BARDET-BIEDL SYNDROME IN NEWFOUNDLAND: MOLECULAR GENETICS OF A RARE RECESSIVE DISORDER IN A SMALL ISOLATED POPULATION

MICHAEL O. WOODS

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BARDET-BIEDL SYNDROME IN NEWFOUNDLAND: Molecular Genetics of a Rare Recessive Disorder in a Small Isolated Population

by

© Michael O Woods

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Abstract

Bardet-Biedl syndrome (BBS) is characterized by retinal dystrophy, dysmorphic extremities, renal structural abnormalities, obesity, and hypogenitalism in males. This autosomal recessive disorder is genetically heterogenous with four identified loci, *BBS1-4* (11q, 16q, 3p and 15q respectively). BBS is a relatively rare disorder, but it is approximately ten times more prevalent in Newfoundland than in northern European populations.

To investigate the high incidence of BBS in the Newfoundland population, members of 17 BBS families were analyzed by haplotype and linkage analyses. Initially, linkage of five families to *BBS1*, one each to *BBS2* and *BBS3* and exclusion of six families from the four known BBS loci was observed.

A large consanguineous Newfoundland BBS family, excluded from the four known BBS loci, was used to identify a fifth BBS gene locus (*BBS5*) on 2q31 in a genome-wide scan. However, this gene did not segregate in any other of the five unlinked families. Therefore, another genome scan was implemented on a consanguineous family excluded from the five BBS loci. Evidence of a sixth BBS gene (*BBS6*) on 20p12 was established and the critical interval narrowed to 2 cM using five other unlinked families. Located within this region is a putative chaperonin gene (*MKKS*) involved in McKusick-Kaufman syndrome, a disorder with an overlapping phenotype with BBS. When *MKKS* was screened for mutations in six Newfoundland BBS families,

one missense and two frameshift mutations were identified. Thus, *MKKS* was the first gene identified to cause BBS. Remarkably, one family could be excluded from all six BBS loci, indicating the existence of a seventh BBS gene (*BBS7*).

By mutational and/or haplotype and linkage analyses, it was possible to assign 14 of the 17 Newfoundland BBS families to known BBS loci. Six families had mutations in *MKKS/BBS6*, five families were associated with the *BBS1* locus, and one family to each of the *BBS2*, *BBS3* and *BBS5* loci. Additionally, one family was excluded from the six known BBS loci. The discovery of *MKKS/BBS6* should aid in the ascertainment of other BBS genes and contribute to the basic understanding of the manifestations of BBS.

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Acknowledgment of Collaboration

As approved by Memorial's School of Graduate Studies, some of the work presented in this thesis was previously documented in the PhD thesis of Terry-Lynn Young. The work completed by Dr. Young, in which I collaborated, consists of the *BBS1* linkage disequilibrium study, which narrowed the *BBS1* locus to 1 cM (Young *et al.*, 1999b); the genome scan of family B9, which identified a fifth BBS locus (Young *et al.*, 1999); and the fine-mapping of family B2, which narrowed the *BBS3* locus to 6 cM (Young *et al.*, 1998). It is included in this thesis to provide continuity to the account of the search for the genetic basis of BBS in Newfoundland. I performed the initial genetic survey of the 17 Newfoundland BBS families (Woods *et al.*, 1999); the genome scan of family B13, which identified a sixth BBS locus; the subsequent microsatellite analysis of the *BBS6* locus on the unlinked families; the screening of *MKKS* for BBS-causing mutations, which demonstrated mutations in *MKKS* cause BBS (Katsanis *et al.*, 2000); and the exclusion of all six BBS loci in family B6, which suggested a seventh BBS locus (Beales *et al.*, 2001). Also, I performed the analyses of the *BBS5* and chromosome 18 loci on the unlinked families previous to the identification of *BBS6*. The mutation analysis was performed in Dr. Jim Lupski's laboratory at the Baylor College of Medicine in Houston, Texas with the support of Drs. Nicholas Katsanis and Phillip L. Beales.

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Chapter 1 Introduction

1.1 A Brief Introduction to the Thesis

The island of Newfoundland, where the subjects studied in this thesis originate, has long been regarded as isolated from the rest of Canada. Newfoundland has a population of approximately half a million persons, a quarter of which are located in the capital city of St. John's. The remaining majority of the populace is spread over an area of 111 390 km^2. Such a sparse population density has contributed to the many small, isolated communities which sprung up on the shores of the island. These communities were traditionally founded on the coastal regions because fishing was the primary industry of the Newfoundland people. An environment such as this may be partially responsible for the increased prevalence of a particular autosomal recessive condition in Newfoundland - Bardet-Biedl syndrome (BBS).

Bardet-Biedl syndrome, a relatively rare condition worldwide, is a systemic disorder. The primary characteristics include retinal dystrophy, renal abnormalities, limb malformations, obesity and hypogenitalism in males. At the origin of this study, only the approximate chromosomal locations (spanning regions ≥9 cM) of four BBS genes (*BBS1-4*) were known. Newfoundland was considered a suitable community to determine more information about the genetics of this rare disease because of: (1) the relatively higher

incidence of BBS in Newfoundland (2) the well characterized nature of the disease in 17 BBS families on the island (3) the highly cooperative nature of the families involved and (4) the unique population structure of the province. It is the purpose of this thesis to genetically characterize the 17 BBS families and attempt to identify one or more of the genes causing BBS in these kindreds.

1.1.1 The Settling of Newfoundland

To understand the genetic disposition of the inhabitants of the island of Newfoundland, elucidation of the origins and migratory patterns of the founders of our contemporary population is necessary. The peopling of Newfoundland was unique in North America in the maintenance of its isolation and the restriction of founding communities to the coastline. Settlements have been comparatively isolated until modern times. As well, most immigrants originated from one of two highly localized areas: the southwest of England and the southeast of Ireland.

At first, in the late 16th and early 17th centuries, there was a migratory fishery conducted by European nations who discouraged permanent settlement. This continued until the late 18th century. Seasonal fishermen stayed during the summer to work on the inshore fishing ships and returned home in autumn. Later, a few remained over the winter for one or two years, some being accompanied by women and children. This

began the transition to permanent settlements. However, during the 17th and 18th centuries, less than 5% of temporary British migrants became permanent settlers (Mannion, 1986). This slow rate of colonization would be a familiar theme over the following centuries.

The first permanent British settlements established in the early 17th century were ones erected by the will of the merchants, to harvest the fruits of the land and sea - furs and seals. Colonies in Conception Bay and the Southern Shore of the Avalon Peninsula were created. However, by the end of the 17th century, there were only approximately 3 000 colonists in 30 communities spanning the area between Bonavista Bay, in the north, and Trepassey, in the south. In the second quarter of the 18th century, the settlement rate of the English intensified and small colonies were founded increasingly westward in the south, and northward and westward in the north, due to the increase in resource diversification during the non-fishing months. Between 1725 and 1775, a relatively large growth spurt occurred because of increasing demand for cod in Europe. There was an increase in women and children arriving on the shores of Newfoundland at this time, but there still remained a great number of unattached adult males in the population, indicating that the seasonal and temporary sectors remained active (Mannion, 1986).

Migration and permanent settlers from southwest England and southeast Ireland increased dramatically during the early 19th century and continued to do so until the mid 1830s. In these three decades, primarily due to influx of migrants, the population of Newfoundland almost quadrupled, from 19 000 to 75 000 people. Of the 45 000 persons

who came off the British ships at this time, approximately 75% were Irish, 23% English and 2% from the Channel Island of Jersey. However, it is impossible to know how many of these remained permanent residents of Newfoundland. Interestingly, there were two major migrations of Irish during this time, one between 1811 and 1816 and the other between 1825 and 1833. The English, on the other hand, maintained a relatively constant rate of immigration and, since they had been the earliest inhabitants, there was now an even ratio of English to Irish on the island. A ratio of 53:47 percent, favoring the English, not only described the derivations of the inhabitants but also their religious affiliation, as almost invariably English was synonymous with Protestantism, and Irish with Catholicism. Although the migration of peoples increased during this era, what contributed most profoundly to the lasting population was the marked increase in women and children. Now there was an availability of female spouses for the many young single men (Mannion, 1986).

Unlike most other New World colonizations, the majority of settlers to Newfoundland can be pinpointed to merely a few localized origins in England and Ireland over the history of the migration. English emigrants came from the contiguous counties of Devon and Dorset and the neighboring areas of Somerset and Hampshire in the southwest of England. These were the source areas that contained the ports involved in the Newfoundland migratory cod fishery. In the southeast of Ireland the situation was similar. The majority of Irish involved in the cod fishery originated within a 30 mile radius of the city of Waterford, including regions of Wexford, Kilkenny, Tipperary, Cork

and County Waterford. Up until the mid to late 17th century there was intermingling of the two nationalities because almost all persons were transported by English ships. Originally, the Irish settled in the same areas as the English, between Trepassey and Bonavista Bay. However, over time the Irish tended to predominate on the Avalon peninsula and the English elsewhere. This pattern can still be recognized today (Mannion, 1986).

Once the major migrations had concluded after the 1840s, natural increase became the dominant mechanism of population growth. By the 20th century approximately 221 000 people inhabited Newfoundland and Labrador, and the population doubled again by the early 1960s, slowly increasing until it reached a maximum of 581 800 in 1984 (Census of Canada, Census of Newfoundland). The steady increase in population resulted in the establishment of new settlements. Community expansion usually occurred in one of three ways: by partitioning ancestral land among heirs; by movement to nearby uninhabited lands inside the settlement core; and by migration further west on the north and south borders of the colony. This eventually led to settlements further along the west and north coasts of the island. The former two mechanisms were, by far, the most common and kept related families close together. This was a strong theme in the peopling of Newfoundland (Mannion, 1986).

During the 19th century, the cod fishery remained the primary source of income. However, in the 1880s, with an increasing populace in the outports relying on the same resources, the average intake of fish by fisherman had declined to only one quarter of that

harvested in the early part of the century. Due to lack of increase in fish prices and the added expense of imported goods, the economy began to decline and communications and transportation within the island was slowed. Also, in the last two decades of the 19th century, growth of the population decreased substantially. This was attributed to the emigration of many persons to the United States. These were primarily Irish Newfoundlanders, who heard from relatives that there was work to be obtained for artisans and laborers in the New England states. Due to the stagnancy of the economy, the outport communities became increasingly isolated from the outside world and from each other (Mannion, 1986).

Scenarios were present throughout Newfoundland's colonization and subsequent continued habitation that were unique in the New World, creating a population that was isolated, homogenous and partitioned into small, separate communities. Firstly, Newfoundland is separated from mainland centers by hostile waters, historically making travel to and from the island difficult. Secondly, the majority of immigrants originated, over almost the entire period of settlement, from only a few distinct locales in England and Ireland. The settlement of the island transpired at a slow pace over centuries as seasonal fisherman became temporary inhabitants and eventually permanent. However, during this time there were two periods of punctuated immigration - during the third quarter of the 18th century and early 19th century. From these migrations came most of the present population. As well, communities were set up primarily on the coast and remained relatively small, isolated entities. In 1982, approximately 50% of the

population lived in communities of fewer than 2 500 inhabitants and 41% in communities fewer than 1 000 people (Bear *et al.*, 1987). Such a social climate and geographical distribution of peoples may have lead to a static gene pool. Families were isolated in small communities, constructed by environmental and socio-economic conditions that encouraged kinships to remain adjacent to one another. Perhaps for these reasons, one hereditary recessive disease, Bardet-Biedl syndrome, is approximately ten fold more prevalent here than in the original Northern European populations (Beales *et al.*, 1997).

1.2 Phenotype of Bardet-Biedl Syndrome

Initially, the cardinal manifestations of Bardet-Biedl syndrome were considered to be retinal dystrophy, polydactyly, obesity, mental retardation and hypogonadism (Cockayne *et al.*, 1935; Bell *et al.*, 1958; Klein and Amman, 1969). Recently, an additional cardinal feature has been added - renal abnormalities (Churchill *et al.*, 1981; Cramer *et al.*, 1988; Bruford *et al.*, 1997; Beales *et al.*, 1999). Also, Green *et al.* (1989) suggested that mental retardation should not be included in the cardinal manifestations, and that hypogonadism and polydactyly should be modified to hypogonadism in males and dysmorphic extremities, respectively. The discrepancies in the essential features of BBS may be due to the different ethnic origins of the patients included in the studies. Also, the completeness of the clinical evaluations on the patients and their families,

particularly regarding renal imaging, has been a factor. Other problems include the lack of follow up of affected children who have not yet developed particular features; and, in the case of testing for mental retardation, the absence of an appropriate exam for the visually impaired. However, for consideration of BBS in this thesis, an individual must have had at least four of the six cardinal manifestations, which include: retinal dystrophy, dystrophic extremities, obesity, learning disabilities, hypogonadism and renal dysfunction (Green *et al.*, 1989; Beales *et al.*, 1999). Beales *et al.* (1999) recently proposed a new diagnostic scheme which stated BBS patients must have four of the six cardinal features, or three cardinal features and two secondary features. The latter includes speech disorder or delay; strabismus, cataracts or astigmatism; brachydactyly or syndactyly; developmental delay; polyuria or polydipsia; ataxia, poor coordination or imbalance; mild spasticity; diabetes mellitus; dental abnormalities; hepatic fibrosis; and congenital heart disease.

