited usefulness. The other polymorphism is due to a difference in length of DNA fragments between two existing and invariant restriction sites. Such DNA segments usually consist of a series of highly repetitious sequences called VNTR (Variable Number Tandem Repeats) and can be detected by any restriction enzyme. VNTR's are

usually mutilallelic and highly polymorphic (White and Lalouel, 1988).

DNA fragments can be separated by size, using an agarose gel electrophoresis. DNA from the gel then can be transferred to a positively charged nylon membrane (Southern, 1975). To visualize specific DNA fragments on the gel, DNA probes which are radioactively labelled fragments of of known segments of DNA are used. DNA probes which have been cloned from the region of DNA being studied can recognize their complementary sequence and will hybridize with the appropriate DNA sequence in the blot. To be useful for linkage analysis, the sites which are detected by the probes have to be polymorphic. The usefulness of probes for linkage analysis is affected by the frequency of heterozygosity as well as their recombinational distance from the disease gene (Ott, 1983).

Gene localization is typically done by comparing the transmission of a disorder (the "test" gene) to that of a battery of "marker" genes scattered throughout the chromosomes to see if any one of the markers happens to be close to the test gene and show reduced recombination. Since linkage can be detected only if a person carrying mutant and normal alleles of a disease gene also carries two different versions of the markers, two properties of a good marker gene are required: 1) the genotype is readily detectable, as with a blood type; and 2) that the the locus is highly polymorphic. In early studies, there were approximately twenty genetic markers available, and mapping genes was more difficult than now (Morton, 1955). Today hundreds of DNA polymorphisms, found throughout the genome, can be used to extend genetic analysis.

Material and Methods

Family selection and testing

Nineteen families totalling 291 persons were studied. Pedigrees can be found in the appendix. The proband, both biological parents, and at least one sibling over age 7 had to be available for testing in order for the family to be accepted into the study. Furthermore, the pedigree had to show a pattern compatible with dominant inheritance as evidenced by having multiple generations of affecteds and a segregation ratio of approximately 50%.

All families were native English speaking. Clinical testing was done by S.D. Smith and B. F. Pennington. Only individuals with IQ (verbal or performance) greater than 90 were accepted for the study (Pennington, 1985).

The diagnosis of RD depended upon both the results of tests of reading ability and history of reading problems. The two most useful parameters were the Reading Quotient (RQ) and the Specific Dyslexia Algorithm (SDA). The RQ was used as a quantitative measure of the discrepancy between observed reading ability and expected reading ability based on age, education, and intelligence (Pennington, 1983). RQ less than 0.80 was considered diagnosis of RD; an RQ of 0.90 or greater indicated normal reading ability; values between 0.80 and 0.90 were considered as suspicious.

The Specific Dyslexia Algorithm (SDA) score was based on the performance on the spelling test and the performances on reading comprehension, general information, and mathematics tests and was

correlated with reading ability (Pennington, 1983).

For adults, a history of reading disability was determined through interview and a reading questionnaire. From the combined information Pennington defined five categories: unaffected, affected, compensated, obligate heterozygote, and questionable. An unaffected individual was one who was normal on both the RQ and SDA tests and had no history of reading problems. An affected individual was positive on either the RQ or SDA, or both and had a positive history of reading problems. If an adult with negative test criteria but a strong history of reading disability was reported, the individual was considered to have compensated. If an adult was negative on the RQ, SDA, and history, but had a parent or sibling who could be documented as being affected and had an affected child, that person was classified as an obligate heterozygote. Finally, if the RQ and SDA was positive but the history was negative, the diagnosis was considered questionable. For linkage analysis, data from individuals in the questionable category were not used.

Sample collection and processing

Thirty to 50 ml of blood were drawn from all cooperative family members. Blood samples were mailed from Denver and other areas to Boys Town National Research Hospital (BTNRH). After the blood was received, DNA was extracted by the cell lysis method of Kunkel (1978). The DNA extraction method involved Triton/sucrose lysis of red cells. After the white cells were pelleted, they were then incubated with proteinase K at 37°C to rupture the cells and free the DNA. Genomic DNA was purified by repeated phenol/chloroform washing and ethanol precipitation. DNA was

stored at either -80°C or at 4°C until use.

DNA was used to type the restriction fragment polymorphisms. Whole blood was used to determine GLO and BF types.

Chromosomal Heteromorphisms

A chromosome heteromorphism is defined as a difference in size and form between homologous chromosomes. The method that first demonstrated the bands along the chromosomes used quinacrine mustard. This method is called Q-banding staining and results in Q-bands distributed along the length of the whole chromosome. Certain regions of the human chromosomes show variation in the size and intensity of quinacrine induced fluorescence. The short arm and centromeres of the acrocentrics are well noted for such a variation. The method that reveals variation in the size of the centromere area is called C-banding staining (Lubs and McKenzie, 1975).

In this study, Q-banded cells were printed at four levels of intensity so that all levels of florescence could be visualized, and intensity was scored at five different levels. The size of the centrometric C-band was judged as small, medium, or large. Slides were stained sequentially, first for Q-banding and then for C-band. Each homolog was then assigned an allele type based on variations in both Q-banding and C-banding. Work with chromosomal heteromorphisms was originally done by Dr. S. Smith and is continuing by Dr. P. Ing at Boys Town National Research Hospital. Both Dr. Smith and Dr. Ing evaluated the heteromorphism typing independently to reduce observer bias. After the initial scoring, the code was broken to reveal family relationships, then the parental origin of each "marker" was

determined by Drs. Ing and Smith.

RFLP Typing

Two probes were provided by Drs. Leach and White. The two probes, known as 2C5 (D6S8) and 3C7 (D6S9), were originally isolated from the recombinant library that had been constructed with DNA from flow-sorted metaphase chromosomes enriched with chromosome 6 (Boncinelli et al., 1984). The localization of both probes to 6p was done by somatic cell hybridization. Subsequently, both probes were found polymorphic. 2C5 is a 0.9 Kb genomic fragment inserted into pBR322 and it is polymorphic with MspI showing variable bands at 10.0 and 6.5 Kb. 3C7 is a 1.3 Kb genomic fragment inserted into pBR322 and is also polymorphic with MspI showing variable bands at 5.7 and 3.1 Kb (Leach et al., 1986).

Marker typing for DNA was carried out as follows: Five to 10 μg of each DNA sample was digested with MspI, one of several restriction enzymes which only cut specific sequence of DNA. The resulting fragments were electrophoresed on 0.6% to 1.0% agarose gels in TBE (Tris-Borate-EDTA) buffer for 18 hours at 1.5 to 2.0 V/cm. The gels were denatured, neutralized, and passively transferred to Gene Screen Plus nylon membranes (Southern 1975). After overnight transfer, agarose clinging to the filters was removed by washing in 1X SSC (0.15M NaCl, 0.15M NaCitrate) and the filters were air dried. Filters were prehybridized in 0.2% polyvinyl-pyrrolidone (M.W.40,000) 0.2% ficoll (M.W.400,000), 0.2% Bovine serum albumin, 0.05 M Tris pH 7.5, 1 M NaCl, 0.1% $Na_4P_2O_7 \cdot 10H_2O$, 1% SDS, 10% dextran sulfate (M.W.500,000), and 100 μg/ml denatured salmon sperm DNA at 65°C for 18 hours.

Prehybridization filters were washed extensively at room temperature in 3 X SSC to remove the SDS. Filters were placed individually in heat-sealing bags. Whole plasmid DNA was nick translated with dCTP$^{\alpha 32}$P to specific activities of 1 to 5 X 10^8 cpm/μg using a commercial kit (Bethesda Research Laboratory). Unincorporated dCTP$^{\alpha 32}$P was removed through a column of Sephadex G-50 equiliberated in STE (0.1 M NaCl; 0.01M Tris, pH 7.5; 0.001 EDTA, pH 8.0). A specific probe was added to each bag which was sealed and placed at 65°C with shaking for 24 to 48 hours. Filters were removed from the bags and washed at 65°C for 30 minutes each in 3 X SSC, 2 X SSC, 1 X SSC, and 0.3 X SSC. The filters were then dried and exposed to X-ray film (Kodak XR 5) at -70°C with a Dupont Cronex intensifying screen for 1 to 4 days.

Red cell enzyme GLO was determined by eletrophoresis using starch gels (Harris and Hopkinson 1976). Serum protein BF was performed using a agarose gel with appropriate antibody-containing overlay (Weitkamp et al., 1977).

The usage of LINKAGE program

Linkage analysis was performed with the computer program LINKAGE using reading disability as a qualitative phenotype with gene frequency of 0.01 (Lathrop, et al. 1984). This implies that the frequency of inherited RD is about 2% which roughly corresponds to the frequency often given for the severe form of this disorder. Linkage was tested between RD and the chromosomal heteromorphisms and both RFLP probes and two classical markers. MLINK from the LINKAGE package can perform two point tests, providing linkage information between two loci. LINKMAP

from the LINKAGE package can perform multilocus tests. In principle, the calculation of the likelihood is the same for three loci as it is for two, since both basically calculate the sum of likelihoods of all possible genotypes. The multilocus test must, however, consider a much greater number of genotypes. The number of these genotypes is given by S(S+1)/2 where for n loci $S = S_1 \times S_2 \times \ldots S_n$ (the total number of different haplotypes), with S_i equal to the number of alleles at the ith locus.

With two point analysis, the order of three or more genes cannot necessarily be inferred by comparing LOD scores and recombination fractions given by each pair of loci. With three point analysis, the order of three loci is determined by the magnitude of the true recombination fractions θ_{AB}, θ_{AC} and θ_{BC}. The largest of these indicates which of the three loci are farthest apart. In a phase known situation, the gene order with the smallest associated sum of recombination is taken as the most plausible one.

LOD scores were computed with the sexes combined in this study. In some two point linkage investigations, a recombination fraction in females appears higher than that in males (Weitkamp, 1972). However, in most linkage analysis it is not usually possible to establish a statistically significant differences between male and female recombination fractions (Falk and Edwards, 1970). Also, differences between male and female recombination fraction depend on the region of the genome. A large sample size is required to detect the relatively small differences in recombination due to sex (Morton 1956). Since linkage analysis in HLA region has not show a significant difference in recombination fraction

between males and females (Olaisen et.al., 1987) and the sample size of this research is relatively small, it is permissible to assume that the recombination fraction is equal between males and females.

Results

RFLP Typing

Example of autoradiographies of probes D6S8 and probe D6S9 are shown in Figures 5 and Figure 6 respectively. Figure 5 shows the band pattern of 2C5 MspI polymorphism. Band sizes are 10.0 and 6.5 Kb. Gene typing can be directly done from the photograph in the figure. For example, the type shown in lane 5 is heterozygous, Ff. Lane 7 shows a homozygote, FF, and the type in lane 8 is the other homozygote, ff. The low molecular weight bands seen in lanes two and four may represent a third allele at this locus.

Figure 6 shows the band pattern of 3C7 MspI polymorphism. Bands are seen at 5.7 and 3.1 Kb. Gene typing is done in the same way which is described in the explanation of Figure 5. For example, lane 4 shows a homozygote, SS; lane 8 shows the other homozygote, ss; the type in lanes 1, 2, and 6 are heterozygous, Ss.

Photographs of Glyoxylase (GLO) and Properdin factor (BF) are not included in this text. (All the work with GLO and BF was done by Judy Kenyon of BTNRH).

Two Point Linkage Analysis

Only one family was informative for linkage analysis with chromosome 6 heteromorphism. The other 18 families were either not tested or uninformative. The results of the analysis (Table A1) excluded close linkage between RD and the centromere of chromosome 6, out to a recombination fraction at 0.086.

The LOD scores from the linkage analysis between RD and BF are

shown in Table A2. Fourteen families were informative and close linkage was excluded between RD and BF out to a recombination fraction of 0.15.

The results of the linkage analysis between RD and GLO are shown in Table A3. Fourteen families were informative. A maximal LOD score of 0.902 was obtained at a recombination fraction of 0.22. Since family 6432 showed positive linkage to chromosome 15, a LOD score of 2.19 was obtained at a recombination fraction of 0.21 without family 6432.

The results of the analysis between RD and D6S8 are shown in Table A4. Nine families were informative. A LOD score was obtained as 1.367 at a recombination fraction of 0.1.

The results of the analysis between RD and D6S9 are shown in Table A5. Nine families are informative. The results excluded close linkage between RD and D6S9 out to a recombination fraction of 0.16. Since D6S9 is now known not to be on chromosome 6, this is not a surprising finding.

Multilocus mapping and order of markers

For the first step of multilocus mapping, it was necessary to determine the order of the markers being used. Table A6 and Table A7 present the information regarding the order of these markers.

Table A6 shows the results of testing the order of D6S9 with BF and GLO. The distance between BF and GLO was assumed to be 10% (This assumption was made for all the analysis). The first row shows the LOD score for the hypothetical position of D6S9 to the left side of BF. The close linkage is excluded out to 19% from that region. The second row shows the LOD score for the hypothetical location of D6S9 between BF and GLO. Linkage is excluded from the area between BF and GLO. The third row

shows the LOD score for the hypothetical position of D6S9 to the right side of GLO and recombination given relative to GLO. Linkage is excluded at 23% away from that region. No evidence indicates that D6S9 is within or near the BF-GLO complex.

The Markers GLO, BF, and D6S8 were known to be on the short arm of chromosome 6, and the order of these three genes on chromosome 6 were believed to be D6S8-BF-GLO (Olaison et al., 1987). The results of this investigation rule out the order D6S8-BF-GLO with a high degree of likelihood; however, we can not distinguish the order of BF-GLO-D6S8 and BF-D6S8-GLO with any certainty. The results indicate that the best position for D6S8 is close to the marker GLO. Table 7 shows the results that pertain to the position of D6S8 in the BF-GLO complex. The assignment of the order is not significant in this context. Since D6S8 showed no crossing over with GLO, these two loci could even be considered as one locus for practical purposes of this research.

Multilocus mapping of RD

The localization of RD was the main purpose of this research. Table A8 and A9 provide information for mapping of RD relative to the other markers.

Table A8 shows the results of an attempt to find position of RD relative to GLO and centromere of chromosome 6. The distance between GLO and centromere was assumed to be 0.05. The third row shows that RD could be excluded at 0.093 away from centromere towards long arm. The first and second row of the table do not provide much information for mapping of RD in those regions.

Table A9 shows the results of an attempt to find position of RD relative to markers BF, GLO and D6S8. The order of these markers were determined as BF-D6S8-GLO in the first step of this analysis. The first row shows that RD can be excluded from the left side of BF toward the end of short arm. The second row shows that RD can be excluded at the region between BF and D6S8/GLO. The third row shows that RD is excluded at the region between D6S8 and GLO. In the fourth row, a maximum LOD score of 1.486 is obtained at a 21% recombination with GLO. A maximum LOD score of 2.645 is obtained at a 22% recombination when family 6432 is not included. As mentioned above, this family showed strong linkage to chromosome 15.

Figure 4 summarizes the results of Table A8, and Table A9, combining the findings from three multilocus linkage analysis.

3C7 was believed to be 50% away from BF locus, however, now 3C7 is known not on chromosome 6. Therefore, 3C7 locus actually contributes no information to the localization of RD on chromosome 6. RD is excluded from the left region of BF toward the end of short arm because of the negative LOD score. The location score for linkage of RD in the interval between BF and GLO shows that RD can also be excluded from the region between BF and GLO. The location score, however, rises up to a maximum of 1.486 for the location of RD about 21% away from GLO towards the centromere. Additional information indicates that RD gene can not be close to the centromere. Unfortunately, the distance between centromere and GLO is unknown, thus the results are not very precise.

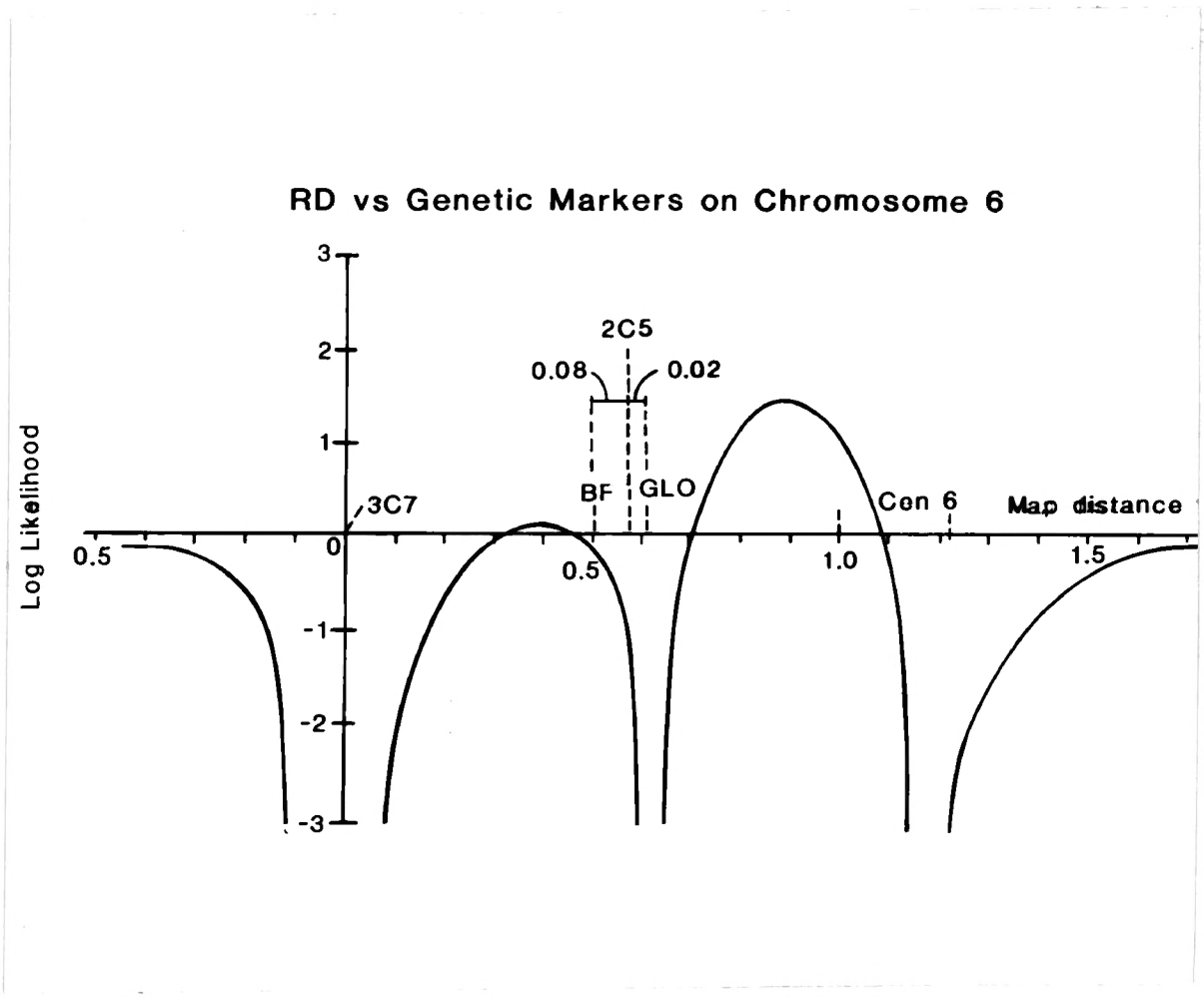


Figure 4. Multilocus linkage results of RD versus five markers on chromosome 6.
LOD score below -2 excludes the gene from that region. This result excludes RD from the region between BF and GLO and from centromere area. A maximum LOD score of 1.486 is obtained at 21% away from GLO towards the centromere, suggesting that RD is more likely located between GLO and centromere if it is on chromosome 6. The distance between RD and GLO can not be decided by this analysis. The analysis assumed that the order of five markers being used are 3C7-BF-GLO-D6S8-Centromere.

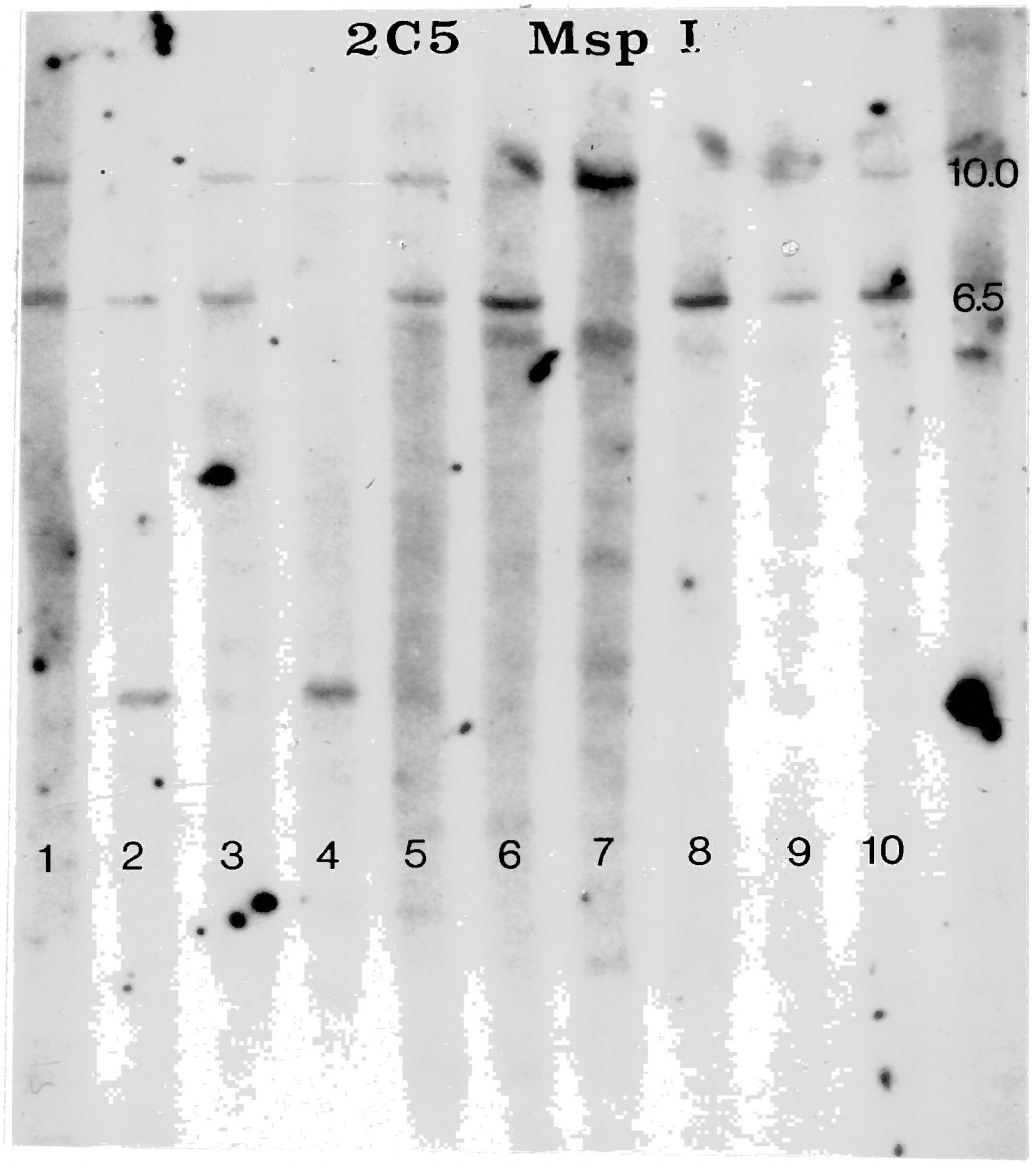


Figure 5. Autoradiograph of the 2C5 (D6S8) MspI polymorphism.
Band sizes are 10.0 and 6.5 Kb. Gene typing could be directly done from the photograph in the figure. For example, the type in lane 5 is heterozygote, Ff. The type in lane 7 is homozygote, FF, and the type in lane 8 is the other homozygote, ff. The low molecular weight bands seen in lane 2 and 4 may represent a third allele at this locus.

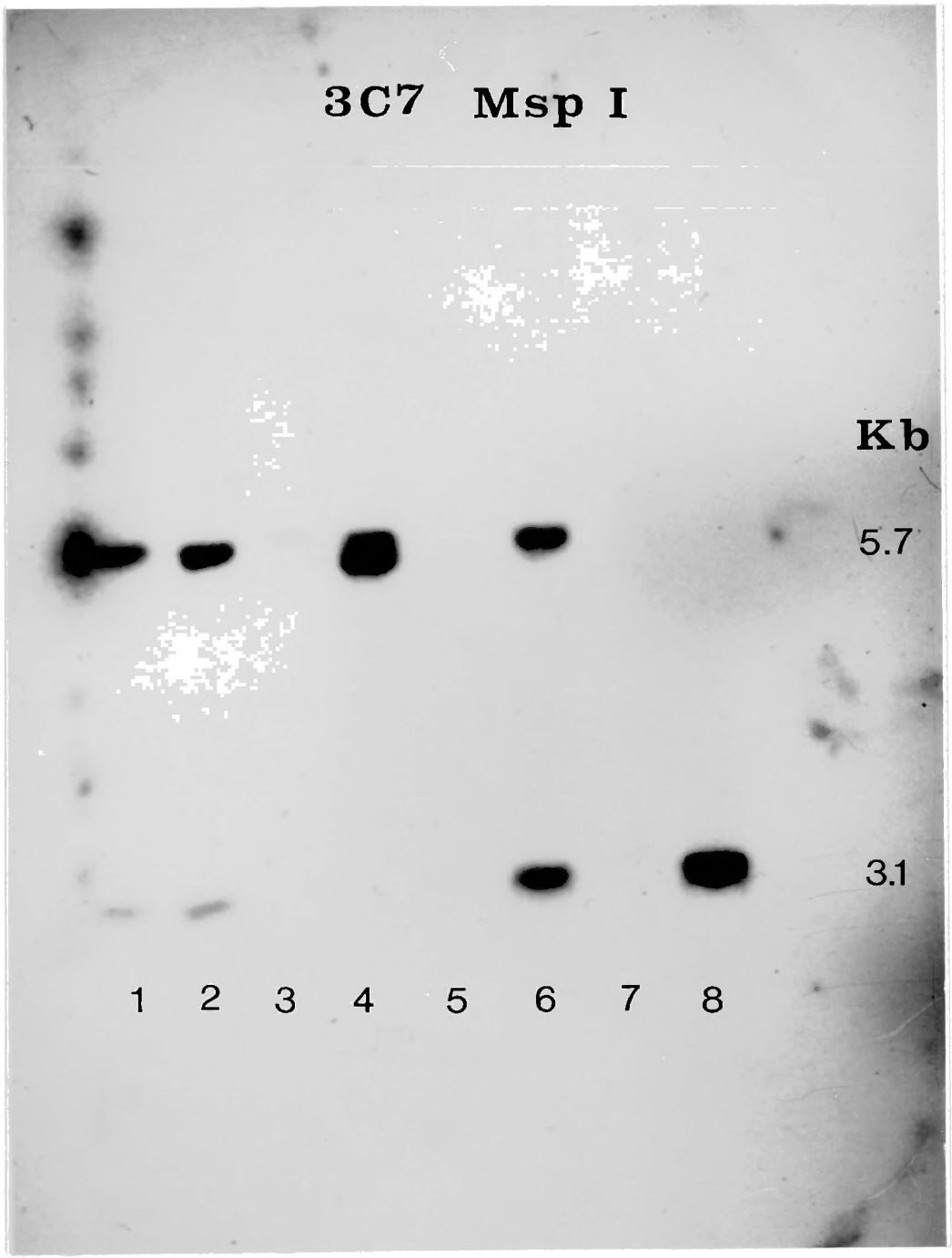


Figure 6. Autoradiograph of the 3C7 (D6S9) MspI polymorphism. Figure 6 shows the band pattern of 3C7 with MspI restriction enzyme. Bands are seen at 5.7 and 3.1 Kb. Gene typing is done in the same procedure described by Figure 5.

Discussion

In recent years, genetic studies of RD have focused on the variability of symptoms and the delineation of subtypes. The results indicate that RD is a heterogeneous language disorder with several subtypes (Omen et al., 1978). Some subtypes may have a genetic etiology and the others may not. It is also likely that more than one genetic form of the disorder exists. Furthermore, such multiple genetic and environmental factors may interact to create a series of overlapping causes of symptoms.

One way to detect the genetic heterogeneity is to study the different types of RD separately with regard to the location of these genes. Each subtype of RD could be verified by the gene localization approach. If different locations are found for different subtypes, the hypothesis of heterogeneity is proven. Confirmed genetic heterogeneity will then provide a more accurate diagnosis and lead to a more specific clinical study in the future.

Early studies have shown a distinct possibility that chromosome 15 may harbor a gene for RD and that not all families have RD due to the same gene. Thus, it seemed worthwhile to search other regions of the genome for a second RD gene.

The study reported here is one part of a collaborative project to screen the genome for other genes which cause RD. This study indicates a possibility of linkage of some RD families with markers on chromosome 6. First, The result of two point analysis with RD-GLO indicates a suggestive linkage between RD and GLO, which is accentuated when family 6432 is not included (See Table A3). Next, a multilocus analysis was done with RD

versus BF/GLO/D6S8. Our results indicate that order of these three markers is either as BF-D6S8-GLO or BF-D6S8-GLO. As mentioned above, the difference between these two orders was not important for this analysis. Although, a order of D6S8-RD-GLO was reported in earlier studies (Leach et al.,1986), the order of these BF-D6S8-GLO was confirmed by Leppert et al. (HGM9, 1987).