1.2.1 Retinal Dystrophy and Other Ocular Manifestations

Retinal dystrophy in Bardet-Biedl syndrome has been well documented due to its prevalence, inherent complexity and ease of evaluation. In three large reviews of BBS, the presence of retinal dystrophy was reported in at least 91% of the patients, making it the most prevalent manifestation, and one that will most likely lead to a successful

diagnosis of this disorder (Bell, 1958; Klein and Ammann, 1969; Beales *et al.*, 1999). However, there is a large degree of variability in the age of onset of blindness and in the spectrum of the retinal disease. Also, there is a lack of consensus on the mechanism of the retinal dystrophy.

Many studies have shown the heterogeneous nature of retinal abnormalities in BBS patients (Klein and Amman 1969; Bergsma and Brown, 1975; Green *et al.*, 1989; Leys *et al.*, 1988; Jacobson *et al.*, 1990). Regardless of the variety of retinal dystrophy, electroretinogram (ERG) results have been shown to be abnormal before pigmentary changes are apparent; thus it is a useful aid in early diagnosis (Francois *et al.*, 1954; Prosperi *et al.*, 1977; Campo et al., 1982; Fralick *et al.*, 1990; Lavy *et al.*, 1995).

In some studies, retinal dystrophy consisting of rod-cone degeneration was a predominant finding in patients who had had an ERG performed (Klein and Amman, 1969; Campo *et al.*, 1982; Jacobson *et al.*, 1990; Lavy *et al.*, 1995). These investigations suggested that the retinopathy is a widespread receptor disorder affecting the peripheral rods initially and later affecting the cones in the macula and periphery. This progression has been termed typical retinal dystrophy. Iannaccone *et al.* (1996) went as far as to hypothesize that the rods are congenitally non-functioning and the cones degenerate shortly after birth.

However, there is some evidence that cone dysfunction occurs prior to rod dysfunction in some BBS patients (Schachat and Maumenee, 1982; Rizzo *et al.*, 1986; Yagasaki and Jacobson, 1989; Jacobson *et al.*, 1990). It should be mentioned that no case

of BBS in which an abnormal ERG was observed has been followed by a normal ERG.

Typical retinitis pigmentosa with the presence of bone spicules is found only in approximately 15% to 20% of cases, with fundus changes in the majority being 'atypical' (Klein and Ammann, 1969; Ehrenfeld *et al.*, 1970; Runge *et al.*, 1986). Wrinkling, hypopigmentation, bull's-eye lesions and geographic atrophy of the macula have also been noticed in patients (Campo *et al.*, 1982; Jacobson *et al.*, 1990; Hrynchak, 2000). The heterogeneity observed in these studies may reflect a spectrum of disease within the syndrome or the description of patients at different points of development of the disease.

Despite varied presentations of retinal dystrophy, severe visual loss always occurs. Studies have shown that visual acuity is moderately reduced at the beginning of teenage years, with rapid deterioration by the third decade (Klein and Ammann, 1969; Runge *et al.*, 1986; Leys *et al.*, 1988; Riise *et al.*, 1997). Visual evoked responses (VER) were often normal in childhood but deteriorated with age (Ehrenfeld *et al.*, 1970; Campo *et al.*,1982; Jacobson *et al.*, 1990; Lavy *et al.*, 1995; Beales *et al.*, 1999). Compared with isolated typical retinitis pigmentosa, BBS patients have a more rapid progression of visual loss, with a mean of seven years from diagnosis to blindness (Beales *et al.*, 1999).

Unfortunately, retinal dystrophy is not the only ocular abnormality occurring in BBS. Beales *et al.* (1999) noted astigmatism, strabismus, cataracts, color blindness, macular oedema and degeneration and optic atrophy. Ehrenfeld *et al.* (1970) observed cataracts in one of their patients as did Schachat and Maumenee (1982). Myopia, ptosis and microphthalmia have also been reported (Rizzo *et al.*, 1986). Nystagmus was

documented in twins with BBS (Gottlob and Helbling, 1999), and in a Scandinavian study cataracts and myopia were frequently observed (Riise *et al.*, 1997).

Ocular histopathology in BBS has not been investigated extensively. In the few studies done, patients were over 18 years of age and had end stage retinal degeneration (Klein and Ammann, 1969; Bisland, 1951; Lahav *et al.*, 1977), or the patients were so young they may not have had time to develop pigmentary retinopathy (McLoughlin and Shanklin, 1967). However, the eyes of a four year old boy considered to have Bardet-Biedl syndrome, who died shortly after a renal transplant, showed photoreceptor cell degeneration without significant changes in the retinal pigment epithelium (RPE) (Runge *et al.*, 1986). The authors noted severe lesions in the macula with both rod and cone degeneration, although no underlying mechanism for these features was determined. There was a build-up of lipofuscin and other granular inclusions in the RPE cells that were hypothesized to have accumulated because of a problem in lysing and voiding of an ingested phagocytic load. The authors suggested the problems in the RPE could be secondary to biochemical anomalies within the outer segments (e.g. an inability of the photoreceptor cell membranes to evoke a proper phagocytic response). The conclusion that the photoreceptors were the primary problem was strengthened with the observance that the RPE was normal throughout the tissue, but the overlying photoreceptors were either normal, absent or damaged. Some problems with this study were the small sample size and the fact that the deceased may not have had BBS. Some of the manifestations described in the boy, like the mild mental retardation and the mild ataxia, may have been

the result of his chronic renal failure. There was also an extensive family history of renal disease. In addition, obesity and hypogonadism were not observed, although often these characteristics do not present at such a young age.

More recently, work on membrane fatty acids has shown an enrichment of polyunsaturated fatty acids in the cell membranes of BBS patients that may indicate increased lipoperoxidation ultimately resulting in the development of retinal degeneration (Corrocher *et al.*, 1989). Abnormal retinal vascularity has also been observed which could be a nonspecific response to the metabolic imbalance following degeneration of photoreceptors or RPE (Campo *et al.*, 1982; Bek *et al.*, 1995).

In the Newfoundland BBS population, all patients presented with severe retinal dystrophy (Green *et al.*, 1989). All tested patients had markedly constricted visual fields, severe abnormalities of color vision, raised dark-adaptation thresholds and extinguished or minimal rod-cone responses on ERG (Green *et al.*, 1989). Of 28 patients examined, two (8%) had a typical retinitis pigmentosa with dense bone spicule pigmentation, pale optic disks and attenuated vessels. Eighteen patients (69%) had an atypical retinal dystrophy with sparse pigmentation, central and peripheral atrophy, attenuated vessels and mild to severe optic disk pallor. Six patients (23%) had severe macular dystrophy with only sparse pigment clumping in the mid-periphery. However, four of these six patients had night blindness at the time of study, indicating rod involvement. In two other patients, the retinas were not visible because of dense cataracts. In the ten families with more than one affected individual, the retinal dystrophy was similar in all affected family

members.

In 1993, eight additional patients were ascertained and examined (O'Dea *et al.*, 1996). At this time, 86% of the 36 patients examined were legally blind, compared with none of the 45 siblings evaluated. The age range of recorded legal blindness was 5 to 29 years. Twenty-five percent were legally blind by the age of 13, 50% by 18 and 100% by age 30. The five patients not legally blind at last examination were all under the age of 12 years.

Other ocular abnormalities which are often associated with retinal dystrophies were also observed, including myopia, astigmatism, nystagmus, glaucoma, posterior sub-capsular cataracts and mature cataracts or aphakia (O'Dea *et al.*, 1996).

1.2.2 Limb Abnormalities

Postaxial polydactyly (PAP) is often the first indication of Bardet-Biedl syndrome, being observable at birth. However, PAP is not a universal finding. Reports of the proportion of patients with this manifestation have varied from 45% (Riise *et al.*, 1997) to about 70% (Bell, 1958; Klein and Ammann, 1969; Beales *et al.*, 1999). Polydactyly may occur in only one limb or as many as all four (Klein and Amman, 1969). However, the lower limbs seem to be more often affected than the upper: Klein and Ammann (1969) found isolated hexadactyly affecting the feet twice as often as affecting the hands, and

Beales *et al.* (1999) observed polydactylous toes three times as often as polydactylous fingers.

Only one study has investigated the skeletal anomalies in a large group of BBS patients through radiography (Rudling *et al.*, 1996). In this report, 43 Scandinavian BBS patients were examined. Thirty-three of these patients (77%) had PAP of the hands, feet, or both. Short finger and toe bones were frequently seen and a high percentage of patients had flat rather than rounded distal joint surfaces of metacarpals and metatarsals. There was no difference in right and left limb findings and no typical polydactyly 'patterns' were found within families. Interestingly, Beales *et al.* (1999) also observed a lack of any familial pattern, emphasized by a set of monozygous twins in which one had polydactyly of three limbs and the other had no polydactyly.

Green *et al.* (1989) reported PAP in 18 of 31 (58%) BBS patients in Newfoundland. Seven of 18 (39%) patients had polydactyly of both the hands and feet, nine had polydactyly of the feet only, and two of the hands only. Thirteen of 26 (50%) patients tested exhibited brachydactyly using anthropomorphic measurements, but 14 of 15 (93%) had brachydactyly with the use of metacarpophalangeal profiles. Most patients (90%) had syndactyly, polydactyly, or both. Both Beales *et al.* (1999) and Green *et al.* (1989) reported that in families with more than one affected member, there were patients with and without polydactyly.

1.2.3 Obesity

Obesity, from mild to severe degree, is the second most common manifestation in Bardet-Biedl syndrome patients, after retinal dystrophy. It is uncommon in children younger than three years old, usually beginning in childhood and increasing in severity with age (Dekaban *et al.*, 1972; Bauman and Hogan, 1973). However, patients can reduce their weight with diet and exercise regimes. In adults, the obesity is usually restricted to the trunk and proximal limbs and less frequently to the face (Hrynchak, 2000), but it has been described as diffuse and non-specific in distribution during early life (O'Dea *et al.*, 1996).

Methods of measurement of obesity have changed since the first large scale studies of BBS took place. Klein and Amman (1969) based their obesity estimates on weight alone, comparing these with the normal population distribution, and calling weights over the 50th percentile as obese. Using this evaluation of obesity, 96% of their patient population was obese. Similarly, Bell (1958) observed obesity in 91% of her patients. In more recent studies, the body mass index (BMI) of an individual (weight/height^2) has been calculated. In a study of 25 Scandinavian BBS patients, 22 of 25 (88%) individuals were regarded as obese, with obesity defined as a BMI greater than 28 kg/m^2 (Riise *et al.*, 1997). Beales *et al.* (1999) reported that 72% of their cohort of 109 patients were obese, a lower proportion than previously observed. They suggested this was a result of their more stringent criteria (a BMI > 29 kg/m^2 was considered obese).

The criteria for obesity in the Newfoundland cohort of Bardet-Biedl patients was weight for height greater than the 90th percentile in relation to average adult Canadian men and women (Green *et al.*, 1989). With this standard, 22 of 25 (88%) patients were obese. Three were considered to be of normal weight, two of whom were previously obese. Five patients lost substantial amounts of weight on calorie-restriction diets. Twelve patients (48%) were considered grossly obese, with weight well above the 95th percentile. Female Bardet-Biedl patients were more obese than their affected brothers (O'Dea *et al.*, 1996).

1.2.4 Renal Abnormalities

Renal abnormalities were a frequent finding in early BBS studies (McLaughlin and Shanklin, 1967; Alton and McDonald, 1973; Falkner *et al.*, 1977; Linne *et al.*, 1986), and recently have been considered a sixth cardinal feature of BBS (Churchill *et al.*, 1981; Cramer *et al.* 1988; Harnett *et al.*, 1988; Green *et al.*, 1989; Anadoliiska and Roussinov, 1993; Beales *et al.*, 1999). Early diagnosis of renal disease was initially made by computed tomography, but now, with the use of high resolution ultrasonography, there has been a marked increase in reports of prenatal and perinatal renal findings (Ritchie *et al.*, 1988; Garber and Bruyn, 1991). Structural or functional renal anomalies have been reported in 100% of BBS patients in one series (Harnett *et al.*, 1988). Renal involvement

is progressive and dictates the clinical outcome in approximately 50% of reported cases (Dippell and Varlam, 1998).

In early BBS studies, the renal involvement was detected because of symptomatic disease in late childhood or adolescence (Dippell and Varlam, 1998). Polydipsia, polyuria, reduced concentrating ability and aminoaciduria as symptoms of tubular dysfunction are often the first renal clinical signs in patients with BBS (Tieder *et al.*, 1982; Fralick *et al.*, 1990). Other indications of renal involvement such as hypertension and urinary tract infections are less common symptoms (Anadoliiska and Roussinov, 1993). Unfortunately, renal involvement was usually identified when chronic renal failure or end stage renal disease (ESRD) developed (Alton and McDonald, 1973; Hurley *et al.*, 1975; Linne *et al.*, 1986).

Many histological changes of the kidney have been noted in BBS including mesangial proliferation and sclerosis, cystic dilatation of the tubules, cortical and medullary cysts, chronic cell infiltration and peri-glomerular and interstitial fibrosis (Hurley *et al.*, 1975; Price *et al.*, 1981). Pathological studies of kidneys from patients with BBS suggest that renal disease originates mainly from the medullary cyst involvement (Dippell and Varlam, 1998). The tubulo-interstitial changes and the cyst formations may be related to underdevelopment and defective connection of the tubular systems in the fetal kidney (Anadoliiska and Roussinov, 1993).

The spectrum of renal disease has been comprehensively documented in the Newfoundland BBS population (Harnett *et al.*, 1988; O'Dea *et al.*, 1996), without the

referral bias which has beset other studies (Hurley *et al.*, 1975), because almost all patients have been ascertained through ophthalmologic records. Almost all Newfoundland BBS patients had either structural and/or functional renal abnormalities. Initially, 23 patients with BBS, ranging in age from three to 52 years, underwent renal imaging with ultrasound and urography (Cramer *et al.*, 1988). Twenty-two of 23 (96%) patients had calyceal clubbing or blunting, and calyceal cysts/diverticula were evident in 17 patients (74%). Vesicoureteral reflux was only observed in one patient, and thus was not the cause of the calyceal changes in most patients, as had been suggested previously (Hurley *et al.*, 1975). Rather, these abnormalities are probably dysplastic in nature. Twenty-one patients (91%) had fetal lobulation of the kidney and diffuse cortical loss was evident in six patients (26%), of which three (13%) had impaired renal function. Fetal lobulation has been observed in other BBS studies (McLaughlen and Shanklin, 1967; Alton and McDonald, 1973; Beales *et al.*, 1999), and is normally seen in the fetus and neonate but usually dissipates during maturation. From these findings Cramer *et al.* (1988) proposed that the combination of calyceal clubbing and diverticula, and fetal lobulation may well be diagnostic of BBS.

Among the Newfoundland patients, 17 (74%) had concentrating defects and seven (30%) had acidification defects, but there was no relationship to the severity of the calyceal or cystic changes on the intravenous urograms (IVU). This lack of correlation between the structural and functional abnormalities suggests that microscopic changes may be responsible for the altered function rather than obvious structural ones (Cramer *et*

al., 1988). They concluded that IVU is the best method for defining the calyceal and cystic changes, but sonography will reveal cortical changes.

O'Dea *et al.* (1996) reevaluated the importance of renal impairment in the natural history of BBS in Newfoundland. A total of 38 patients, ranging in age from 1 to 63 years, and 58 unaffected siblings were studied in 21 families. Renal impairment was observed in 9 of 36 (25%) BBS cases, with the earliest age of onset being two years. By age 48, 25% of BBS cases had chronic renal insufficiency whereas only one unaffected sibling had mild renal impairment. This lower frequency of ESRD in BBS patients contrasts with previous reports (Alton and McDonald, 1973; Hurley *et al.*, 1975; Linne *et al.*, 1986), but these previous studies may be biased. The patients studied in previous investigations were referred because of a specific renal problem, and thus may represent the subset of BBS patients who have more severe renal disease.

Twenty-five of 38 (66%) Newfoundland BBS patients were hypertensive, compared with only 5 of 45 (11%) of the unaffected siblings. Longevity was significantly reduced, as 25% of affected individuals died by the age of 44, compared with only 2% of unaffected siblings. Of the eight patients who died by last follow-up, six (75%) had developed renal failure at time of death, while only one unaffected sib had died, and that was of a myocardial infarction. Renal transplantation was successful in one individual from this series. A few other incidences of renal replacement therapy have also been reported (Linne *et al.*, 1986; Williams *et al.*, 1988; Norden *et al.*, 1991; Crocker *et al.*, 1994; Beales *et al.*, 1999).

In the Newfoundland BBS cohort, which was ascertained through ophthalmological records, 100% of patients had renal abnormalities. From these observations, the combination of calyceal clubbing and diverticula, and fetal lobulation were considered to be diagnostic of BBS. Thus, renal manifestations were considered a sixth cardinal feature.

1.2.5 Hypogonadism in Males

The presence of small testes and very small penis has been documented in most males with Bardet-Biedl syndrome. The incidence of hypogenitalism ranges from 74% (Bell, 1958) to 96% (Beales *et al.*, 1999) in the large BBS review studies. Almost all the literature on hypogenitalism is based on a qualitative definition of the trait. Very few endocrine studies have been undertaken, thus the origin of male hypogonadism is not determined (Toledo *et al.*, 1977; Green *et al.*, 1989). In one investigation, hypothalamic-pituitary-gonadal function was evaluated in three male siblings with BBS, and testicular biopsies were performed (Toledo *et al.*, 1977). The authors suggested an evolving gonadal disorder that progresses throughout adult life. Mozaffarian *et al.* (1979) reported a patient with germinal aplasia in one testis, incomplete spermatogenesis in the other, and a failure of the genitalia to respond to 11 months of testosterone treatment.

Seven of eight (88%) Newfoundland male patients had small testes and very small

penises (Green *et al.*, 1989). Three (38%) patients had high basal follicle stimulating hormone (FSH) levels and, in all eight, FSH responded to pituitary stimulation. Two (25%) had low testosterone levels. Three had supranormal responses to gonadotropin releasing hormone, but in the remaining five, response was normal. One patient (13%) had high basal luteinizing hormone levels. Male fertility has been reported in just one instance - in an Iranian patient having seven offspring (Ghadami *et al.*, 2000).

1.2.6 Other Observed Manifestations in Bardet-Biedl Syndrome

Many abnormalities other than the cardinal manifestations considered above have been associated with Bardet-Biedl syndrome. In the past, hypogonadism in both sexes and mental retardation were considered primary characteristics of BBS. The clinical features described below are important when considering BBS as a diagnosis.

1.2.6.1 Cognitive Deficit

Initially, mental retardation was regarded as a cardinal manifestation of Bardet-Biedl syndrome (Biedl, 1922; Cockayne *et al.*, 1935; Bell, 1958; Klein and Amman, 1969). However, during this period, intelligence tests, if any were employed, did not take

into account severely impaired vision. Also, most often, BBS cases were not diagnosed until visual loss had become debilitating and it was too late to address the situation educationally or socially. Under these circumstances, most, if not all, individuals would 'appear' mentally retarded. The social implications of being "mentally retarded" at these times would, of course, be an additional barrier in obtaining educational aid. Today, with earlier diagnosis and an appropriate environment, many people with BBS are capable of much more independent lives resulting from increased education (Green *et al.*, 1989; Riise *et al.*, 1997; Beales *et al.*, 1997). Riise *et al.* (1997) reported that the majority of their 25 Scandinavian patients functioned within the normal range of intelligence with no obvious differences from their unaffected sibs. Beales *et al.* (1997) results were less favorable, but still reassuring, with 8 of 28 (28%) children capable of remaining in mainstream education, although with classroom aids. Another 28% went to special schools based on educational needs apart from vision loss.

In the Newfoundland BBS cohort, only a minority of patients (41%) were diagnosed mentally retarded when appropriate verbal and performance IQ tests for the visually impaired were used (e.g. the Haptic Intelligence Scale). Performance IQ tests results were usually better than verbal scores, especially in patients with a good formal education. Six patients achieved grade 9, one a university degree, and another takes computer courses (Parfrey *et al.*, 1997). Although learning deficiency varies among individuals with BBS, and indeed within families, some learning difficulties are present in many cases.

1.2.6.2 Hypogonadism in Females

As discussed above, hypogenitalism was originally determined to be a primary trait of Bardet-Biedl syndrome (Biedl, 1922; Cockayne *et al.*, 1935; Bell, 1958; Klein and Amman, 1969). It has now been relegated to the category of 'other features'. The incidence of hypogonadism in females, which is more difficult to detect, is not as high as in males. Unlike in males, there have been several cases of reproductive success in females (Bell, 1958; Green *et al.*, 1989; Riise *et al.*, 1997; Beales *et al.*, 1997). Studies on females with BBS have failed to identify a consistent primary or secondary endocrine dysfunction (Leroith *et al.*, 1980; Campo and Aaberg, 1982; Lee *et al.*, 1986). However, Green *et al.* (1989) observed that 12 patients of reproductive age had irregular menstrual periods, one patient (8%) had hypogonadotropic hypogonadism, and two (16%) were hypoestrogenemic, but with high or normal gonadotropin levels. Several women had abnormally high luteinizing hormone levels and high normal prolactin levels, which are characteristic of polycystic ovary syndrome. However, their ovaries were normal on ultrasound, although, in some cases, visualization was limited by obesity.

There have also been reports of structural urogenital abnormalities in female BBS patients (McLoughlin and Shanklin, 1967; Klein and Ammann, 1969; Nadjimi *et al.*, 1969; Campo and Aaberg, 1982; Srinivas *et al.*, 1983). Stoler *et al.* (1995) reviewed the literature for structural anomalies and reported two cases of vaginal atresia in women with BBS. Additionally, Mehrotra *et al.* (1997) and Oguzkurt *et al.* (1999) reported three

more cases of vaginal atresia. Stoler *et al.* (1995) found 11 instances of female structural genital abnormalities including those identified in the Newfoundland population (which, in two cases, they counted twice) (Cramer *et al.*, 1988; Green *et al.*, 1989). The majority of these patients had hemato- or hydrometrocolpos. More subtle findings, such as abnormally placed urethra or asymptomatic vaginal atresia, are likely under reported because often a complete pelvic examination is not performed. These structural genital abnormalities are an important part of the phenotype of BBS in females.

1.2.6.3 Diabetes Mellitus

In Newfoundland, diabetes mellitus was reported in 12 of 38 (32%) BBS patients, compared with none of the 45 unaffected sibs (O'Dea *et al.*, 1996). Twenty-five percent of patients were diabetic by age 35 and 50% by age 55. All patients had large increases in serum insulin levels after a glucose load, suggesting insulin resistance. Thus, diabetes in this cohort was probably type II (Parfrey *et al.*, 1997). This was a much higher rate than was found by Klein and Ammann (14%; 1969).

Seven patients (6%) had non-insulin dependent diabetes mellitus (NIDDM) in a study in the United Kingdom (Beales *et al.*, 1999), and three patients (12%) of Scandinavian origin were also reported to have diabetes mellitus (Riise *et al.*, 1997). The former study stated that NIDDM in BBS was a consequence of severe insulin resistance.

Obesity is likely involved in the pathogenesis, by creating a reduction of cellular insulin receptors, which leads to a decreased insulin sensitivity and an increase in insulin levels (Parfrey *et al.*, 1997). In at least one study, diabetes has been observed as a direct cause of death (Escallon *et al.*, 1989).

1.2.6.4 Cardiac Manifestations

McLoughlin *et al.* (1964) reviewed the literature of 330 published cases of BBS and noted nine cases of congenital heart defects. Heart malformations have occurred mostly in males (Moench, 1954; Blumel and Kniker, 1959; Spigolon *et al.*, 1959). Acquired heart disease has been noted frequently in association with hypertension and renal disease in patients with BBS. Twenty-two BBS patients of Bedouin descent were subjected to echocardiography (EKG) in a recent study (Elbedour *et al.*, 1994). Seven patients (32%) showed an EKG abnormality, and three of these (43%) were determined to have a definite congenital anomaly. Beales *et al.* (1999) also reported congenital heart defects in eight patients (7%).

1.2.6.5 Additional Presentations

Dental anomalies, first documented in 1960 (Magnusson), were frequently found in Bardet-Biedl syndrome patients when an appropriate oral assessment was performed (Borgstrom *et al.*, 1996; Beales *et al.*, 1999). The most significant findings were hypodontia, small teeth, enamel hypoplasia, short roots and a thickened mandible, as well as a high arch palate. Twenty-seven percent (29 of 109) of patients in the Beales *et al.* (1999) study had dental problems. Kobrin *et al.* (1990) reported the first case of oligodontia (the lack of a large number of teeth) in a BBS patient.

Hepatic fibrosis has been reported previously (Ross *et al.*, 1956; Meeker and Nighbert, 1971; Pagon *et al.*, 1982; Nakamura *et al.*, 1990) but has not been properly assessed in most BBS studies. Thus, the true frequency of hepatic fibrosis is unknown.

Early onset asthma was reported in 28 of 109 (25%) patients in the Beales *et al.* (1999) study, all of whom were from kindreds linked to the *BBS1* locus. This prevalence is three times as high as the United Kingdom general population prevalence (7%).