The results suggested RD is more likely to be located between GLO and the centromere if it is present on chromosome 6 (See Figure 4).

A maximal LOD score of 2.645 was obtained from the multilocus analysis when family 6432 was removed. This suggests the possibility of heterogeneity since family 6432 shows a strong linkage to chromosome 15. Is there a gene on chromosome 15 which causes one type of RD and one on chromosome 6 which causes another? We examined the possibility of heterogeneity with a program HOMOG, but the results turned out to be not statistically significant (Kimberling, Pers, Comm). The efficiency of the analysis is believed to be too low to have detected heterogeneity at the level of recombination observed.

Factors having a possibility of reducing efficiency of linkage analysis need to be discussed. Besides heterogeneity, other factors that may affect the efficiency and the accuracy of linkage analysis are; missing data, reduced penetrance (Ott, 1985), misdiagnosis of RD, and marker typing errors.

Missing data is only a small problem in this data set. Some critical family members are absent and this has reduced informativeness. Unfortunately, there is little one can do about missing data since this is due

either to death or non cooperation.

Penetrance refers to the clinically affected proportion among susceptible individuals. It is not necessary that all the genotypically susceptible individuals are affected, because environmental factors may interact with genetic factors to decide if the genotype will be expressed. Penetrance can range from 0 to 1. Penetrance can be estimated by studying the frequency of obligate carriers who do not express the trait. For example, individual 5 of family 6491 (Figure A8) is presumed to be affected but she does not show any symptoms of RD. The fact that this person carries the RD gene is evidenced by the observation that her two sons are affected. Thus she is considered to be an example of nonpenetrance. If such a person has no children, he or she will have been considered as a normal homozygote and the efficiency of analysis could have reduced. There is a way to join the estimation of recombination fraction with penetrance in analysis to improve accuracy (Ott, 1985). A minimum estimate of penetrance (Decker et al., 1980) could be estimated simply as the number of obligate heterozygotes over the total number of affected individuals and obligate heterozygotes (non-penetrant individuals). The computer program LINKAGE can incorporate the penetrance parameter to offer a more accurate result. Such analysis will be performed in a future study.

Misdiagnosis can mislead linkage analysis by rejecting linkage at a certain area where linkage actually exits. False negative diagnosis is equivalent to nonpenetrance and can be handled as discussed above. False positive diagnosis poses a different problem. This can be taken into account

by introducing another parameter for phenocopies. However, the proportion of phenocopies in the population has not been accurately estimated.

Typing errors in lab procedures is another possible source affecting the efficiency of analysis. Typing tests are redone whenever there are doubts regarding their accuracy. Genetics provides a good internal check on the accuracy of these results.

Relative efficiency of three point and two point linkage analysis tests were performed by Lathrop and colleagues (1984). Three point analysis showed more power in the combination of information from different families and in the estimation of the order of used markers. The more markers that are available, the more likely the data will be informative. In this research, we used two point analysis to screen for linkages. It is quick and robust since results do not depend on assumption of gene order. A LOD score of 0.886 was obtained when analysis of RD versus GLO was done by pairs. Multilocus mapping was used to confirm preliminary analysis and to enhance the statistical scores by allowing pooling data from linked markers. A LOD score of 1.213 was obtained when analysis of RD-GLO-D6S8 was performed.

The most important factor in the analysis is the distance between markers and disease locus. If there truly is a second gene for RD between GLO and centromere, then additional markers for this region are needed. Thus, the future direction of this research will be to study other DNA markers between GLO and the centromere of chromosome 6 to confirm the RD location.

If some day an RD gene could be localized on chromosome 6, the next step will be to isolate and clone it. The possible gene product could be determined and its function could be investigated.

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Appendix

Table A1. LOD scores from a two point linkage analysis of RD with a heteromorphism of the centromere of chromosome 6.

	Recombination Fractions (θ)				
Family	0.0	0.1	0.2	0.3	0.4
6372	- ∞	-1.190	-1.027	-0.540	-0.219

Table A2. LOD score from a two point linkage analysis of RD with genetic marker BF (Properdin factor).

	Recombination Fractions (θ)				
Family	0.0	0.1	0.2	0.3	0.4
7	- ∞	-0.961	-0.325	-0.072	-0.015
8	- ∞	-0.440	-0.192	-0.075	-0.012
102	0.835	0.666	0.477	0.272	0.084
6375	0.058	0.033	0.017	0.007	0.002
6432	- ∞	-1.634	-0.812	-0.384	-0.134
6484	0.125	0.100	0.065	0.032	0.008
6576	- ∞	-0.380	-0.132	-0.043	-0.008
8001	- ∞	0.092	0.188	0.158	0.084
8005	0.125	0.084	0.049	0.022	0.006
8006	- ∞	-0.440	-0.192	-0.075	-0.017
8007	- ∞	-0.440	-0.192	-0.075	-0.017
8008	- ∞	-0.440	-0.192	-0.075	-0.017
8009	0.601	0.464	0.317	0.169	0.048
8010	- ∞	-0.104	-0.055	-0.024	-0.006
Totals	- ∞	-3.400	-0.979	-0.163	0.006

Table A3. LOD scores from a two point analysis of RD with genetic marker GLO (Glyoxylase).

	Recombination Fractions (θ)				
Family	0.0	0.1	0.2	0.3	0.4
7	- ∞	0.141	0.084	0.039	0.010
102	1.430	1.100	0.755	0.398	0.107
6372	- ∞	-0.238	0.254	0.149	0.040
6375	0.057	0.033	0.017	0.007	0.002
6432	- ∞	-2.786	-1.258	-0.557	-0.134
6484	1.406	1.138	0.858	0.565	0.266
6491	0.300	0.214	0.123	0.064	0.017
6576	- ∞	-0.344	-0.191	-0.133	-0.077
8001	- ∞	-0.920	0.188	0.158	0.084
8002	- ∞	-1.773	-0.028	-0.005	0.004
8005	0.124	0.052	0.010	-0.005	-0.003
8008	- ∞	-0.440	-0.192	-0.075	-0.017
8009	0.800	0.464	0.317	0.169	0.048
8010	- ∞	-0.104	-0.055	-0.024	-0.006
Totals	- ∞	-1.147	0.882	0.750	0.341

Table A4. LOD scores from a two point analysis of RD with D6S8 (2C5).

	Recombination Fractions (θ)				
Family	0.0	0.1	0.2	0.3	0.4
7	0.203	0.141	0.084	0.039	0.010
8	0.896	0.639	0.397	0.191	0.050
6372	0.601	0.509	0.407	0.291	0.149
6375	-1.617	0.112	0.144	0.083	0.230
6432	0.241	0.121	0.059	0.026	0.011
6484	0.382	0.286	0.284	0.000	0.106
6576	0.234	0.141	0.084	0.039	0.010
8001	- ∞	-0.666	-0.252	-0.086	-0.084
8002	0.125	0.084	0.049	0.022	0.006
Totals	- ∞	1.367	1.256	0.605	0.276

Table A5. LOD scores from a two point analysis for RD with D6S9 (3C7).

	Recombination Fractions (θ)				
Family	0.0	0.1	0.2	0.3	0.4
8	- ∞	-0.440	-0.192	-0.075	-0.012
102	- ∞	-0.382	-0.155	-0.056	-0.012
6372	-0.174	-0.026	0.014	0.015	0.005
6432	- ∞	-0.236	-0.091	-0.039	-0.012
6484	-0.380	-0.011	-0.002	-0.001	0.002
6576	-2.273	-0.289	-0.044	0.047	0.055
8002	- ∞	-0.104	-0.055	-0.024	-0.066
8005	0.125	0.084	0.049	0.022	0.006
8009	- ∞	-1.829	-0.984	-0.516	-0.209
Totals	- ∞	-3.233	-1.460	-0.627	-0.243

Table A6. LOD scores from a multilocus analysis of D6S9 with BF and GLO*.

	Recombination Fraction (θ)				
Order	0.0	0.1	0.2	0.3	0.4
D6S9-*BF*-GLO	- ∞	-2.859	-1.973	-0.836	-0.463
	0.00	0.04	0.06	0.08	0.10
BF-D6S9-GLO	- ∞	-6.044	-6.799	-7.981	-8.325
	0.0	0.1	0.2	0.3	0.4
BF-*GLO*-D6S9	- ∞	-4.435	-2.372	-1.227	-0.136

*The recombination between BF and GLO is fixed at 0.10. LOD scores are given at recombination fractions between D6S9 and BF in two rows, and D6S9 and GLO in the last row.

Table A7. LOD scores from a multilocus analysis of D6S8 with BF and GLO*.

	Recombination Fraction (θ)				
Order	0.00	0.02	0.04	0.06	0.08
D6S8-*BF*-GLO	- ∞	-2.350	-1.897	-1.163	-0.434
	0.10	0.08	0.06	0.08	0.00
BF-D6S8-GLO	4.825	4.497	4.113	3.623	2.886
	0.02	0.016	0.008	0.004	0.000
BF-*GLO*-D6S8	4.824	3.896	2.783	1.163	0.434

*See Table A8 for future explanation.

Table A8. LOD scores from a multilocus analysis of RD with GLO and Centromere *of chromosome 6.

	Recombination Fractions (θ)				
Order	**0.0**	**0.1**	**0.2**	**0.3**	**0.4**
RD -*GLO*-Cen6	- ∞	-0.228	0.828	0.659	0.226
Order	**0.0**	**0.1**	**0.2**	**0.3**	**0.4**
***GLO*-RD-Cen6**	0.024	0.052	0.797	0.543	0.168
Order	**0.0**	**0.1**	**0.2**	**0.3**	**0.4**
GLO-Cen6-*RD*	- ∞	-1.908	-1.029	-0.541	-0.220

*The recombination between GLO and the centromere was assumed to be 0.50.

Table A 9. The results of the multilocus analysis of RD with BF, GLO and D6S8*.

	Recombination Fractions (θ)				
Order	**0.0**	**0.1**	**0.2**	**0.3**	**0.4**
RD-*BF*-GLO-D6S8	- ∞	-1.870	0.676	1.063	0.598
	0.000	**0.016**	**0.032**	**0.048**	**0.064**
***BF*-RD-D6S8-GLO**	- ∞	-14.514	-12.628	-12.799	-15.026
	0.000	**0.004**	**0.008**	**0.016**	**0.020**
BF-*D6S8*-RD-GLO	- ∞	-21.396	-19.963	-21.751	- ∞
	0.0	**0.1**	**0.2**	**0.3**	**0.4**
BF-D6S8-*GLO*-RD	- ∞	1.351	1.482	0.750	0.630

*The recombination between BF and D6S8 was assumed to be 0.08 and between D6S8 and GLO to be 0.02.

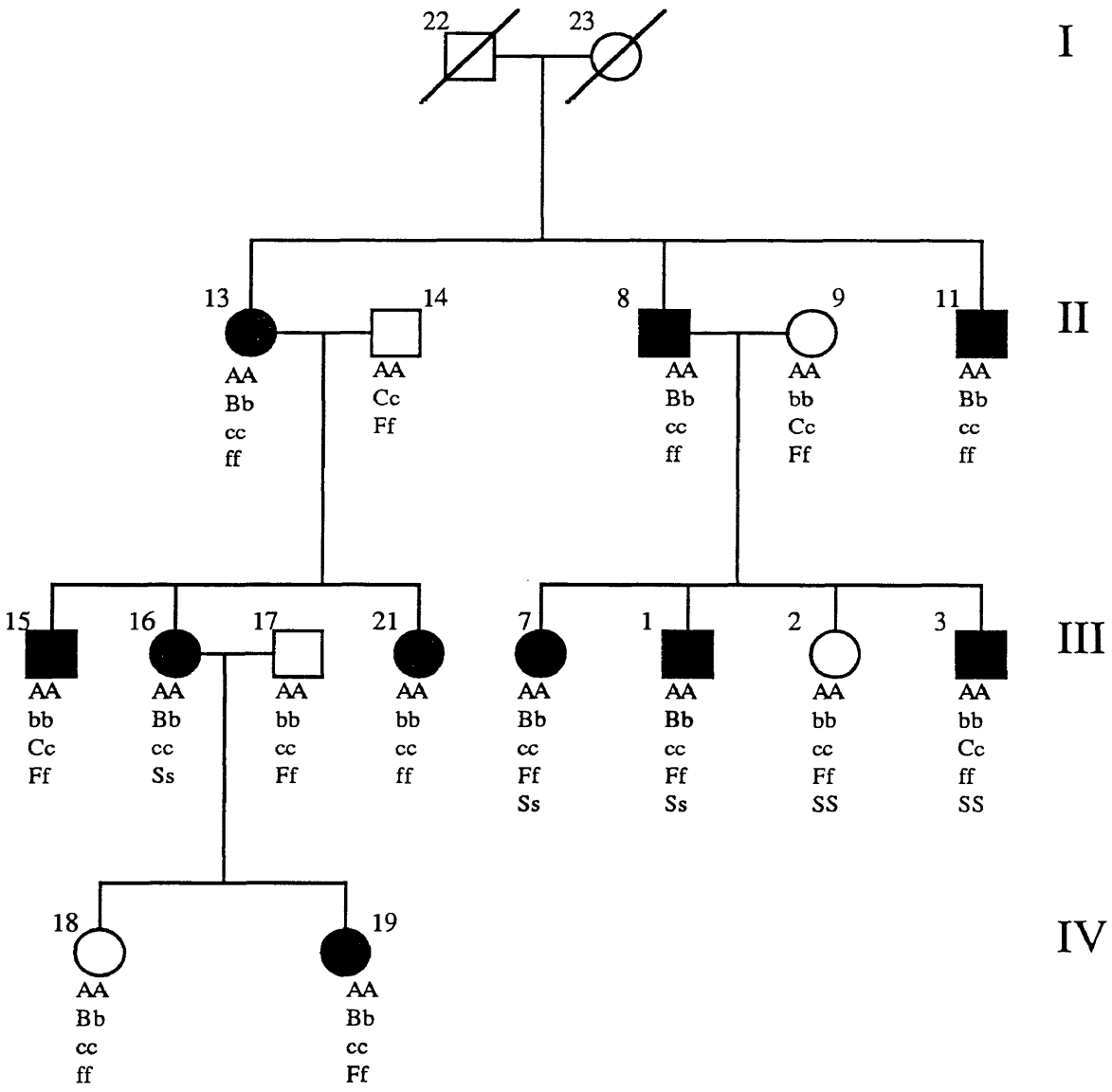

Figure A1. Pedigree of family 7 with RD. A-Centromere of chromosome 6. (Cen 6), B-BF, C-GLO, F-D6S8, S-D6S9.