Other traits which have been observed in patients with Bardet-Biedl syndrome include: Hirschsprung disease (Maeda *et al.*, 1984; Radetti *et al.*, 1988; Islek *et al.*, 1996; Parfrey *et al.*, 1997; Beales *et al.*, 1999; Lorda-Sanchez *et al.*, 2000), anal atresia (Biedl, 1922; Kalangu and Wolf, 1994), speech deficit (Beales *et al.*, 1999), behavioral difficulties (Klein and Ammann, 1969; Green *et al.*, 1989; Beales *et al.*, 1999), poor motor coordination and multiple widespread pigmented naevi (Beales *et al.*, 1999).

1.3 Disorders Overlapping in Phenotype with Bardet-Biedl Syndrome

Due to the number and variability of manifestations in Bardet-Biedl syndrome, there are numerous syndromes which overlap with BBS in some manner. However, the disorders most often identified in the differential diagnosis of BBS are Lawrence-Moon, McKusick-Kaufman, Alstrom and Biemond II syndromes (Table 1.1).

1.3.1 Laurence-Moon-Bardet-Biedl Syndrome - An Historical Perspective

The nosology of syndromes combining the manifestations of ocular defects, hypogonadism, obesity, mental retardation and extremity malformations has been fraught with difficulties. The phentype now called Bardet-Biedl syndrome was not originally termed this, nor is its name presently used in a consistent way in medical literature. Initially, Laurence and Moon, in 1866, described four members of one family with short stature, hypogonadism, mental retardation and spinocerebellar ataxia. Also, all four had nystagmus; two had bone corpuscle pigmentation of the fundus, and two had choroidal atrophy. At this point, the described syndrome was called Laurence-Moon. Later, members of the same family were reviewed by Hutchinson (1900) and were noted to have developed spastic paraplegia, which was added as a feature of this disorder. Then in 1920, Georges Bardet described a little girl with infantile obesity, polydactyly of the right

Table 1.1 Syndromes with clinical manifestations which overlap with BBS (+, present; -, absent).

Features	Bardet-Biedl	Laurence-Moon	McKusick-Kaufman	Alstrom	Biemond II
Pigmentary retinopathy	+	+	-	+	-
Renal abnormalities	+	-	-	+/-	-
Obesity	+	-	-	+	+
Hypogenitalism	+	+	-	+	+
Limb abnormalities	+	-	+	-	+
Cognitive deficit	+	+	-	-	+
Deafness	-	-	-	+	-
Spastic paraplegia	-	+	-	-	-
Iris coloboma	-	-	-	-	+
Hydrometrocolpos	+/-	-	+	-	-
Congenital heart disease	+/-	-	+	+/-	-
Mode of inheritance	autosomal recessive	autosomal recessive	autosomal recessive	autosomal recessive	auto. rec. auto. dom.

foot, and retinitis pigmentosa. Soon after, Artur Biedl (1922) added hypogenitalism, mental retardation, anal atresia and skull deformities to the description of Bardet, as well as his name, to form Bardet-Biedl syndrome. Unfortunately, this was not the end of this nomenclature tale. In 1925, Solis-Cohen and Weiss observed four cases in a single family with what would currently be termed Bardet-Biedl syndrome. However, they defined it as Laurence-Biedl syndrome - combining the two syndromes. From there, other forms were fashioned including Laurence-Moon-Biedl and Laurence-Moon-Bardet-Biedl (LMBB), depending on the scientist and what was in vogue.

1.3.1.1 Laurence-Moon Syndrome

Schachat and Muamenee (1982) performed a literature review of the Laurence-Moon syndrome as described by Laurence, Moon, and Hutchinson and discovered 21 cases which fit that criteria. However, as stated above, Bardet-Biedl and Laurence-Moon were lumped together in the 1920s, and called Laurence-Moon-Bardet-Biedl syndrome. It was later acknowledged, by many, that LMBB syndrome actually comprised two separate syndromes, BBS and Laurence-Moon syndrome. The latter is characterized by ocular abnormalities, mental retardation, hypogenitalism and spastic paraparesis (Lancet, 1988). It differs from BBS by the absence of obesity, malformations of the extremities and renal abnormalities, and by the presence of neurological complications. The

recognition of a distinction between the two syndromes is not universal however. Physicians and scientists in North America generally distinguish between the two syndromes, but in the United Kingdom, there is a tendency to refer to BBS as LMBB. Like BBS, Laurence-Moon syndrome exhibits an autosomal recessive inheritance pattern. No genetic locus has yet been identified for Laurence-Moon syndrome, perhaps due to the dearth of identified families.

1.3.2 McKusick-Kaufman Syndrome

McKusick-Kaufman syndrome (MKS) was first described in two Amish families in 1964 by McKusick *et al.*, and the diagnosis confirmed in other families by Dungy *et al.* (1971) and Kaufman *et al.* (1972).

The cardinal manifestations of MKS are hydrometrocolpos (HMC), present in 80-95% of females, and polydactyly present in about 95% of males and females. Additionally, congenital heart disease (CHD) occurs in approximately 10% of all patients, and hypospadias has been documented in males. For females without a family history, HMC with distal vaginal agenesis or a transverse vaginal membrane and PAP are considered sufficient clinical evidence of MKS. Over 90 cases have been recorded to date (David *et al.*, 1999; Slavotinek and Biesecker, 2000).

HMC occurs because of a failure to canalize the junction between the inferior

uterus and the vagina. Usually identified at birth, the accumulation of secretions cause distension of the abdomen and can be associated with respiratory compromise, edema or hydronephrosis (Stone *et al.*, 1998). These complications can be life-threatening.

Until recently, the phenotypic overlap between MKS and BBS had been overlooked in the literature. Polydactyly is the manifestation most frequently occurring in both syndromes. However, vaginal atresia which is frequently present in MKS, but rare otherwise, has been reported in at least 16 female BBS patients. Other instances of lower urinary tract anomalies have also been described in BBS (Verloes *et al.*, 1997; David *et al.*, 1999; Slavotinek and Biesecker, 2000). CHD has also been associated with BBS, although infrequently. In reviews by McLoughlin *et al.* (1964 and 1967), 9 of 330 patients had CHD and a report by Chitayat *et al.* (1987) associated CHD with BBS 9% of the time. More recently, Elbedour *et al.* (1994) examined a series of Bedouin patients having cardiac manifestations including cardiomyopathy, pulmonary stenosis and bicuspid aortic valve in 11 of 22 (50%) BBS patients.

Nine female patients with genital and digital anomalies, and consequently diagnosed with MKS in infancy, were studied in follow-up by David *et al.* (1999). Because of the development of retinal dystrophy, obesity, mental deficiency and/or renal abnormalities, a new diagnosis of BBS was assigned. The authors suggested that MKS is an over-diagnosed condition, that children with polydactyly and HMC do not necessarily have MKS. They also suggested that these children should be followed closely to determine whether other BBS manifestations occur later, especially retinal dystrophy.

Thus, studies of MKS without long term follow-up should be interpreted cautiously.

In a literature review of 49 MKS patients of non-Amish descent, 15 MKS patients of Amish descent and 19 patients with BBS who were mis-diagnosed as having MKS in the neonatal period, Slavotinek and Biesecker (2000) concluded that there were no features allowing reliable differentiation of the two syndromes in the neonatal period. However, it was noted that uterine, ovarian and fallopian tube anomalies (other than HMC) are more common in BBS patients, and these may be useful in discriminating between the two syndromes in the newborn.

In order to map the gene for MKS, Stone *et al.* (1998) employed two large consanguineous Old Order Amish pedigrees, originally identified by McKusick (1978). They performed a genome screen using microsatellite genetic markers, searching for homozygosity by descent (HBD) in affected individuals. Through this method they identified an MKS gene locus on chromosome 20p12. By observance of recombinations in the large Amish families, a region of one centimorgan (cM) was identified as containing the putative *MKKS* gene. An extension of the physical map already assembled by others looking for the Alagille syndrome gene, now known to be *JAG1*, was created and the critical interval for *MKKS* was narrowed to less than 500 kilobases (kb).

As a follow up, Stone *et al.* (2000) sample-sequenced the *MKKS* critical region looking for unique genomic sequences. A six exon transcript with a predicted open reading frame (ORF) of 570 amino acids and two alternative 5' terminal exons was discovered. This transcript was expressed in a wide range of adult and fetal tissues,

including skeletal muscle, heart, testis, brain and kidney. The authors looked for alterations in two MKS families; one was the large Amish family in the previous study and the second a non-Amish sporadic MKS patient. Alterations were found in both. Affected individuals in the Amish family were homozygous for the complex allele H84Y/A242S in exon 3. The sporadic patient was a compound heterozygote for a substitution (Y37C) in exon 3 and a two base pair deletion (delGG) in exon 5. A mouse homolog was cloned, sequenced, and a tissue expression pattern similar to the human one was observed.

Interestingly, three individuals (two females and one male) from the large Amish kindred homozygous for the H84Y/A242S mutation had no apparent manifestations of the disorder. Thus, the authors suggested, MKS could be considered incompletely penetrant, a phenomenon never previously clearly demonstrated for a human autosomal recessive syndrome.

The putative human protein product of *MKKS* was compared with protein database information and showed greatest similarity to the archebacterial chaperonins and the t-complex-related proteins (TCRP). Comparison of the presumed folding pattern of the MKKS protein with the database suggested it was most closely related to the thermosome of *Thermoplasma acidophilum*, a member of the group II chaperonin family (Stone *et al.*, 2000).

1.3.3 Alstrom Syndrome

Obesity, retinal dystrophy, diabetes mellitus and progressive sensorineural deafness were the characteristics which Alstrom *et al.* (1959) first described in three individuals. He and co-workers stated that Alstrom syndrome (ALMS) differs from Bardet-Biedl syndrome in its lack of polydactyly, mental deficiency and genital anomalies. However, in 1969, Klein and Amman added acanthosis nigricans and male hypogenitalism to the list of manifestations of ALMS. Other features which have been associated with ALMS include cardiomyopathy, renal and hepatic degeneration, hypothyroidism, insulin resistance, growth hormone deficiency, progressive baldness, hyperuricemia, hypercholesterolemia, hypertriglyceridemia, gynecomastia and reduced fertility (Russel-Eggitt *et al.*, 1998).

The ocular phenotype of Alstrom syndrome differs from BBS in its severity; nystagmus occurs by one year of age, photophobia is common and the ERG is extinguished or shows cone-rod dystrophy in infancy. Usually in ALMS, a patient's vision is severely impaired by the end of the first decade; bone spicule pigmentation is not common nor is the bull's eye maculopathy (Millay *et al.*, 1986; Russel-Eggitt *et al.*, 1998). ALMS is also differentiated from BBS by its progressive deafness, usually beginning in childhood, and the frequent presence of acanthosis nigricans.

Although there have been only approximately 70 cases of ALMS reported, there have been studies concerning its molecular genetics conducted. An autosomal recessive

mode of inheritance had been presumed, as consanguinity was documented in a number of cases (Alstrom *et al.*, 1959; Klein and Amman, 1969; Charles *et al.*, 1990; Marshall *et al.*, 1997). Collin and associates (1997) examined a large consanguineous French Acadian kindred with ALMS to determine the chromosomal location of the *ALMS1* gene. Firstly, they tested candidate gene regions for linkage. Since the mouse mutant *tubby* shared phenotypical similarities with ALMS including obesity, insulin resistance and retinal and cochlear degeneration, they hypothesized *tub* a good candidate. *tub* is located on mouse chromosome 7, syntenic with human chromosome 11p15. However, no linkage was identified. Then the authors tested linkage to syntenic regions of other mouse obesity genes, including *fat*, *ob*, A^y and *db* and growth-associated candidate genes like growth hormone (GH), GH receptor and GH releasing factor. Once again no linkage was found between the disease and these loci. A genome-wide scan was then implemented and a putative locus covering a 14.9 cM region on chromosome 2p13 was identified. Subsequent confirmation of this locus and its refinement to a 6.1 cM region was made (Macari *et al.*, 1998; Collin *et al.*, 1999). Candidate genes identified in this region include transforming growth factor alpha (*TGFA*), a cell membrane cytoskeleton gene (*ADD2*), the B1 subunit of ATP6 (*ATP6B1*), dynactin 1 (*DCTN1*) and tachykinin 1 receptor (*TAC1R*), all of which have been excluded by mutational analysis (Collin *et al.*, 1997 and 1999) or because they are associated with phenotypes which do not wholly overlap with ALMS (Bianchi *et al.*, 1994; De Felipe *et al.*, 1998; Karet *et al.*, 1999). More recently, a gene from the sodium bicarbonate cotransporter (NBC) family, *NBC4*,

has been cloned and isolated to 2p13 (Pushkin *et al.*, 2000). It has been suggested as an ALMS candidate because of its chromosomal location and expression pattern.

1.3.4 Biemond II Syndrome

Biemond, in 1934, described two siblings with a BBS-like syndrome, consisting of short stature, iris coloboma, mental retardation, preaxial polydactyly and hypogenitalism. He called this group of manifestations Biemond II syndrome (BS2). Hydrocephalus and hypospadias were included later in the syndrome description. Because of the clinical variation between the original report by Biemond and subsequent papers, Verloes *et al.* (1997) reviewed the literature on BS2 and categorized the few existing cases of BS2. The six categories included: (1) BBS with fortuitous iris coloboma or aniridia; (2) BS2 in its most strict form - the initial report; (3) a "new" dominantly inherited form of colobomatous microphthalmia occasionally associated with obesity, hypogonadism and mental retardation; (4) cytogenetically proven Rubinstein-Taybi syndrome; (5) an unclassifiable, early lethal genetic syndrome; (6) a "new" coloboma-zygodactyly-clefting syndrome.