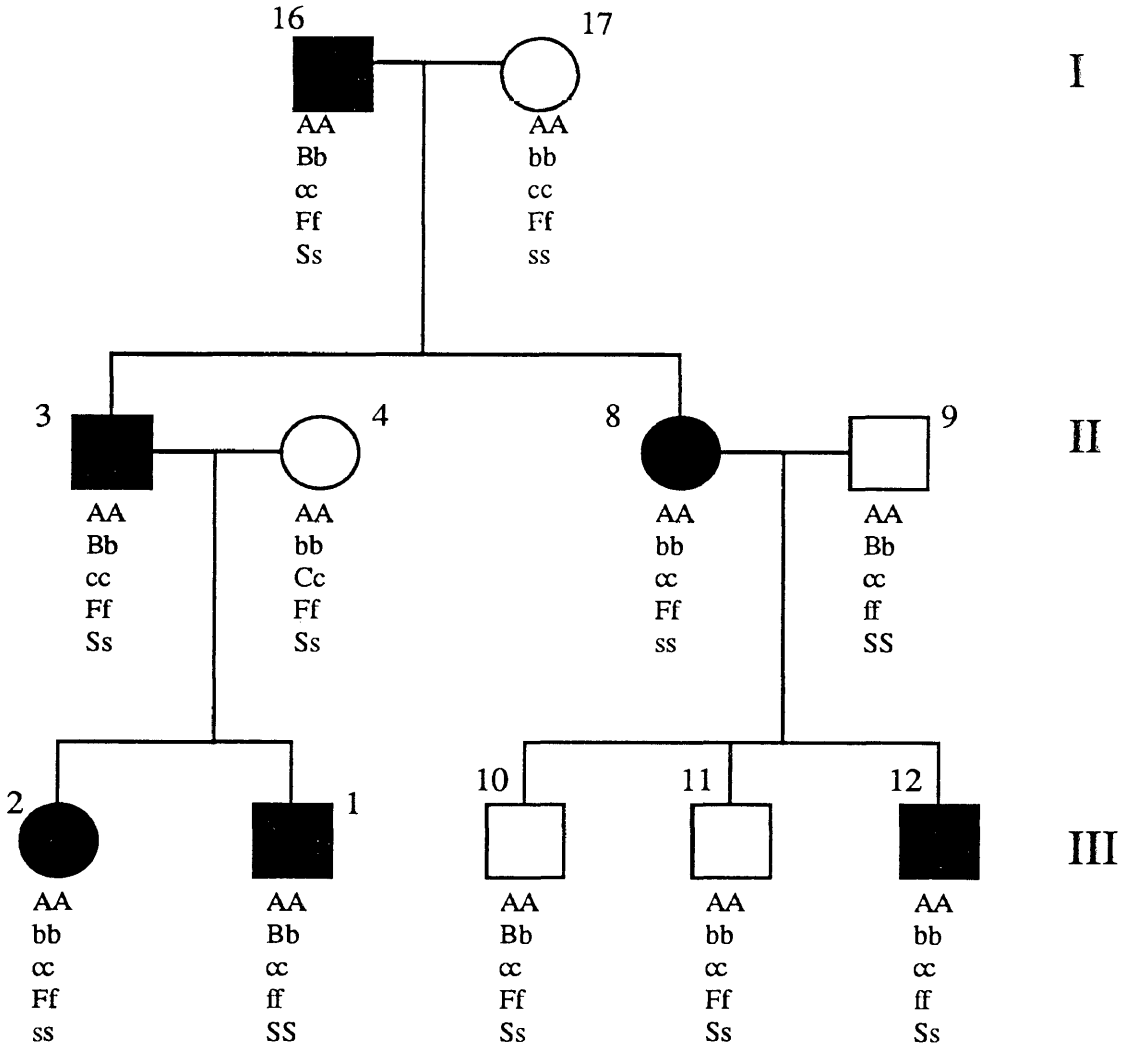

Figure A2. Pedigree of family 8 with RD. A-Cen6, B-BF, C-GLO, F-D6S8, S-D6S9.

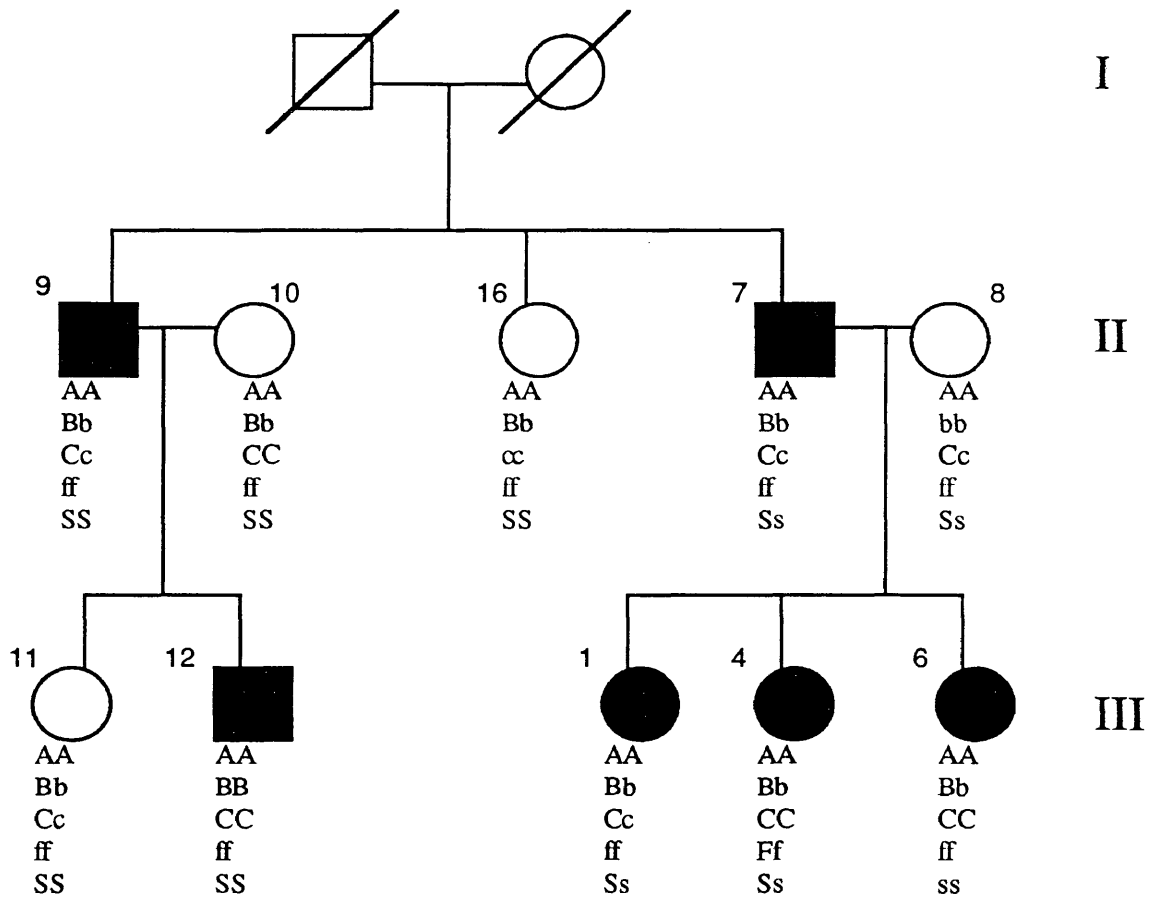

Figure A3. Pedigree of family 102 with RD. Information of first generation is not available. A-Cen6, B-BF, C-GLO, F-D6S8, S-D6S9.

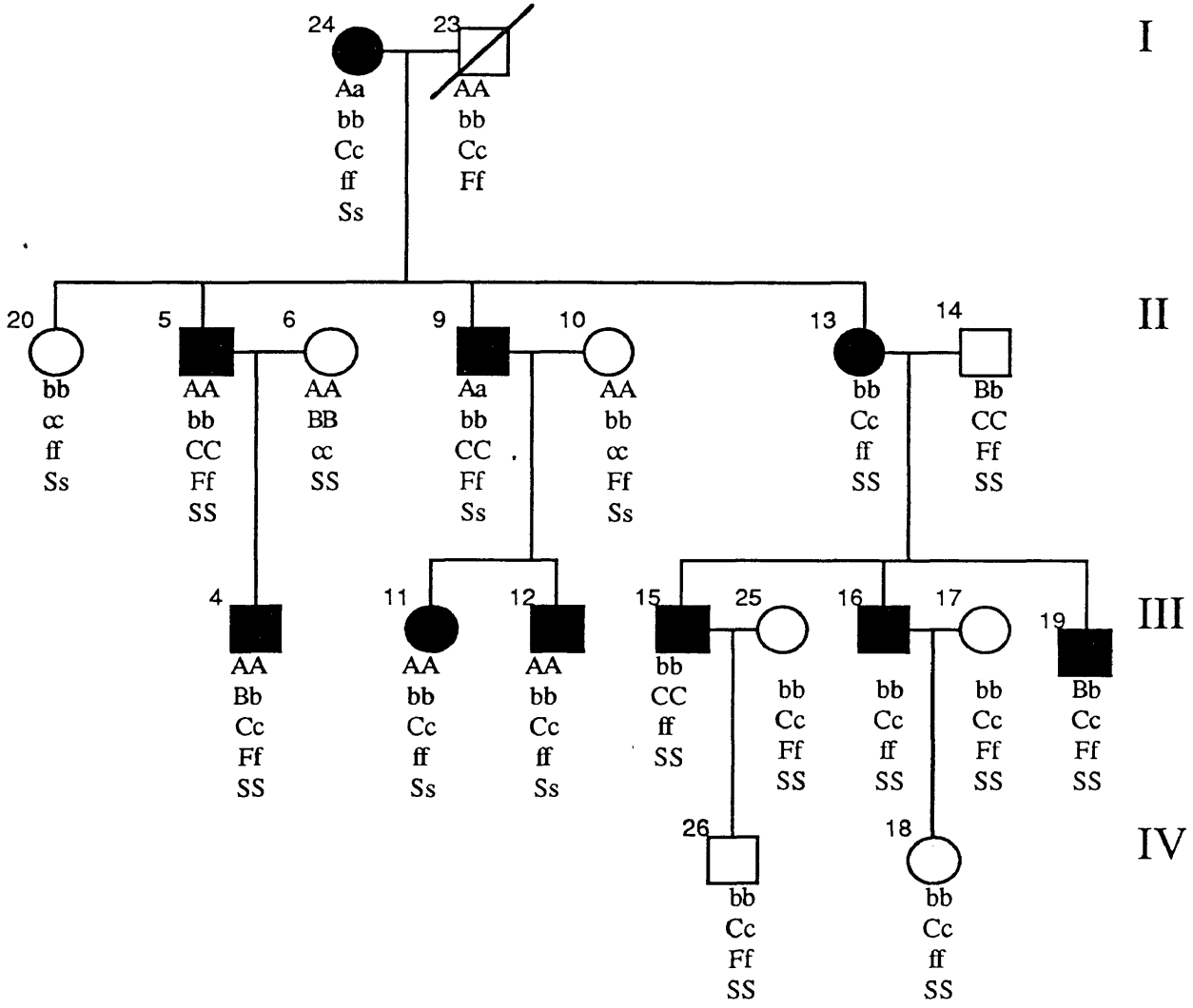

Figure A4. Pedigree of family 6372 with RD. Information of individual 23 was estimated according to the data of second generation. A-Cen6, B-BF, C-GLO, F-D6S8, S-D6S9.

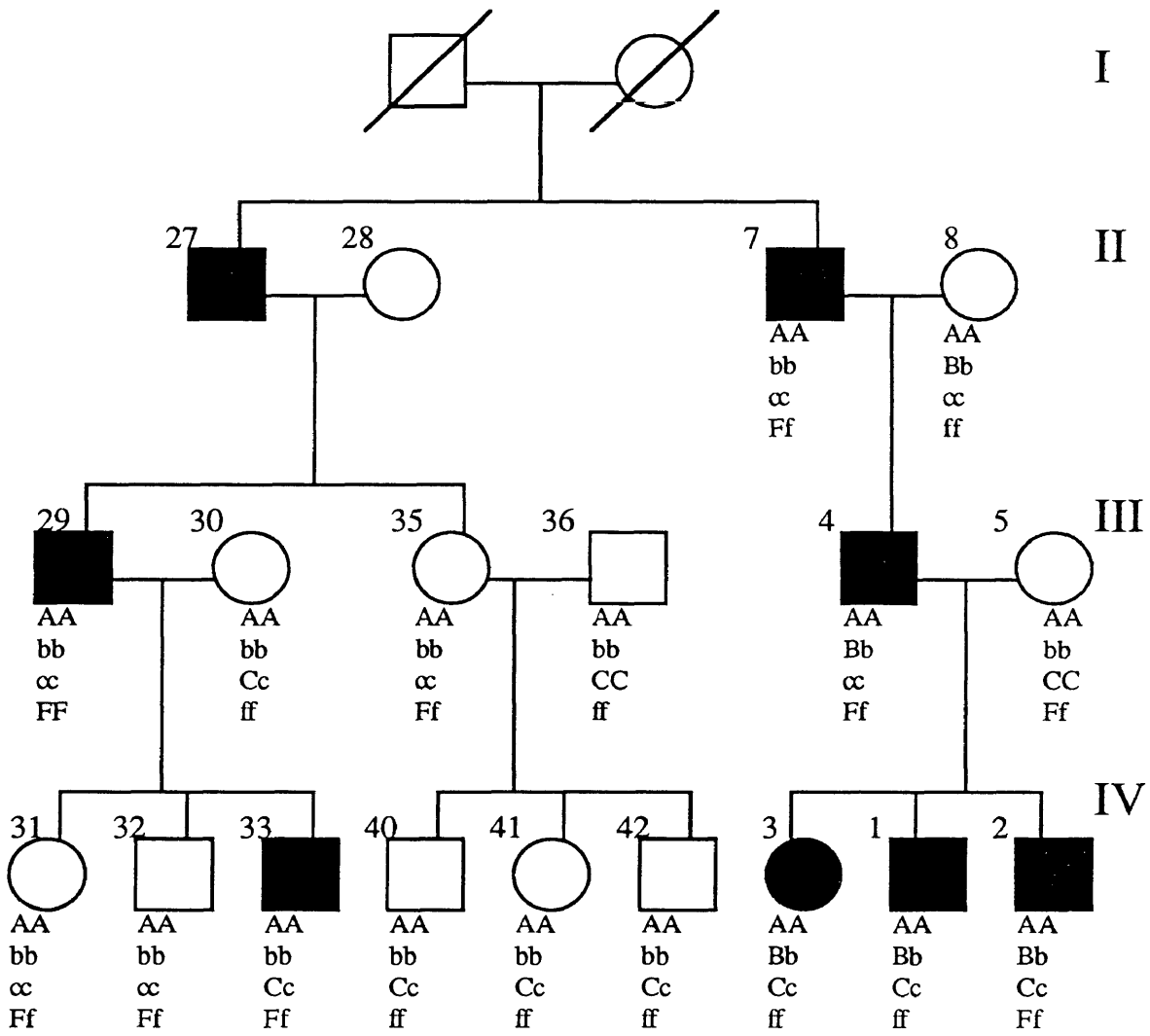

Figure A5. Pedigree of family 6375 with RD. Information of first generation is not available. A-Cen6, B-BF, C-GLO, F-D6S8.