For most families, the mode of inheritance appears to be autosomal recessive; however, there have been reports of autosomal dominant inheritance in two families (Grebe, 1953; Blumel and Kniker, 1957). Also, Verloes *et al.* (1997) diagnosed three

patients that could be grouped into the dominantly inherited coloboma-obesity-hypogenitalism-mental retardation syndrome (category 3 above). Due to the paucity of cases and the ambiguity of the phenotype, it is uncertain if BS2 actually exists, or is an allelic form of other related syndromes, such as BBS.

1.4 Prevalence of Bardet-Biedl Syndrome

The prevalence of Bardet-Biedl syndrome varies greatly among the populations studied thus far. Klein and Ammann (1969) estimated a prevalence of 1 in 160 000 in Switzerland; in Norway it is 1 in 128 000 (Lofterod *et al.*, 1990) and in Denmark, 1 in 59 000 (Haim, 1992). The frequency in the United Kingdom was later estimated at 1 in 125 000 (Beales *et al.*, 1997). However, some smaller populations have a much higher prevalence. The Bedouin of Kuwait have a prevalence of approximately 1 in 13 500, the highest known rate in the world (Farag and Teebi, 1989). Newfoundland also has a similarly high prevalence of 1 in 17 500 (Green *et al.*, 1989). These higher figures may be explained by higher coefficients of inbreeding in smaller populations. For example, in a United Kingdom study, 8% of affected patients had consanguineous parents (Beales *et al.*, 1999); while in Israel, Switzerland and Newfoundland, which are more homogenous populations, a higher proportion of patients had consanguineous parents (50%, 48% and 35%, respectively)(Amman, 1970; Ehrenfeld *et al.*, 1970; Green *et al.*, 1989). Bell

(1958) estimated that 39% of patients in a study of 273 BBS patients from around the globe had consanguineous parents.

The sex ratio for an autosomal recessive disorder is expected to be a 1:1 ratio. For BBS, there seems to be a slight male preponderance. Among 462 patients examined in four major studies, a 1.3:1 ratio was observed, favoring males (Bell, 1959; Klein and Ammann, 1969; Green *et al.*, 1989; Beales *et al.*, 1999). A breakdown of these studies showed that the Newfoundland BBS population, consisting of 38 individuals, exhibited a perfect 1:1 ratio of males to females (O'Dea *et al.*, 1996). A smaller study of BBS patients in Scandinavia, consisting of 25 patients, exhibited a higher male predominance, with 16 affected males and nine females (Riise *et al.*, 1997).

Due to the identification of four BBS loci, the distribution of BBS could be described by loci. In three studies examining North American/European populations (Beales *et al.*, 1997; Bruford *et al.*, 1997; Katsanis *et al.*, 1999), BBS1 families accounted for between 36% and 56%, BBS2 between 24% and 27%, and BBS4 between 32% and 35% of BBS pedigrees. Only one family had been mapped to the *BBS3* locus at the start of this thesis (Sheffield *et al.*, 1994).

1.5 Genetic Heterogeneity of Bardet-Biedl Loci

Originally, only a single BBS locus was predicted because of the rarity of the

syndrome and complexity of the phenotype. However, this turned out to be an erroneous assumption, as four BBS loci had been mapped by 1995.

1.5.1 Bardet-Biedl Syndrome 1

Using 28 North American kindreds of Northern European descent, as well as three Hispanic families, a locus for Bardet-Biedl syndrome was identified on chromosome 11q13 (Leppert *et al.*, 1994). Genetic markers spanning the genome were used to locate a possible disease locus in 219 individuals, including 67 affected persons. Statistical significance for linkage between the disease in these families and markers at two loci on chromosome 11q was demonstrated. One marker, *PYGM*, is the gene for human muscle glycogen phosphorylase and the other, *D11S913*, is anonymous. The additive lod score for all 31 families was 4.31 (θ=0.15) at *PYGM* and 4.02 (θ=0.13) at *D11S913*. Multi-point linkage analysis using *PYGM* and the closest nearby marker, *INT2*, increased the total lod score to 4.59 (θ=0.20). Seventeen of the 31 families exhibited positive lod scores at *PYGM* and 17 showed negative lod scores, indicating locus heterogeneity.

Taking the 17 families giving a positive lod score at *PYGM*, the authors localized the gene to a 12.8 cM region by analyzing additional markers around *PYGM* and *D11S913*. No recombinations occurred between *PYGM* and the disease gene, and thus, it was the favored position for a BBS gene. The confidence interval, based on the 1 lod

difference, extended approximately 1 cM proximal to *PYGM* and 2 cM distal to *PYGM*. At this time, two possible candidate genes were reported in the critical region of *BBS1*. Genes for Best Vitelliform Dystrophy, a juvenile macular dystrophy, and *ROM1*, which encodes a protein present in the rod outer segment of the retina, were both excluded as *BBS1* because they were expressed solely in the retina.

1.5.2 Bardet-Biedl Syndrome 2

Not intuitively, the discovery of the *BBS2* locus, in 1993, occurred previous to that of *BBS1* (Kwitek-Black *et al.*, 1993). Two large consanguineous Bedouin families, containing a total of 21 patients, were utilized to map a BBS locus to chromosome 16q21. Both families were clinically distinguished from Laurence-Moon syndrome through their lack of spastic paraplegia and presence of polydactyly and obesity; from Alstrom syndrome, by lack of deafness; and from Biemond syndrome by absence of iris colobomata.

Before performing a genome wide scan on these kindreds, the authors examined candidate loci for possible linkage with the disease. Loci included as candidates were retinitis pigmentosa loci on chromosome 8, 7p and 7q; Usher syndrome loci on 1q, 11p, 11q, and 14q; the rhodopsin locus on 3q; and the Best Vitelliform Dystrophy locus on 11q. Once these loci were excluded, the authors proceeded with the screen. Linkage was

detected on chromosome 16 in one family. A statistically significant lod score of 4.2 (θ=0) was observed at *D16S408*. After further genotyping, a region of homozygosity was shown to extend 18 cM, between *D16S419* and *D16S265*. A significant multi-point lod score was obtained [5.3 (θ=0)] with the best location for the disease gene at *D16S408*. In conclusion, they presented strong evidence for a BBS gene on chromosome 16q21. When additional genetic markers became available between *D16S419* and *D16S265* this family was used to narrow the critical region to 1.5 Mb (Kwitek-Black *et al.*, 1996).

The second Bedouin kindred, genealogically unrelated to the former kindred, was also studied with additional chromosome 16 markers. However, BBS in this family was excluded from linkage to *D16S408* and adjacent markers by statistically significant negative lod scores of less than -2.0 (θ=0). Thus, evidence for genetic heterogeneity of BBS was provided.

1.5.3 Bardet-Biedl Syndrome 3

With the Bedouin kindred excluded from the *BBS2* locus above, Sheffield *et al.* (1994) genotyped over 200 microsatellite markers in an effort to find a third BBS locus. Success was found with markers on the short arm of chromosome 3. Microsatellite *D3S1753* provided the highly statistically significant two point lod score of 7.52 (θ=0). Additional markers were typed around *D3S1753*. By haplotype analysis, a critical region

of 11 cM was obtained. This interval extended between *D3S1254* and *D3S1302* on 3p13-p12.

1.5.4 Bardet-Biedl Syndrome 4

A fourth BBS locus was identified with yet another large, consanguineous Bedouin kindred (Carmi *et al.*, 1995b). Once again there was no known relationship between this kindred and the others used to identify the *BBS2* and *BBS3* loci. The three known BBS loci were excluded by haplotype and linkage analyses before a genome scan was implemented using pooled DNA samples. A statistically significant lod score [4.66 (θ=0)] was obtained on chromosome 15 and the critical interval of *BBS4* was estimated at 9 cM. It was remarked that no retinopathy or obesity genes had been identified in this region at the time. Confirmation of this locus came in 1997 by Bruford *et al.* who observed 32-35% of their 29 BBS families were linked to 15q22.3-q23. Through recombinations in a single consanguineous family, these authors reduced the *BBS4* critical interval to 2 cM, between *D15S131* and *D15S114*. Thus, three separate genes caused the same disease in the Bedouin population of the Middle East.

1.6 Clinical Variation of Bardet-Biedl Syndrome

Although BBS is characterized by a complex phenotype, there has been documented variation of affected individuals within and between families. The few studies which have assessed the inter-locus variation have not been conclusive, probably due to the paucity of families having been genetically categorized to date. Also, there have been some conflicting results between studies. However, there was some suggestion that weight, vision, cognitive deficit and limb malformations may vary between BBS loci.

Obligate carriers of BBS genes have also been studied to determine if heterozygotes have a particular BBS-related phenotype. However, once again, these investigations were inconclusive due to the lack of available molecular data and the small size of the studies.

1.6.1 Intra-familial Variation

The intra-familial variability of Bardet-Biedl syndrome has been noted frequently (Klein and Ammann, 1969; Bergsma and Brown, 1975; Escallon *et al.*, 1989; Lavy *et al.*, 1995; Rudling *et al.*, 1996; Leppert *et al.*, 1994). Riise *et al.* (1997) addressed intra-familial variation in BBS directly by examining 11 Scandinavian families having two or more affected individuals. Genetic analysis was performed previously (Bruford *et al.*,

1997); however, statistically significant lod scores were obtained for only two chromosome 15 families. All other kindreds were not informative. Thus, probabilities were assigned to each of three loci (*BBS1*, *BBS2* and *BBS4*) which could not be excluded. Variation of expression of obesity, skeletal abnormalities of the extremities, hypogenitalism, short stature, paraplegia, dental abnormalities and the course of retinal dystrophy was apparent within families. In particular, the retinal dystrophy varied widely with respect to age of onset and course of disease.

A Newfoundland cohort of BBS families was also observed to exhibit intra-familial variation (Green *et al.*, 1989). Three affected individuals from one family differed in the degree of polydactyly, mental retardation, endocrine dysfunction and renal abnormalities. Similar observations occurred in six of ten other families studied with multiple affected siblings. Within these families the following features showed less intra-familial variability: presence of obesity, type of retinal dystrophy and presence of abnormal renal calyces.

1.6.2 Inter-locus Variation

An important consideration in the assessment of Bardet-Biedl syndrome patients is the degree of clinical variation that can be attributed to different BBS genes. The first opportunity to consider such a possibility came with the discovery of the *BBS1* locus

(Leppert *et al.*, 1994). However, within the BBS1 subset, the authors did not observe a combination of traits, variation of particular traits, or variability of severity of a trait which could distinguish these families from families mapping to other loci.

In a study of three large Arab-Bedouin kindreds, three gene loci were compared [chromosome 16 (*BBS2*), chromosome 3 (*BBS3*) and chromosome 15 (*BBS4*)] to determine if there were differences among families segregating separate BBS loci. Carmi *et al.* (1995) found no appreciable differences with regard to renal or cardiac abnormalities. However, differences were observed among families with regard to polydactyly. Affected individuals in the *BBS3* family had the anomaly in all four limbs; those in the *BBS4* family had polydactyly only on the hands, and those in the *BBS2* kindred had a phenotype somewhere between the two others. Syndactyly had been reported previously in affected individuals (Amman *et al.*, 1970; Green *et al.*, 1989); however, it was not seen in the three families of this study. Therefore, the authors suggested syndactyly was caused by mutations in BBS genes different than those segregating in their families.

Carmi *et al.* (1995) observed statistically significant differences in the mean BMI of the chromosome 15 and 16 families. Also, the patients in the chromosome 3 kindred tended to have a higher BMI compared with those in the chromosome 16 family. However, there was no statistical significant difference. No sex differences were found within families, although, when males were compared between chromosome 3 and 15 families and between chromosome 16 and 15 families, there were significant differences

in mean BMI.

In summary, Carmi *et al.* (1995) suggested there were significant differences among families concerning the distribution of a sixth digit, albeit no statistically significant results were presented. For the case of obesity, it was suggested that the chromosome 16 family was the 'leanest'; the chromosome 15 family was the most obese, and the chromosome 3 kindred had a pronounced progression toward morbid obesity after puberty. Lastly, because the males in the chromosome 15 kindred were significantly more obese than in the other two families, which was not the case for the females, there may be a sex-dependent obesity effect.

A follow-up study of the Carmi *et al.* (1995) paper, by Heon *et al.* (2000), concentrated on the retinal characterization of three Arab-Bedouin kindreds. ERGs were found to be abnormal in all affected individuals. Age at which ERG abnormalities were noted was variable between families, with the chromosome 15 family's retinal function preserved longer. The authors also regarded the chromosome 3 family as the most severely myopic.