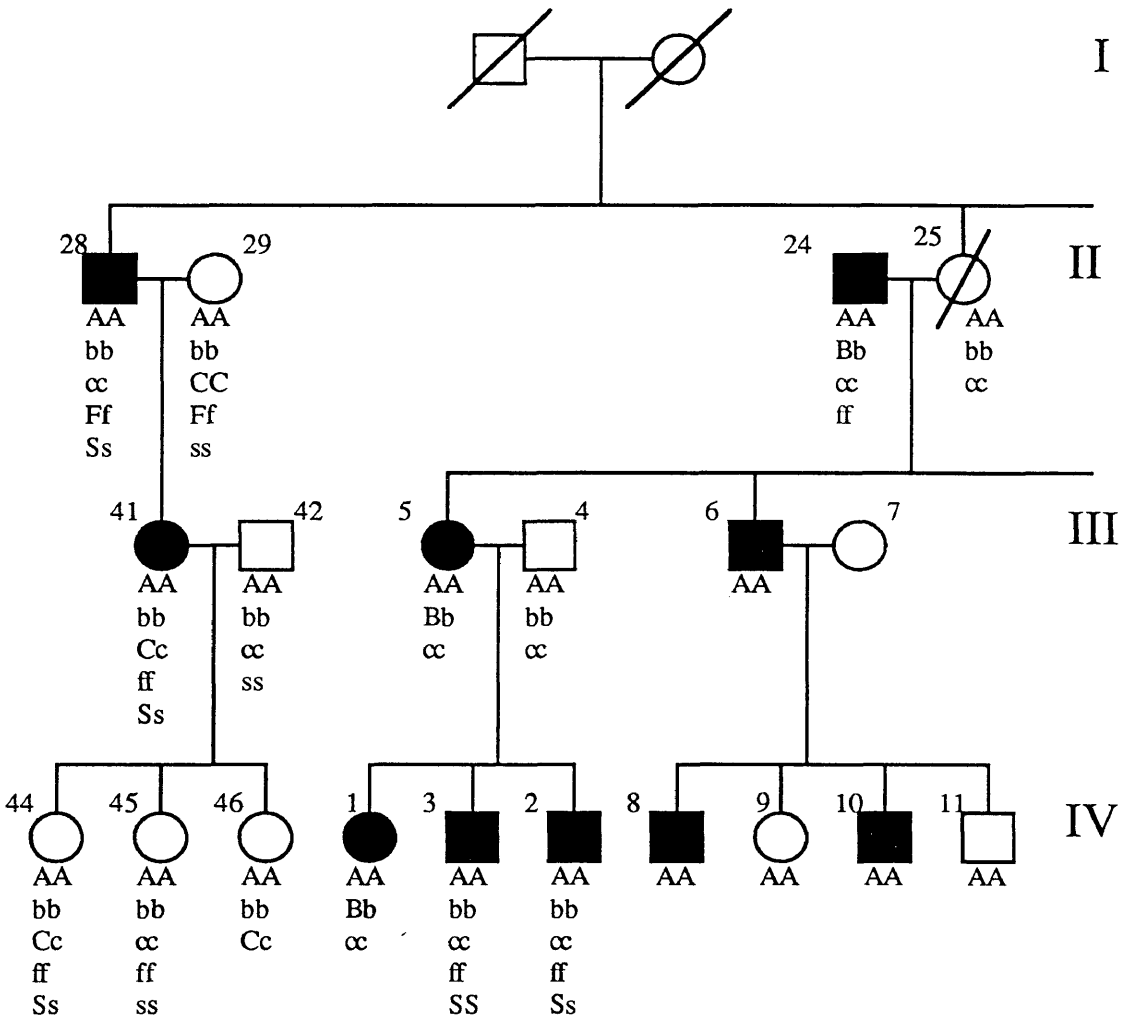

Figure A6. Pedigree of family 6432 (page 1 of 2) with RD. Information of first generation is not available. Information of individual 25 was inferred from the data of next generation. A-Cen6, B-BF, C-GLO, F-D6S8, S-D6S9.

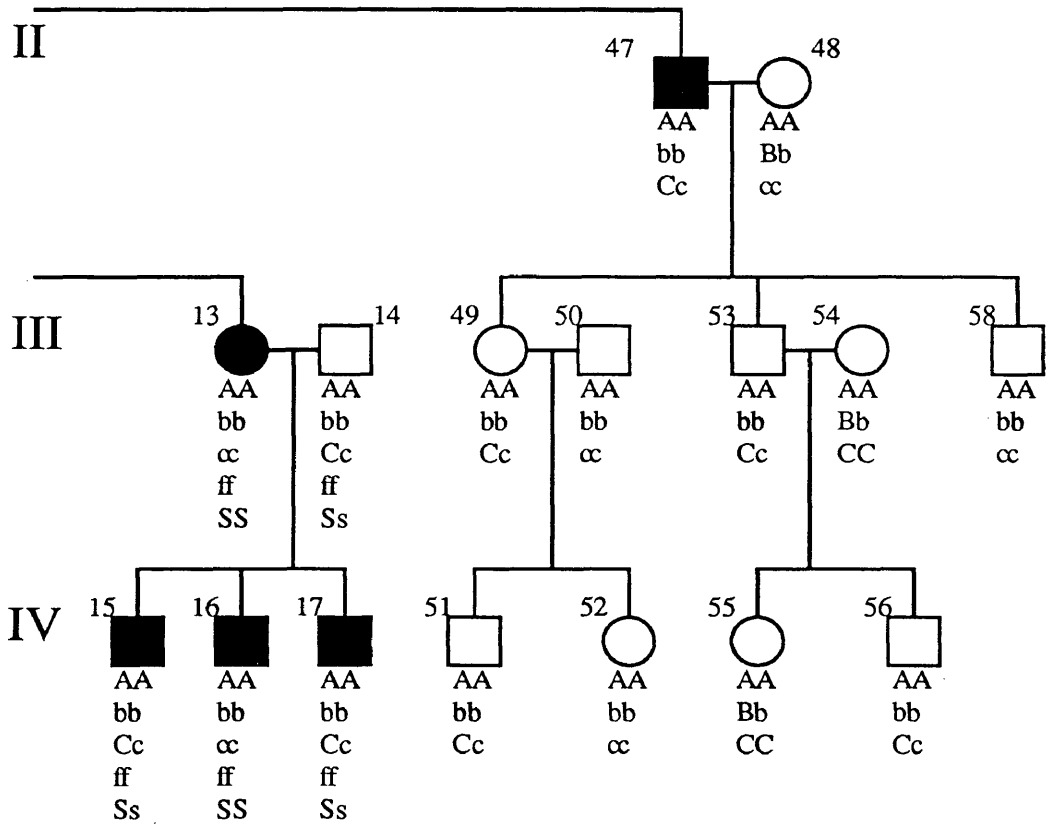

Figure A6. Pedigree of family 6432 (page 2 of 2) with RD. Information of first generation is not available. Information of individual 25 was inferred from the data of next generation. A-Cen6, B-BF, C-GLO, F-D6S8, S-D6S9.

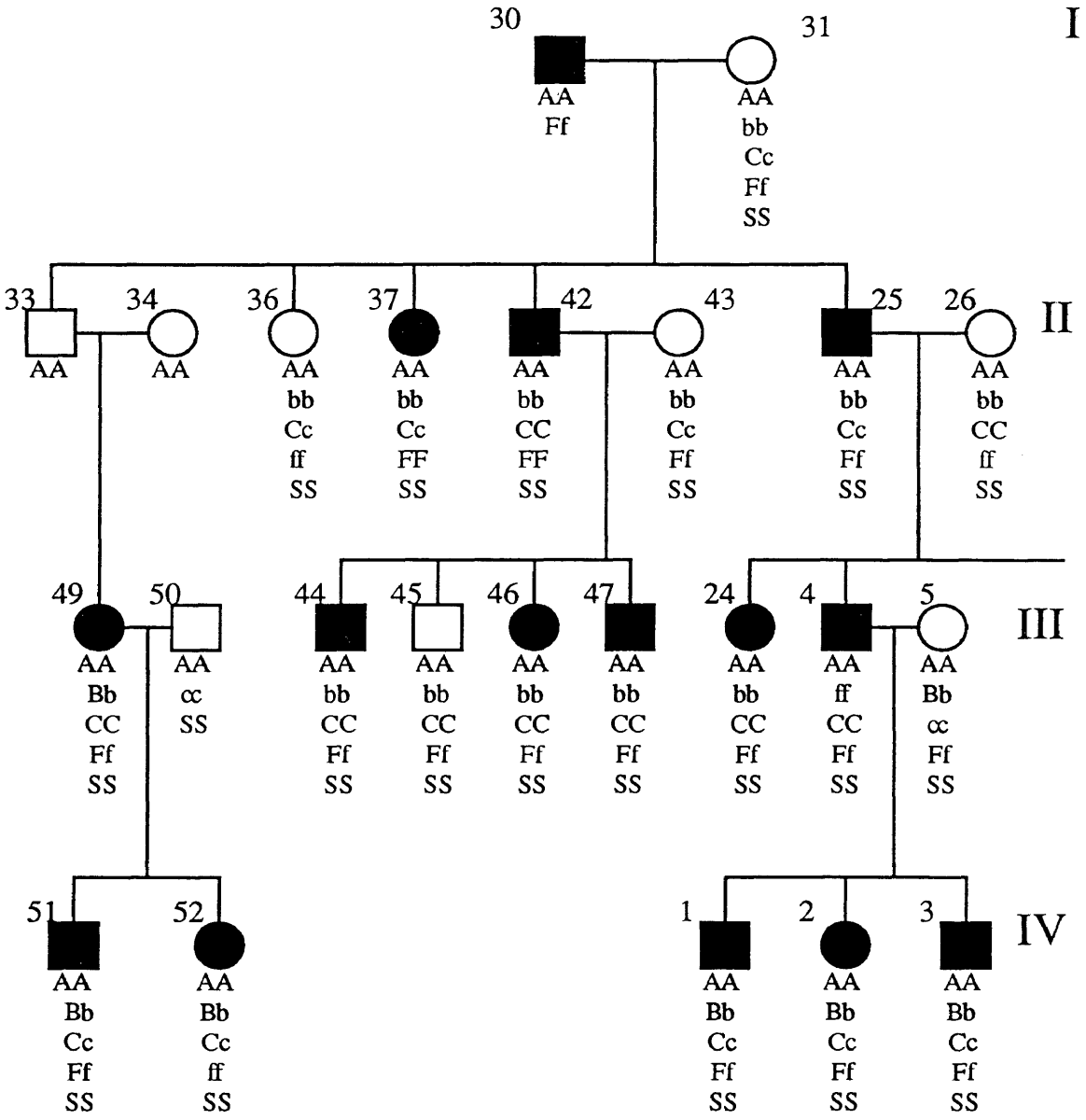

Figure A7. Pedigree of family 6484 (page 1 of 2) with RD. A-Cen6, B-BF, C-GLO, F-D6S8, S-D6S9.

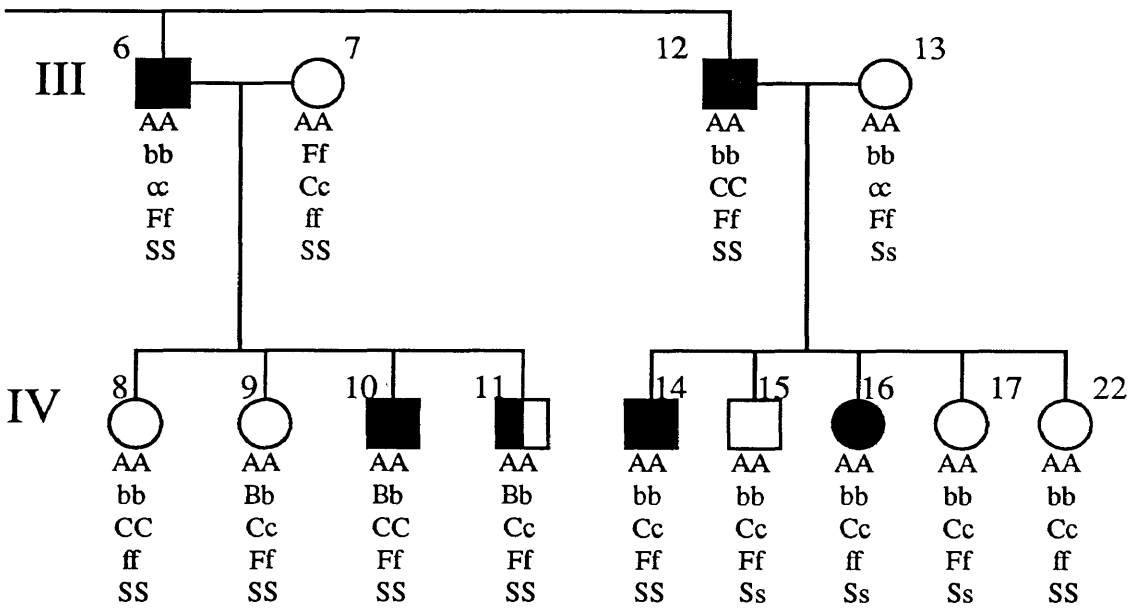

Figure A7. Pedigree of family 6484 (page 2 of 2) with RD. Diagnosis of individual 11 is unsure. A-Cen6, B-BF, C-GLO, F-D6S8, S-D6S9.