In the Beales *et al.* (1997) paper, one BBS4, three BBS2 and eight BBS1 kindreds, of various ethnic backgrounds, were studied with regard to inter-locus phenotype comparisons. They found a statistically significant difference in mean age of onset of night blindness between locus categories: BBS1 < BBS4 and BBS2 < BBS4. Also, a significant difference was obtained between males and females in BBS1 families regarding the mean age of onset of night blindness - males had an earlier mean age of

onset (12.2 years vs. 15.5 years). The authors suggested hormonal influences may have caused this trend. Polydactyly was not observed in the chromosome 15 family and there were no differences in the distribution of polydactyly between family types. Mention was also made of the lack of excess lower limb polydactyly, previously commented on by Green *et al.* (1989).

Interestingly, in this same study by Beales *et al.* (1997), unlike previous observations, the height of affected offspring was not significantly shorter than the parents, and for BBS1 patients, it was the reverse. Both the affected sons and daughters were significantly taller than their fathers and mothers, respectively. The opposite was true for BBS2 daughters, and the sons had no appreciable difference compared with their fathers. When weights of parents and affected offspring were compared for both sexes, there was a significant increase over the parents at all loci except in the BBS2 sons. BMIs were also compared by locus and sex. The overall BMIs significantly increased in offspring compared with parents. However, sons did not differ significantly from their fathers, but daughters did. The difference was most pronounced in the BBS4 family. Beales *et al.* (1997) also commented that the 28% of their patients who needed special educational needs were mostly from the BBS2 and BBS4 families, the latter being more severely affected. Additionally, the 24% of patients having asthma were all from BBS1 families.

Bruford *et al.* (1997) studied 29 families from nine countries and found no clinical distinctions between families linked to *BBS1*, *BBS2*, *BBS4*, or among unlinked kindreds.

A comparison of two chromosome 15 families indicated no common BBS4 phenotype (Riise *et al.*, 1997). In one family, polydactyly was localized to the feet or totally absent, and obesity was morbid. For the other family, polydactyly was isolated to the hands, and the BMI was just above normal. This conflicts with the Carmi *et al.* (1995) findings of polydactyly on predominantly the upper limbs in their chromosome 15 kindred.

Some observations were similar in the Beales *et al.* (1997) and Carmi *et al.* (1995) papers. In both studies there was a propensity for the BBS2 group to be the leanest and the BBS4 families to have the lowest occurrence of polydactyly.

However, there has been no statistically consistent pattern of manifestations that distinguish among BBS loci in the few studies that have been performed to date. This could be due to the small patient sample sizes employed in these studies.

1.6.3 Heterozygous Carriers of a Bardet-Biedl Gene

A study of one five generation family was undertaken to determine if Bardet-Biedl heterozygotes are also predisposed to manifestations of BBS such as obesity, renal anomalies and other symptoms (Croft and Swift, 1990). Medical information for members of this large consanguineous family with two clinically diagnosed cases was obtained through questionnaires and examination of medical records of 23 living relatives and 52 deceased persons (information for these being provided by the closest living

relative). Because the parents of the affected individuals were consanguineous, the BBS gene could be traced through a number of obligate carriers. However, no molecular analysis was available.

From the data collected, and the review of previously reported BBS families, the authors tried to determine possible clinical effects of the BBS gene in heterozygotes. They extrapolated that the Bardet-Biedl syndrome gene may be a possible candidate for one of the genes predisposing to obesity in the general population. Also, hypertension might be a clinically important consequence of BBS heterozygosity, if the renal effects of being a heterozygote are milder than in the homozygotes. As well, the observance of diabetes in seven of the blood relatives of the affected persons in this study lead the authors to suggest that heterozygosity predisposes to diabetes. In addition, they postulated that the gene either directly caused the manifestations of renal disease, hypertension and diabetes, or these were secondary to gene-associated obesity. Furthermore, the authors stated that the heterozygosity frequency in the general population was likely to be at least 1%.

A follow-up study was undertaken to further investigate obesity in heterozygous carriers (Croft *et al.*, 1995). Health questionnaires and medical records were assessed on 34 parents of BBS patients, all of whom were Caucasian. Height and weight information was self-reported. The authors compared the proportion of severely overweight heterozygote fathers (26.7%) with that of a U.S. population group matched for age, sex and race (8.9%) and noted for BBS heterozygotes there was a three-fold greater

prevalence. However, only one mother was severely overweight. Heterozygotes were also significantly taller than men and women in the appropriate control groups. Mean height of homozygotes was greater than for the controls as well, but not significantly. No excess in hypertension or diabetes mellitus was reported. Again the authors assumed the heterozygous prevalence in the general population to be 1%. Using their prevalence ratio (3.0) regarding the excess risk of obesity for BBS heterozygotes, they inferred that 2.9% of all severely overweight males in the general population are BBS heterozygotes.

To address the phenotype of heterozygotes in the Newfoundland population, O'Dea *et al.* (1996) examined the unaffected sibs of BBS patients and observed 57% of them had a BMI greater than 27 (considered obese in this study). Twenty-five percent were hypertensive by age 49, only one had mild renal impairment, none had diabetes mellitus, and there was only a 2% mortality rate by age of 50. Meanwhile, 25% of affected individuals died by the age of 44. The authors suggested the rate of hypertension in the unaffected sibs could be artificially high because they were assessed only once in the study (on repeat testing blood pressure may not have remained elevated). The authors concluded that heterozygous sibs are unlikely to have a clinically important phenotype.

However, like the Croft *et al.* studies (1990 and 1995), there were no molecular analyses performed, making it impossible to determine which unaffected sibs were carriers and which were not. Therefore, a heterozygous phenotype may be 'diluted out' by the non-carrier sibs. However, Beales *et al.* (1999) reported no excess of obesity among obligate carrier parents.

Larger and more complete studies of individuals having molecular testing is needed to evaluate successfully if carriers of a mutant BBS gene have a particular phenotype regarding obesity, hypertension, diabetes, or height.

1.7 Identification of Disease Causing Genes

The strategies of positional cloning and candidate gene approaches are most often employed to locate, identify and subsequently find mutations in a disease causing gene. In the positional cloning approach, the isolation of a gene starts with knowledge of its genetic or physical location in the genome, and little or no knowledge regarding its function (Ballabio, 1993). Three main steps are involved in positional cloning: (1) determining the chromosomal region that is linked to the disease; (2) identifying all genes within this chromosomal region; (3) screening these genes for mutations that segregate with the disease in multiple families.

Linkage analysis is often the first step in positional cloning. A mode of inheritance is proposed, then families and genetic markers are chosen to start the study. Once a region is identified containing the causative gene, the genes within the candidate region must be identified. Regions greater than one Mb in size often contain an overwhelming number of possible transcripts; therefore, scientists prefer regions smaller than 1 Mb. Often this means the DNA region must be cloned. However, with the

progress of the Human Genome Project, the sequence of interest may be contained within an appropriate database.

If cloning is undertaken, then one or more cloning vectors (bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs), and cosmids) are utilized to create a contig map containing DNA fragments from the region of interest. Next is the identification of the genes in the candidate region. Gene isolation schemes include, among others, sequencing the region, exon trapping, screening of cDNA libraries and a candidate gene approach. The latter is theoretically the simplest method of identifying the appropriate gene, since the gene of interest may have been previously cloned. The existence of genome databases has increasingly facilitated the candidate gene approach, as has the completion of the 'rough draft' of the Human Genome Project. Once the gene is identified, the next phase is identification of mutations in the putative disease-causing gene. Some approaches include direct sequencing, protein truncation testing and the use of non-denaturing gels to identify single-stranded conformational polymorphisms (Papadopoulos, 1995).

Linkage analysis, using genetic markers such as microsatellites, is almost always utilized in positional cloning in lieu of very informative chromosomal abnormalities, or, in the case of cancer genes, loss of heterozygosity. Microsatellites are simple sequence repeats, consisting of a series of several repeats of two to five nucleotides. The most common are CA repeats. There are more than 35 000 informative microsatellites in the human haploid genome, occurring at least once every 100 000 base pairs (bp) (Weber *et*

al., 1990). These polymorphic segments are also relatively evenly dispersed throughout the genome, thus making them very useful in population genetics and the determination of family relationships (Weissenbach *et al.*, 1992).

The repeat length of alleles of microsatellite sequences are polymorphic except at loci with fewer than about five repeat units (Valdes *et al.*, 1993). Because these repeats are highly polymorphic, they are indispensable for linkage analysis. In many instances, if a particular microsatelite is completely linked to a gene, all affected individuals will have the same allele. This should not occur for an unlinked microsatellite marker, as long as the microsatellite was polymorphic enough. To ensure accuracy in the locus determination of a disease-causing gene, more than one microsatellite is usually analyzed.

Once several markers have been identified in a particular region of interest, a 'haplotype' can be constructed. A haplotype refers to a set of alleles of a group of closely linked loci on the same chromosome. Haplotype analysis is a very useful tool in defining a critical region within which a disease-causing gene is located, especially in an area which is densely populated with microsatellites. Within a haplotype, markers that do not have the same alleles among affected individuals due to recombination events can be used to further delimit the critical region. A specific kind of haplotype analysis, homozygosity mapping, is used to determine whether a recessive disease-causing gene is associated with a known locus in a consanguineous kindred. One expects to observe homozygosity for an allele at the disease locus in affected individuals from a consanguineous family exhibiting a rare recessive disease. In a genetically inbred population, as is the case in parts of

Newfoundland, a specific allele set is usually passed on through the generations from a common progenitor. In this case an affected individual has a homozygous haplotype due to identity by descent (IBD) (Sheffield *et al.*, 1994). Because of recombination, the further back the common ancestor is, the smaller will be the homozygous allele set.

Once linkage and haplotype analyses have been executed successfully, the candidate gene approach can be used immediately if the critical region has been narrowed down sufficiently, and/or there is a very good candidate gene in the genomic area of interest. Candidate genes in the critical interval may be selected in one of the following ways: a gene that shows homology to a gene implicated in an animal model of the disease; a gene that displays an appropriate expression pattern or function given the pathogenesis of the disease; or a gene that shows homology, or functional relatedness, to a gene implicated in a similar human disease phenotype. If no such genes are identified within the critical region, then positional cloning and database analyses will have to be undertaken to identify all existing transcripts. Once a candidate is identified, mutation analysis is performed to determine whether or not it is the disease-causing sequence.

Usher syndrome is a genetically and clinically pleiotropic disorder for which the positional candidate approach has been successful. It is an autosomal recessive disorder which is characterized by retinitis pigmentosa and sensorineural deafness. Phenotypically, this syndrome has three major forms, differing in severity of symptoms and time of onset: Usher syndrome type I, II and III. Six genes have been mapped for Usher syndrome type I (USH1), two for Usher syndrome type II (USH2) and one for

Usher syndrome type III (USH3) (Keats and Corey, 1999). One USH1 gene, *USH1B*, has been identified as an unconventional myosin, *MYO7A* (Weil *et al.*, 1995). *USH1B* was demonstrated to link to markers on chromosome 11q13.5, and the critical region was refined through microsatellite analysis and homozygosity mapping of a large inbred Samaritan kindred (Bonne-Tamir *et al.*, 1994). Previously, a mouse deafness gene, *shaker-1* (*sh1*), had been mapped to the *USH1B* homologous murine region on chromosome 7 (Brown *et al.*, 1992) and then was shown to encode an unconventional myosin of the type VII family (Gibson *et al.*, 1995). *MYO7A* was a good functional and positional candidate for USH1B; therefore, it was cloned, and mutations were found in USH1B patients (Weil *et al.*, 1995). Subsequently, mutations in *MYO7A* were discovered to cause an autosomal recessive non-syndromic hearing impairment, DFNB2, and also an autosomal dominant form, DFNA11 (Liu *et al.*, 1997; Weil *et al.*, 1997). It seems that some mutations in *MYO7A* impair the protein function more than others, producing diseases of varying extent and severity.

Identification of a disease-causing gene is the initial step in determining the normal function of the protein and how the associated mutations affect the protein function. Eventually it is hoped that some method of combating the disease can be identified through drug or gene therapy derived from the functional studies. Also, genetic testing can be initiated, aiding in family counseling by medical geneticists.

1.8 Thesis Goal

The purpose of this thesis is to examine Newfoundland families burdened with BBS, a rare autosomal recessive disease, in order to classify the genetic variant of the disease affecting each kindred. It is the ambition of this thesis to identify a possible causative gene(s), through positional cloning and candidate gene approaches, and subsequently demonstrate a mutation(s) in this gene(s) which segregate(s) in these kindreds. The families studied are from a previously well defined and thoroughly clinically investigated population (Harnett *et al.*, 1988; Cramer *et al.*, 1988; Green *et al.*, 1989; O'Dea *et al.*, 1996).