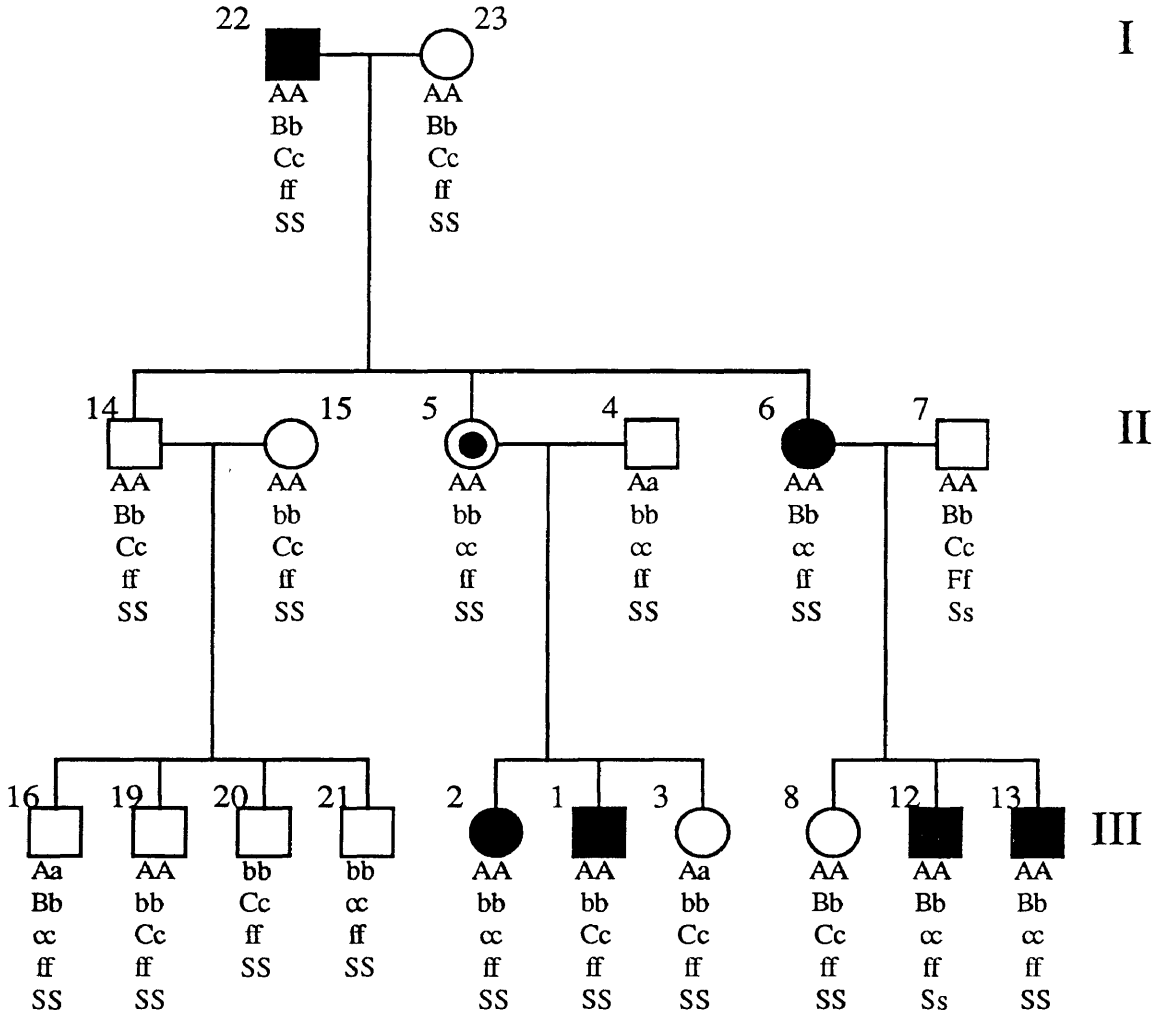

Figure A8. Pedigree of family 6491 with RD. Individual 5 is an obligate heterozygote. A-Cen6, B-BF, C-GLO, F-D6S8, S-D6S9.

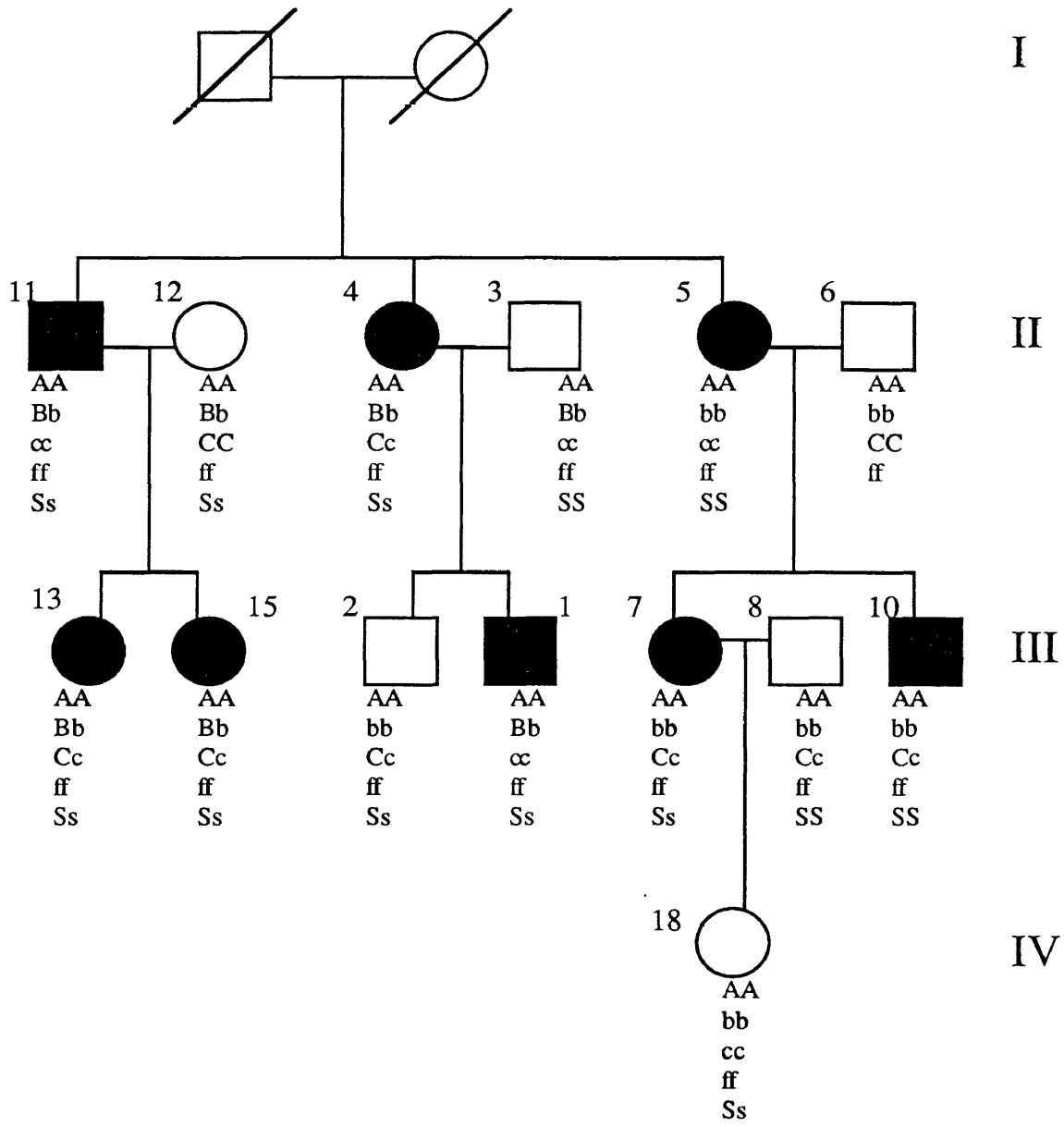

Figure A9. Pedigree of family 6576 with RD. Information of first generation is not available. A-Cen6, B-BF, C-GLO, F-D6S8, S-D6S9.

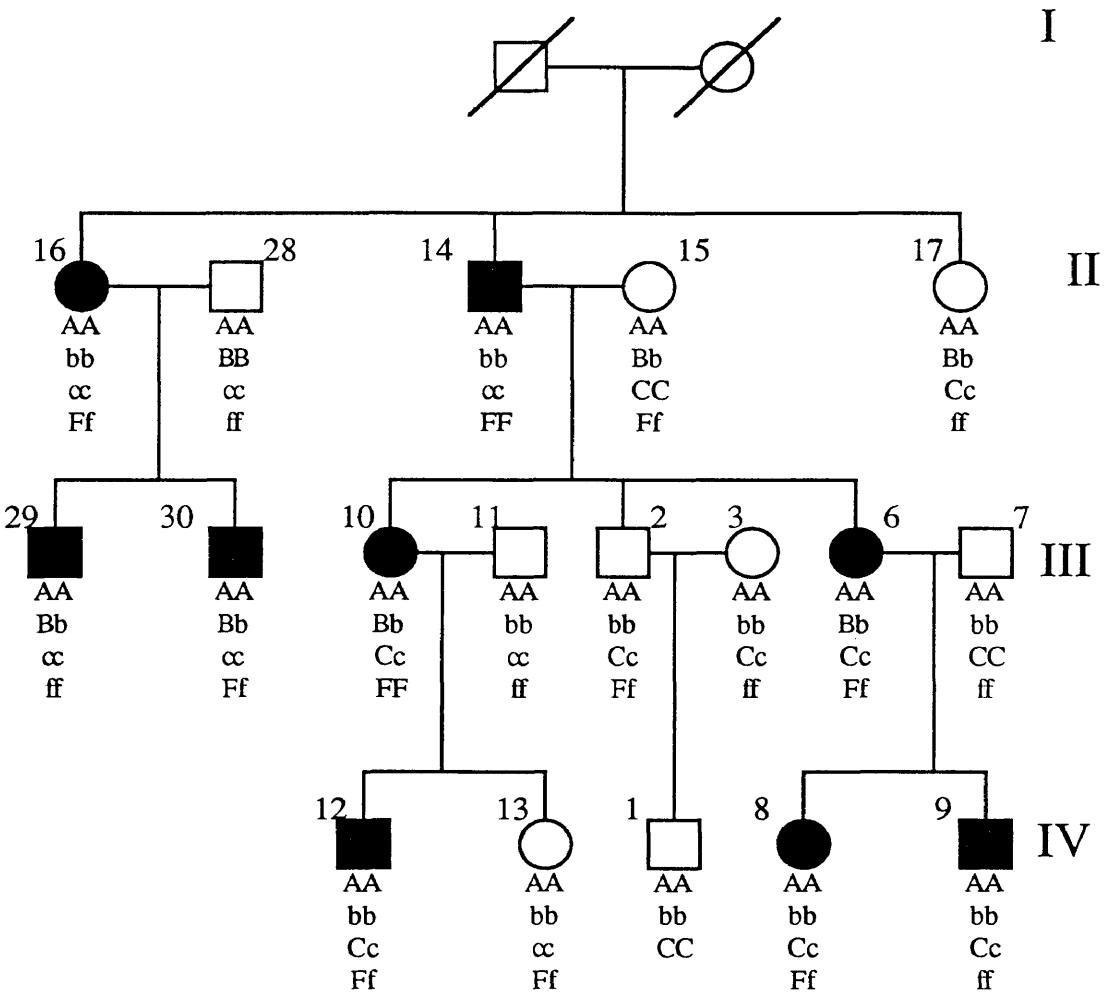

Figure A10. Pedigree of family 8001 with RD. Information of first generation is not available. A-Cen6, B-BF, C-GLO, F-D6S8.

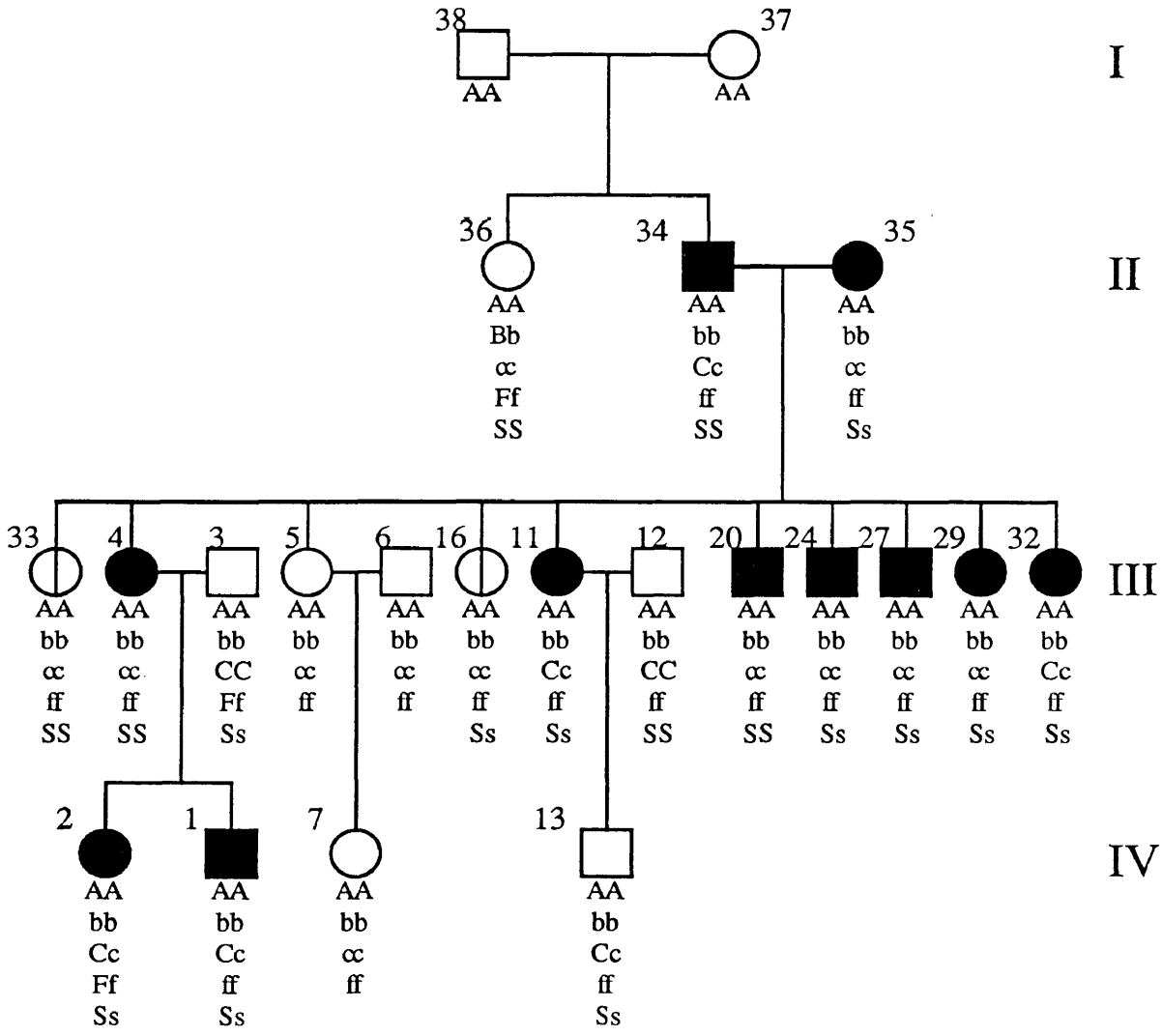

Figure A11. Pedigree of family 8002 with RD. Information of first generation is not available. The diagnosis of individual 16 and 33 is not sure. A-Cen6, B-BF, C-GLO, F-D6S8, S-D6S9.