Chapter 2 Materials and Methods

2.1 Ascertainment and Clinical Analysis of Bardet-Biedl Syndrome Families

There are a total of 22 families known to have BBS in Newfoundland. However, due to the unavailability of DNA in five families, only 17 BBS kindreds were examined as part of this thesis. In the early 1980s, 16 of 17 of these families with BBS were ascertained in one of three ways: from the Ophthalmology Department records of the Health Sciences Centre, St. John's, Newfoundland; from the Canadian National Institute for the Blind (CNIB) Register; and through additional family studies (Harnett *et al.*, 1988; Green *et al.*, 1989). Subsequently, one kindred with BBS was identified by the Nephrology Unit at the Health Sciences Centre, St. John's, Newfoundland in 1993 (O'Dea *et al.*, 1996). In total, 34 patients, consisting of 20 males and 14 females in 17 families, were included in this thesis. Of these, 32 were completely clinically assessed. The remaining two were deceased at the time of evaluation; therefore, only medical records were reviewed. Complete clinical testing was also possible for 45 unaffected siblings. A total of 111 unaffected family members and 34 patients were genetically surveyed from these 17 BBS families.

A protocol for clinical investigation was approved by the Human Investigations Committee of the Faculty of Medicine, Memorial University of Newfoundland, and by

the Medical Advisory Council of the St. John's General Hospital. Appropriate informed consent was obtained from participants in this study. Diagnosis of affected members was based on the following criteria: the presence of retinal dystrophy, obesity or a history of obesity, dysmorphic extremities, and the absence of neurological complications. Further investigations showed that 100% of the BBS patients who were examined by ultrasound displayed fetal lobulation of the kidney, providing direct evidence that renal structural abnormalities are a cardinal manifestation of the disease. Other manifestations of BBS observed included genital hypoplasia in males, and cognitive deficit, but these manifestations were not incorporated into defining a case (Harnett *et al.*, 1988; O'Dea *et al.*, 1996). The above diagnostic criteria agree with the more recent criteria proposed by Beales *et al.* (1999).

On each visit to the clinic by BBS patients or their unaffected siblings, blood pressure was recorded. Blood was drawn for measurement of serum urea, creatinine, electrolytes, glucose, glycosylated hemoglobin, calcium, alkaline phosphatase, albumin, total protein, hemoglobin and complete blood count. Blood was also obtained for measurement of follicle stimulating hormone, luteinizing hormone, prolactin, testosterone and estradiol. Urine samples were obtained and analyzed for presence of blood and protein. Twenty-nine BBS patients underwent ultrasound of both kidneys, and 21 had serial studies. All ultrasounds were interpreted by one radiologist. Unaffected siblings did not undergo ultrasound investigation. The height and weight of 27 adult BBS patients (15 females and 12 males) and 42 adult siblings (21 women and 21 men) were converted

to BMI scores (O'Dea *et al.*, 1996). Measurements were also made of the head circumference, and the length and breadth of the hands and feet in many patients.

Ophthalmological investigations included retinal function testing (color vision testing, perimetry and dark adaptation testing), and when possible electroretinographic studies, photography of the fundus and fluorescein angiography were performed. Psychological testing was conducted, consisting of the administration of the standard Wechsler Adult Intelligence Scale verbal tests and the Haptic Intelligence Scale. A skeletal survey and a complete physical exam was also done (Green *et al.*, 1989). In addition, medical records were reviewed for all patients to obtain confirmation of information concerning age of onset of legal blindness, hypertension, diabetes mellitus, renal impairment and ESRD (O'Dea *et al.*, 1996).

Full family pedigrees were obtained through interviews with individuals in the extended family, and whenever possible this information was confirmed by archival research at the Association of Newfoundland and Labrador Archives (http://www.anla.nf.ca/) and on the Internet in Project 21 (http://www.huronweb.com/genweb/p21/main.html). The latter is the initiative to secure the Newfoundland Census of 1921 in a public electronic database format. Through these methods parental consanguinity was identified in six kindreds and suspected in an additional six families (Fig 2.1). In five of the six kindreds presumed to be consanguineous (B2, B10, B11, B12, B15), there were ancestors from both sides of the family living in the same area and sharing the same surname.

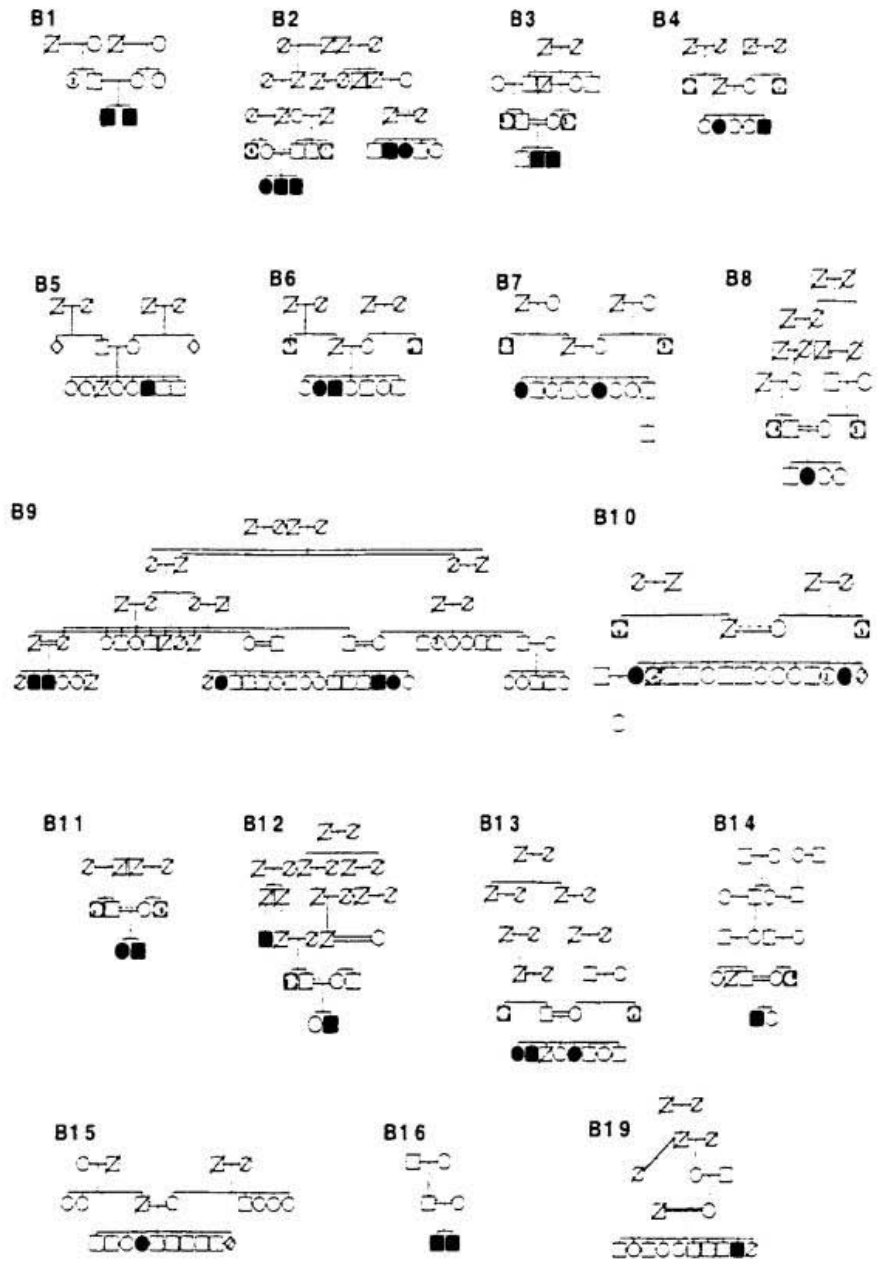


Fig 2.1 Pedigree structure of the 17 Newfoundland families with BBS examined in this thesis.

The BBS families are distributed almost exclusively in small coastal communities throughout the island of Newfoundland (Fig 2.2). The greatest concentration of BBS kindreds is in the Conception Bay area, a large bay located on the northeastern portion of the Avalon Peninsula. However, there is a surprising scattering of families throughout the rest of the island. The population of Newfoundland is 560 000, with 260 000 people living around the St. John's area, and the remaining 300 000 distributed in many coastal communities and in a few larger towns in the interior of the island.

2.2 Extraction of DNA

2.2.1 Extraction of DNA from Whole Blood

All DNA samples but two were extracted from the white cells of venous blood, which was collected in EDTA tubes and processed within one week of blood letting. A simple salting-out method was employed for DNA extraction. Forty-five milliliters of warmed (37°C) NH_4Cl:Tris (900ml of 0.155 M NH_4Cl; 100ml of 0.17 M Tris.HCl, pH 7.65) was added to 5 ml of whole blood in a 50ml tube and the mixture incubated at 37°C for 5 min. This was centrifuged at 2500 rpm for 5 min (1000xg). The supernatant was poured off, leaving a white cell pellet at the bottom of the tube, and 10 ml of saline solution (0.85% NaCl) was added. The 50 ml tube was vortexed briefly and centrifuged

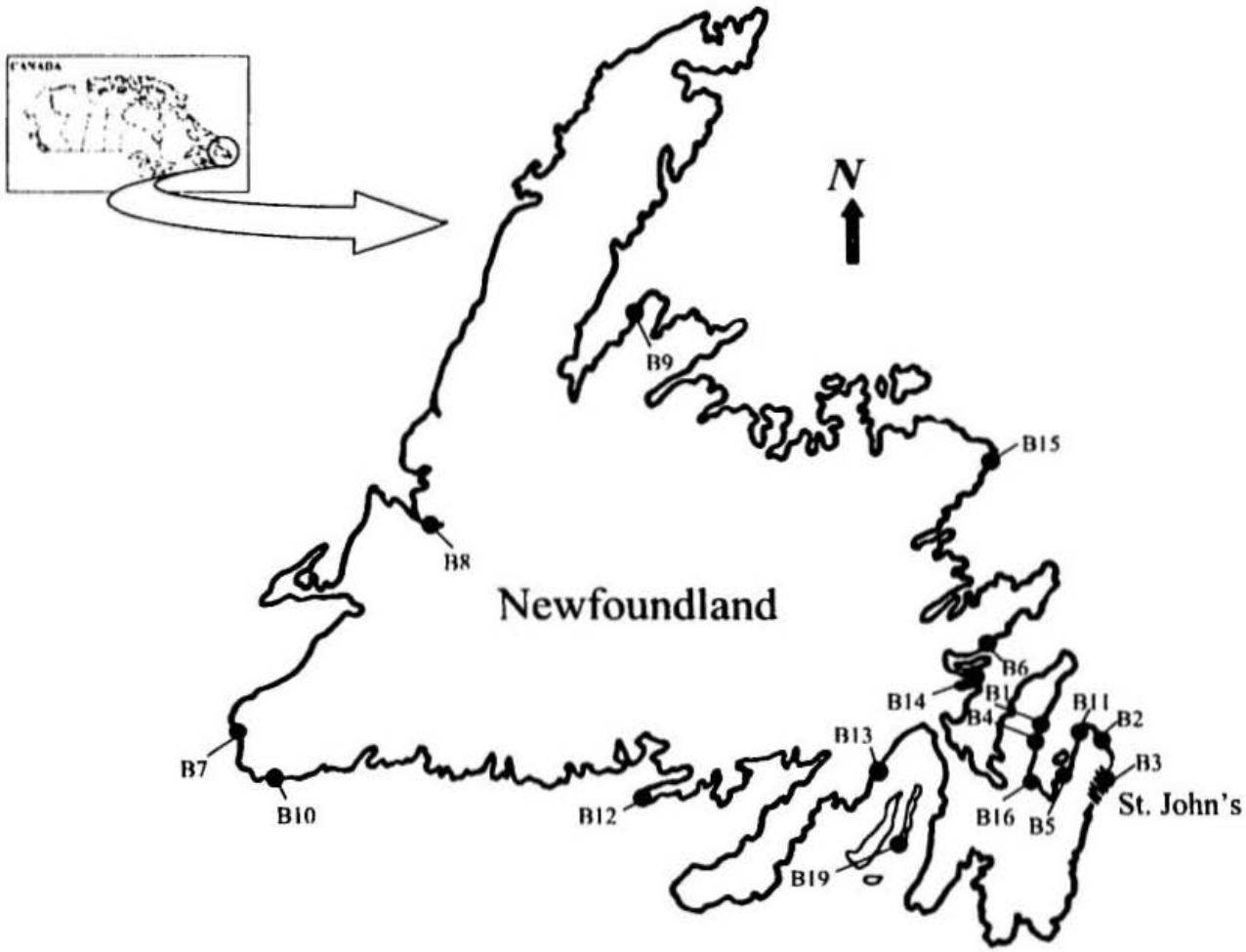


Fig 2.2 Distribution of BBS families on the island of Newfoundland.

again under the same conditions. The supernatant was removed and 3 ml of nuclei lysis buffer (10 mM Tris.HCl, 400 mM NaCl, 2 mM EDTA, pH 8) was added to the pellet. This mixture was briefly vortexed and transferred to a 15 ml centrifuge tube. With the addition of 0.2 ml of 10% SDS and 0.5 ml of pronase E solution (3 mg/ml in 1% SDS, 2 mM EDTA), the mixture was incubated overnight in a 37°C water bath. The next day 1 ml of saturated NaCl was added and the 15 ml tube was shaken vigorously for 15 sec, then centrifuged at 2500 rpm for 15 min (Miller *et al.*, 1988). The supernatant was gently poured off into another 15 ml tube and 2 volumes of absolute ethanol added to the supernatant. The tube was inverted several times as the DNA precipitated. A 9" glass pipette was melted into a hook and cooled, then used to fish out the DNA. Finally, the DNA on the hook was washed several times with 70% ethanol and allowed to air dry. The DNA was dissolved in 300-900 µl TE (10 mM Tris, 1mM EDTA, pH 8) overnight and then gently rotated in an incubator for roughly an hour. DNA samples were labeled appropriately, registered in a DNA bank book and in a computerized database, and then stored at 4°C.