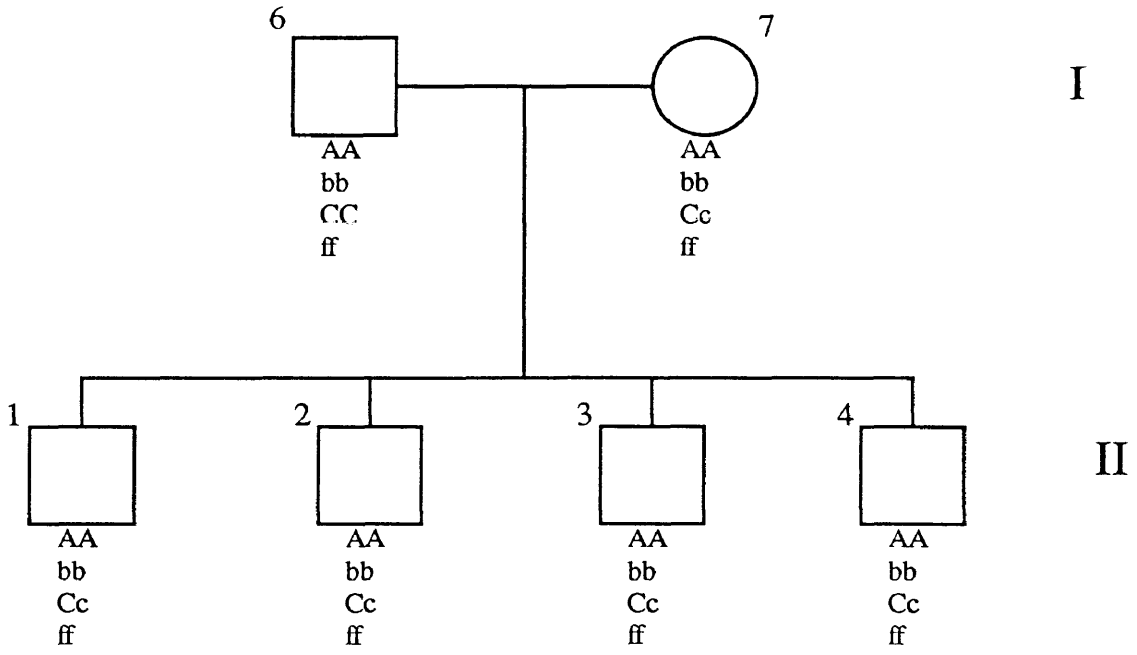

Figure A12. Pedigree of family 8003 without any affecteds of RD. Data of this family provides information for the order of genetic markers used in this analysis. A-Cen6, B-BF, C-GLO, F-D6S8, S-D6S9.

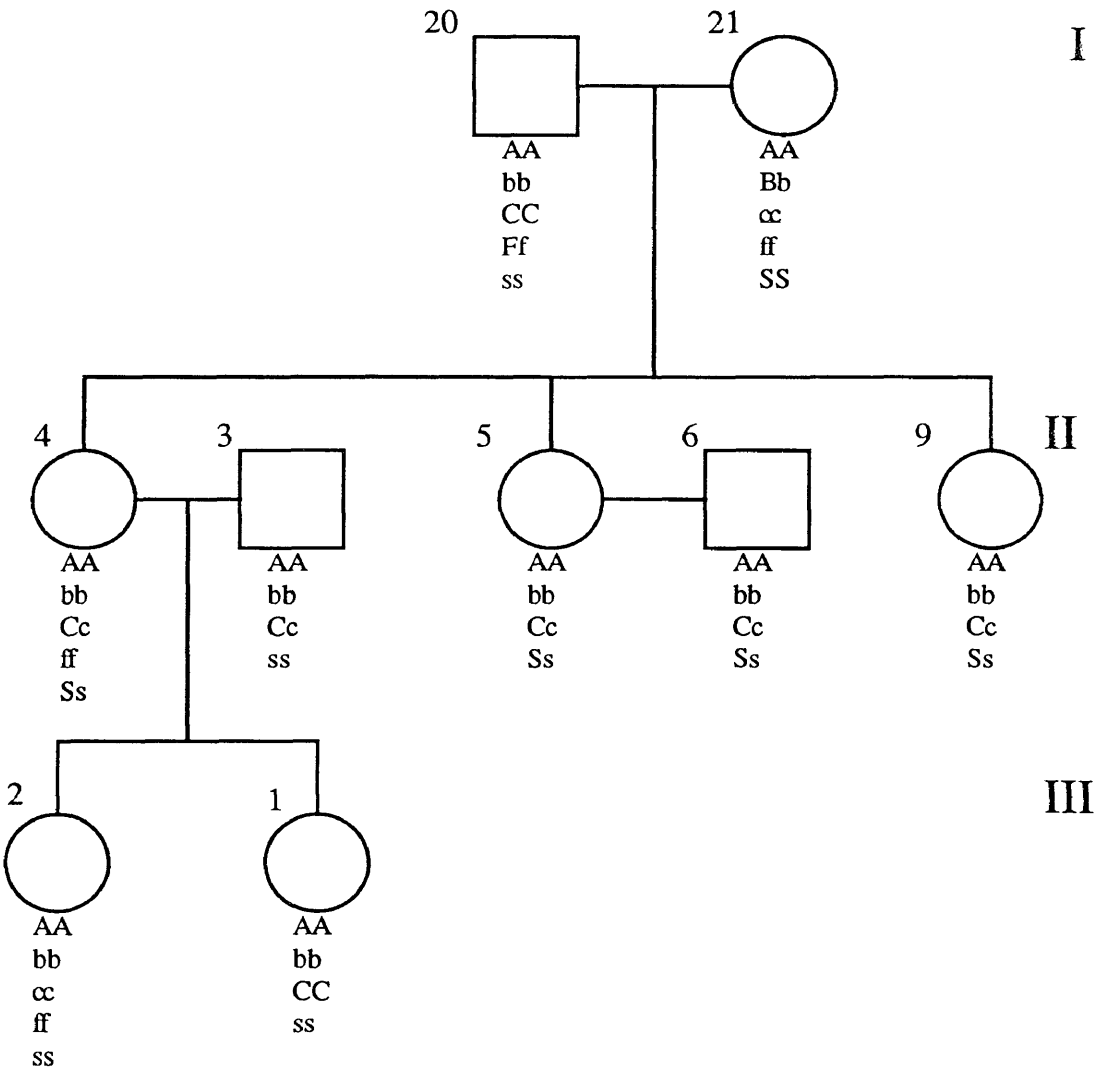

Figure A13. Pedigree of family 8004 without any affected RD individuals. Data of this family provides information for the order of genetic markers used in this analysis. .A-Cen6, B-BF, C-GLO, F-D6S8, S-D6S9.

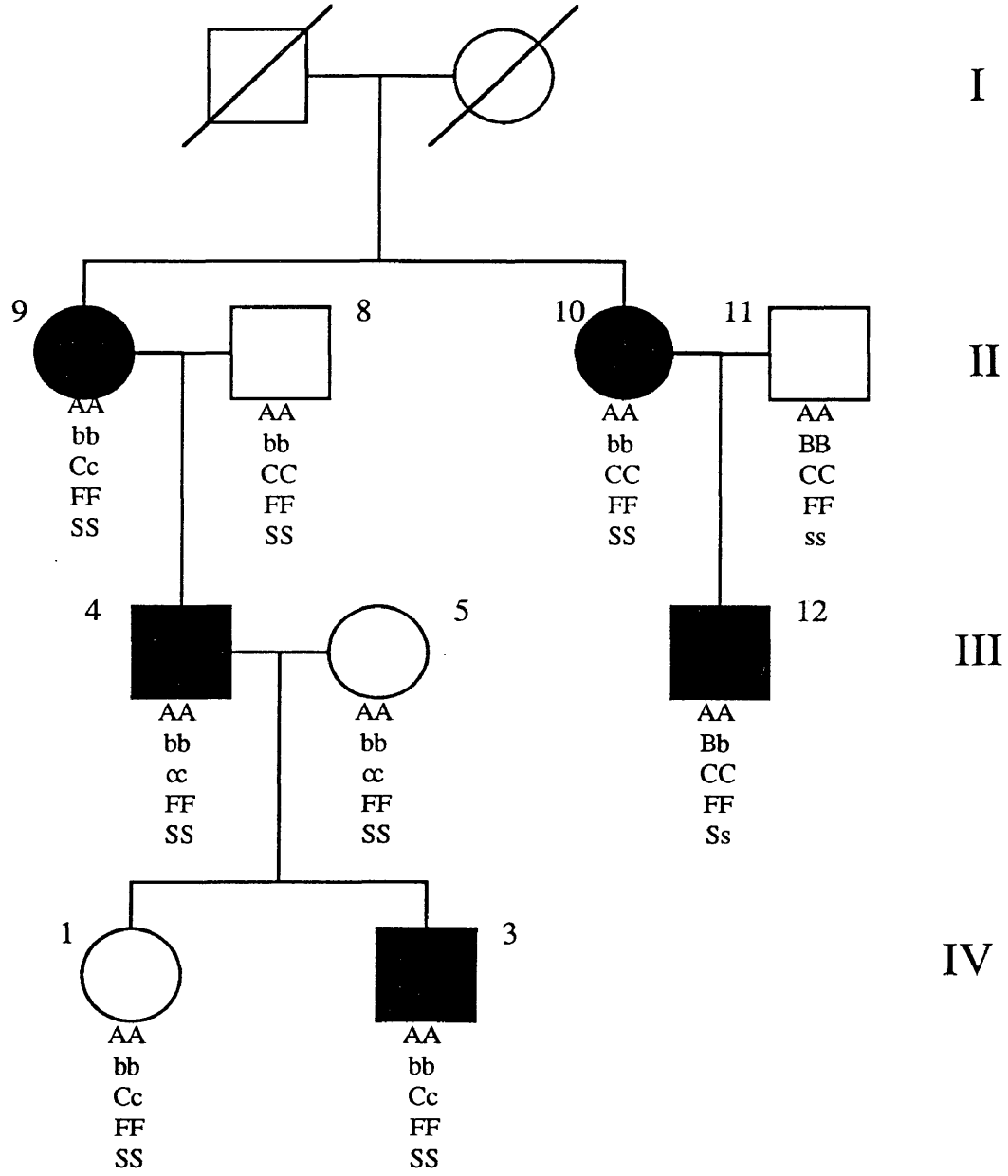

Figure A14. Pedigree of family 8005 with RD. Information of first generation is not available. A-Cen6, B-BF, C-GLO, F-D6S8, S-D6S9.

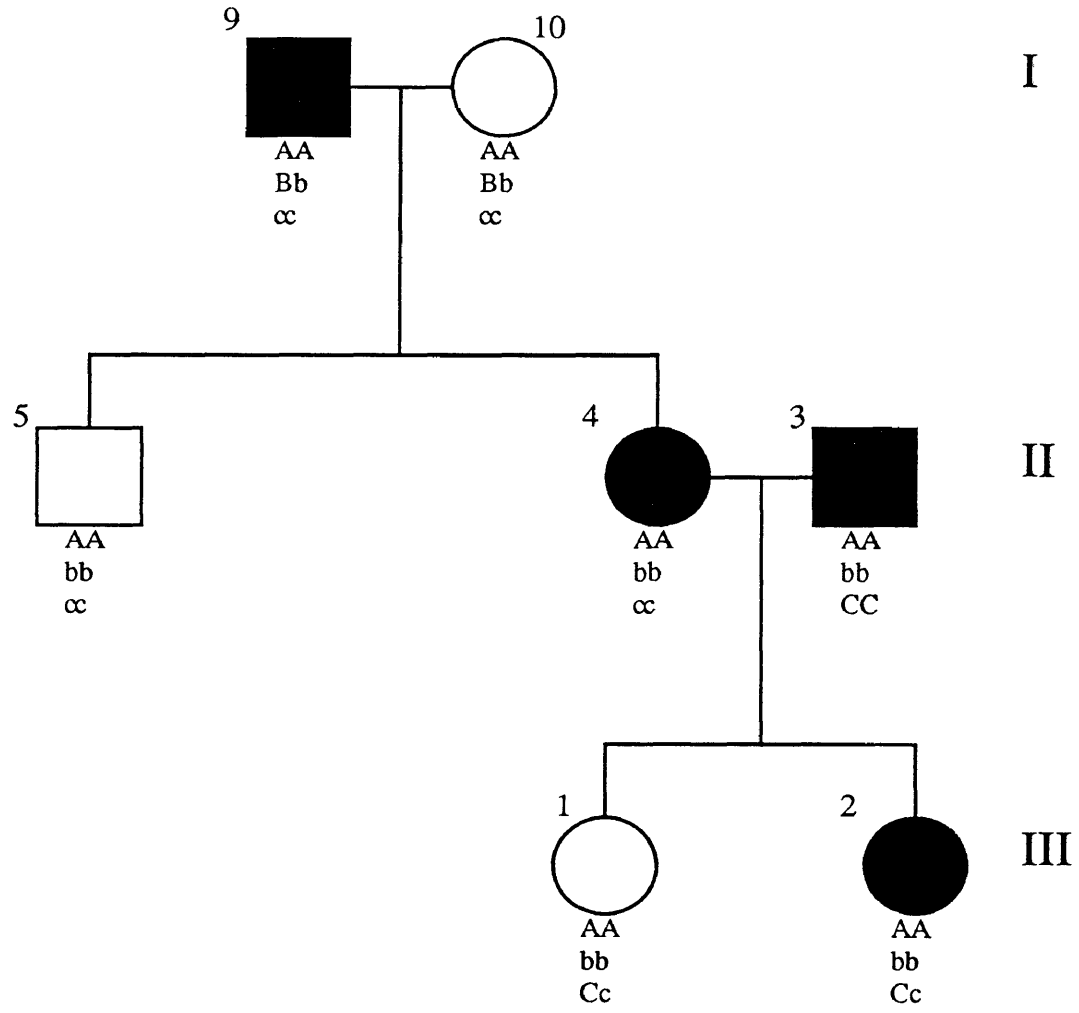

Figure A15. Pedigree of family 8006 with RD. In second generation, individual 3 and 4 are both affected, this type of family does not provide much information. A-Cen6, B-BF, C-GLO.

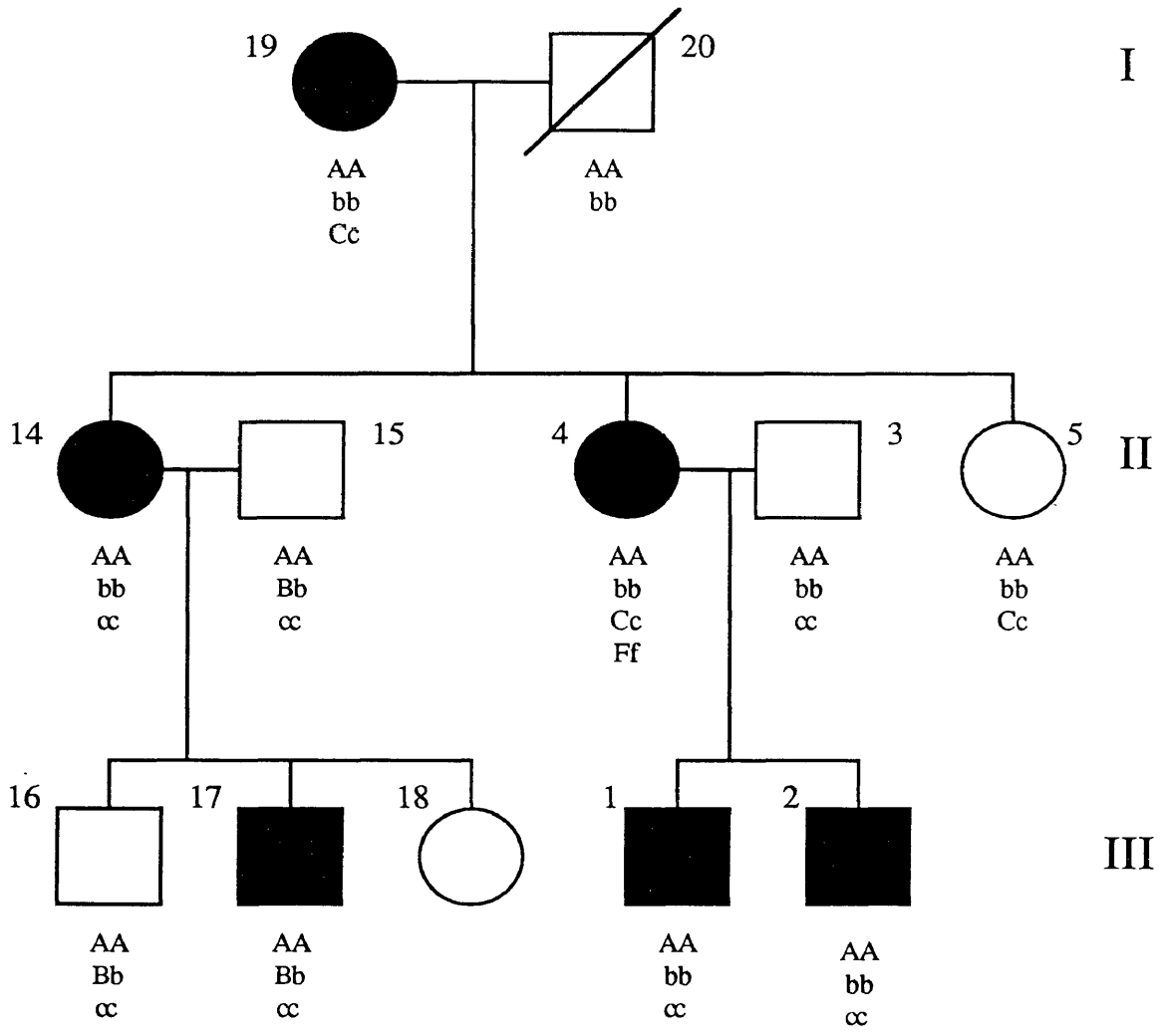

Figure A16. Pedigree of family 8007 with RD. Information of individual 20 is estimated. The diagnosis of individual 14 is not sure. A-Cen6, B-BF, C-GLO.

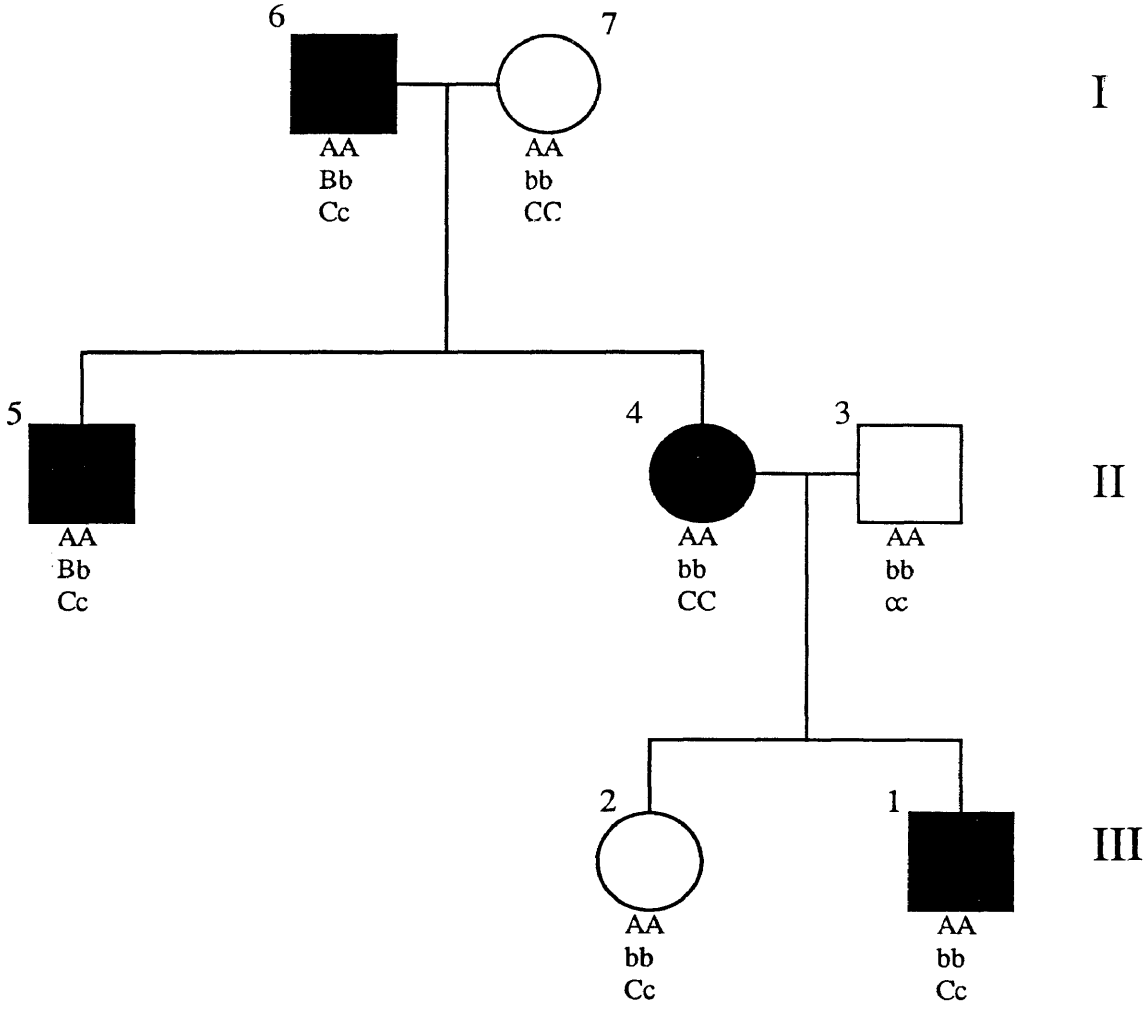

Figure A17. Pedigree of family 8008 with RD. A-Cen6, B-BF, C-GLO.

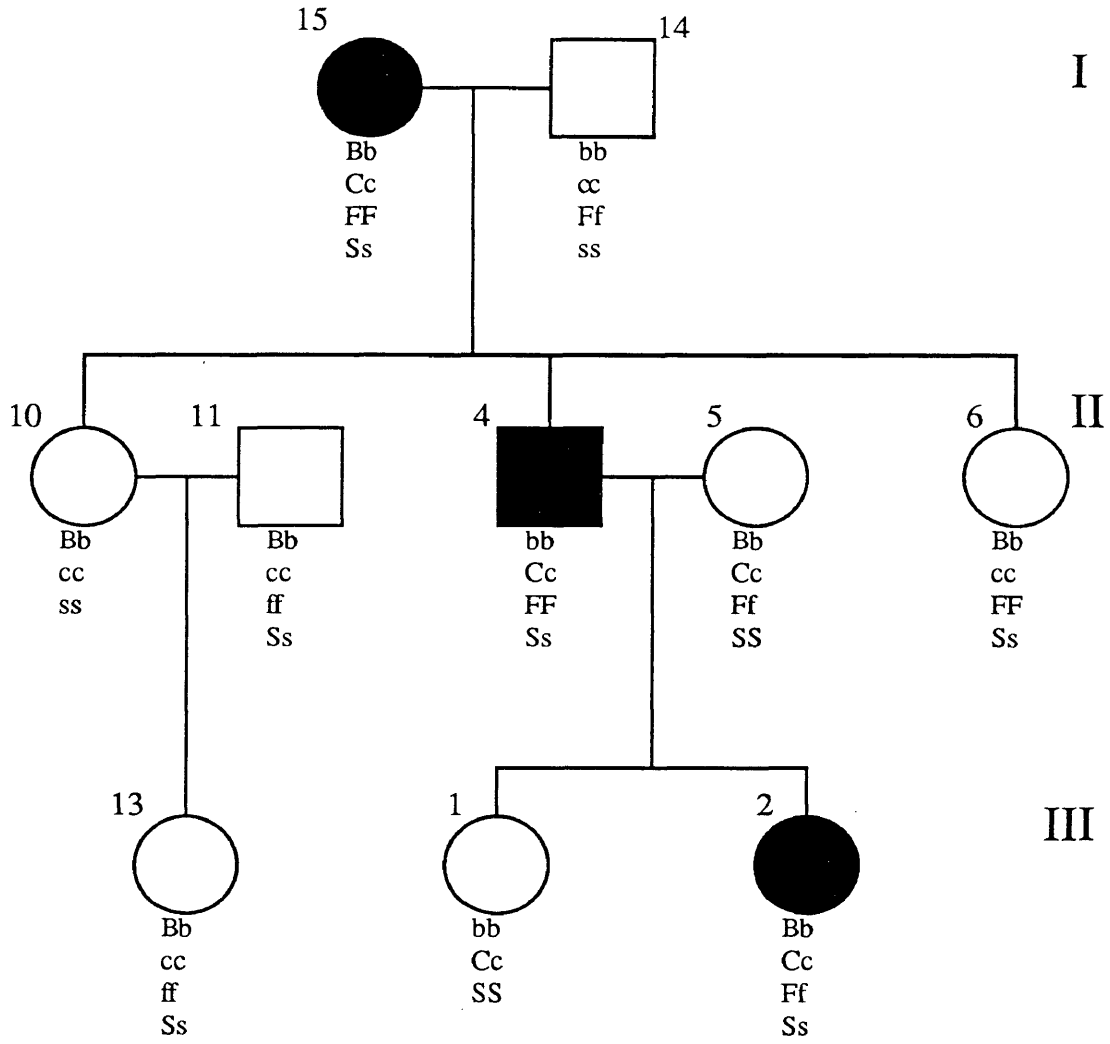

Figure A18. Pedigree of family 8009 with RD. A-Cen6, B-BF, C-GLO, F-D6S8, S-D6S9.

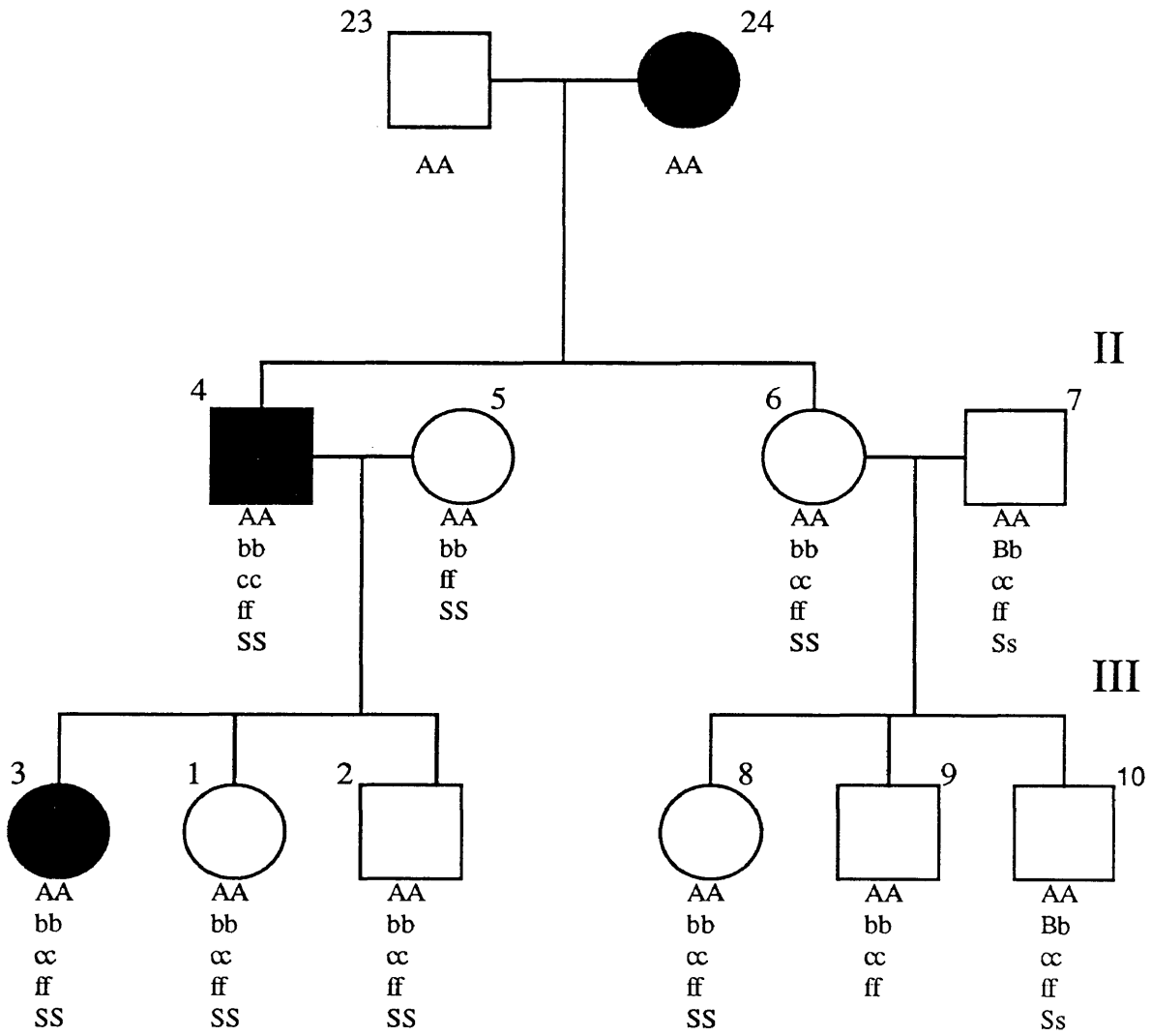

Figure A19. Pedigree of family 8010 with RD. A-Cen6, B-BF, C-GLO, F-D6S8, S-D6S9.