2.2.2 Extraction of DNA from Paraffin Blocks

Two DNA samples (B3, PID 11; B9, PID 24) were obtained through extraction by salting out from archival paraffin blocks of the kidney. Sections of 5 x 10 microns were

obtained using a microtome and individual sections were treated with 1 ml of toluene in a 55°C water bath for 5-10 min. Samples were then microcentrifuged for 5 min and the toluene decanted. The toluene treatment, centrifugation and decantation steps were repeated. Then 1 ml of absolute ethanol was added at room temperature for 5-10 min. Again the sample was centrifuged and decanted. These three steps were repeated and the sample was left to air dry at room temperature for 10-15 min. Digestion with proteinase K (425 µl of 0.5 x TBE buffer; 50 µl of 10% SDS; 25 µl of 0.5 µg/µl proteinase K) took place overnight in a 55°C water bath.

The next day, a 0.27 volume of saturated 6 M NaCl was added and the sample was centrifuged (2000 rpm) for 5 min. The supernatant was transferred to another tube and the original tube was discarded. Then 1.5 ml of 95% ethanol was added. At this point the DNA strands appeared in suspension. DNA samples were labeled appropriately, registered in a DNA bank book and in a computerized database, then stored at 4°C. Calculations of DNA concentrations were not performed.

2.3 Microsatellite Marker Analysis

2.3.1 Genotyping

The polymerase chain reaction (PCR) was conducted using approximately 100-

200 ng of template DNA, with primers purchased from Research Genetics, Inc. Primers were for the amplification of di-, tri- or tetranucleotide microsatellite DNA (**Appendices B-H**). One primer (usually forward) of each pair was end-labeled using T_4 polynucleotide kinase (Pharmacia, Biotech, Uppsala, Sweden) and [γ-^{32}P]ATP (Amersham, Ontario, Canada) (Sambrook *et al.*, 1989). Standard 10 µl PCR reactions containing 1.5 pmol of primer, 200 µM dNTPs, and 0.125 units of Tfl DNA polymerase (Promega, Madison, Wisconsin) were made. Almost all samples were subjected to 33 cycles of 94°C for 30 sec (denaturation), 55°C for 20 sec (annealing) and 72°C for 30 sec (extension), after an initial denaturing step of 2 min at 95°C. Thermocyclers used for all microsatellite amplification procedures were the Perkin-Elmer Gene-Amp 9600, MJ Research PTC-200 and the Biometra Tgradient. DNA samples which did not amplify were subjected to a touchdown cycle consisting of a 2 min denaturation step at 95°C followed by one cycle of 30 sec at 94°C, 20 sec at 65°C and 30 sec at 72°C, then nine additional cycles each with a decrease of one degree of annealing temperature. After these ten cycles, the annealing temperature remained at 55°C for 20 cycles, finishing with a 5 min extension step at 72°C. If this did not work, the initial annealing temperatures were adjusted by one degree in either direction until successful. All cycles concluded with a cooling step which was maintained at 4°C. PCR products were stored in a 4°C refrigerator for up to a week.

Amplified DNA was analyzed on 6%-8% polyacrylamide denaturing gels with or without formamide (Litt *et al.*, 1993). Most often a 60 ml volume of gel solution (6%) was made using 6ml of 10x TBE, 9ml 40% acrylamide, 19 ml 95% formamide, 10ml

dH_20, 20g urea and 480µl of 10% ammonium persulfate. When formamide was used, the gel solution was filtered through Whatman filter paper #1. A casting tray was made using 10 ml of gel solution with the addition of 50 µl of TEMED. The casting tray and gel apparatus were allowed to set for 15 min before the gel was poured. Gels were poured with a syringe into the 21 cm x 40 cm x 4 mm gel apparatus (Bio-Rad) and left to set for at least one hour. They were then pre-heated at a constant 42 W until the front gel plate reached 50-55°C (usually one hour). All excessive urea on the gel front was washed away by a pipette and 2.5 µl of each sample was loaded into the wells of a 36 well comb.

Samples were prepared by adding equal amounts of dye (consisting of 95% formamide, 0.5 M EDTA, 0.05% bromophenol blue and 0.05% xylene cyanole) and sample into a microtiter plate and heating on a 95°C heating block for 5 min, then immediately cooled on ice. Gels were then loaded and run at a constant 42 W for two to eight hours depending on the size of the fragments analyzed (**Appendices B-H**). All fragment sizes were obtained from The Genome Database (http://gdbwww.gdb.org). The gels were then placed on Whatman 3 mm paper and dried in a slab gel drier (Bio-Rad - Model 583) for three hours at a temperature of 80°C. When gels were dry they were put in the dark at room temperature with autoradiography film (Kodak X-Omat Blue XB-1) (Sambrook *et al.*, 1989). Subsequently, the autoradiography film was developed and analyzed. Alleles were scored relative to each other and not size, as no size references were prepared. Alleles were also scored blind with respect to disease status.

2.3.2 Haplotype analysis

Haplotypes were constructed manually for each family at each tested locus using all available microsatellite data, and represented the minimal number of recombination events. Several criteria were used for locus assignment/exclusion based on informative haplotypes. Sharing of haplotypes between affected and unaffected individuals in the same family was used to exclude a locus. Similarly, a locus was excluded if affected individuals in the same family had different parental haplotypes. Support for linkage to a BBS locus was established in consanguineous families if, and only if, the affected individual(s) displayed homozygosity by descent (HBD) (Lander and Botstein, 1987) in the critical region of a known or suspected BBS locus, and their unaffected sibling(s) did not. In suspected consanguineous kindreds, homozygosity by state (HBS), defined as a homozygous haplotype due to parental consanguinity of unknown degree, was taken as support for linkage. In families in which there was no indication of consanguinity, haplotype sharing (HS) at the hypothesized or known BBS critical region in affected individuals, but not in their unaffected sib(s), indicated support for linkage. Using this haplotype analysis protocol (Fig 2.3), a family could be either excluded, linked, or not excluded from a particular locus.

Microsatellite marker orders were obtained from published materials as well as from various electronic databases: GeneMap '99 (http://www.ncbi.nlm.nih.gov/genemap/); The Genome Database

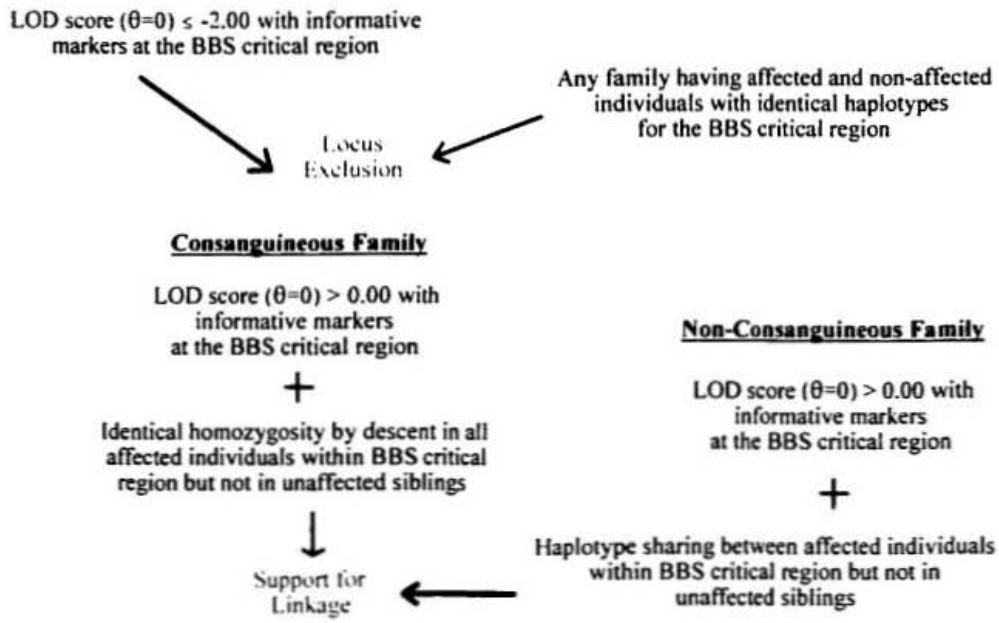


Fig 2.3 Flow chart representing processes of haplotype and linkage analyses regarding the support or exclusion of a particular locus in a BBS family.

(http://gdbwww.gdb.org/); Marshfield Center for Medical Genetics (http://research.marshfieldclinic.org/genetics/); Stanford Human Genome Center (http://www-shgc.stanford.edu/); Whitehead Institute for Biomedical Research/MIT Center for Genome Research (http://www-genome.wi.mit.edu/); and Cooperative Human Linkage Consortium (http://lpg.nci.nih.gov/CHLC/). Using this information, a best fit map was created at each BBS locus tested for linkage.

2.3.3 Whole Genome Screens by Homozyosity Mapping Using Pooled DNA

In instances in which a relatively rare recessive disorder occurs in a genetically isolated inbred population, homozygosity for an allele at the disease locus is expected in the affected individuals, due to HBD from a common progenitor. Based on this assumption, the DNA pooling method is used to facilitate identification of HBD at the disease locus. DNA pools are made of equal amounts of DNA from each affected individual (test pool) and from each unaffected individual (control pool). The pooled DNA samples are analyzed with microsatellite markers using PCR. When DNA pools from controls and affected individuals are compared there will be no differences in allele distribution for markers unlinked to the disease locus except by chance. However, there will be a shift in allele frequencies from the control pools to the affected pools at genetic markers linked to the disease gene (Sheffield *et al.*, 1994).

This method was employed as it was deemed very appropriate for finding BBS loci in the Newfoundland population. The consanguineous family B9 (Fig 2.1), which had been excluded from the four original BBS loci, was used in the initial genome screen implementing homozygosity mapping of pooled DNA. Two control pools of DNA from four living parents and 11 unaffected siblings, as well as a test pool of DNA from the four surviving patients, were subjected to PCR. The total DNA concentrations of each pool were equivalent. The genomic scan was performed with microsatellite markers from the Cooperative Human Linkage Consortium human screening set, Weber version 8 (Research Genetics). The screening set was broken into five subsets corresponding to microsatellite size to ensure that each gel electrophoresis would contain amplification products of similar size. The PCR protocol was 33 cycles of 94°C for 30 sec (denaturation), 55°C for 20 sec (annealing) and 72°C for 30 sec (extension), after an initial denaturing step of 2 min at 95°C. Amplification reactions were not multiplexed. Thermocyclers used for amplification of samples were the Perkin-Elmer Gene-Amp 9600 and the MJ Research PTC-200. Each polyacrylamide gel was loaded with 33 samples, corresponding to three DNA pools, amplified with 11 different primers which gave products of similar size. Gel electrophoresis and autoradiography were conducted as in **Chapter 2.3.1**.

A reduction of multiple alleles in the control pool to one allele in the test pool, at a particular marker, warranted that marker to be run separately on all available pedigree members. Once a marker looked as if it segregated with the disease in the pedigree,

additional markers adjacent to the original marker were identified through the abovementioned databases. These were then tested on the family to create a haplotype at the putative BBS locus. Once again all microsatellites were amplified as in **Chapter 2.3.1**.

Similarly, the consanguineous kindred B13 (Fig 2.1) underwent a genome wide scan once it was excluded from all known BBS loci. Three pools were constructed with the two parents in one control pool, four unaffected siblings in the second control pool and the two affected individuals in the test pool. The genome screen and subsequent microsatellite analyses were performed exactly as with family B9.

2.4 Linkage Analysis

Linkage analysis was carried out to corroborate the haplotype analysis. Two-point linkage analysis was performed using the MLINK (v5.21) subroutine of FASTLINK (v4.0P) and LINKAGE (v5.21) (Lanthrop and Lalouel, 1984; Cottingham *et al.*, 1993; Schaffer *et al.*, 1994). BBS was modeled as an autosomal recessive disorder with a penetrance of 0.95. The disease gene frequency was adjusted to 0.008, based on the disease incidence of 1:17 500 in the Newfoundland population (Green *et al.*, 1989). However, in the linkage disequilibrium study of BBS1 families, the disease allele frequency of 0.0032 was used, adjusted to reflect an estimated 40% contribution of the

BBS1 locus to the overall population frequency. Suspected consanguineous loops were not considered when conducting two-point lod scores. All microsatellites were assumed to have nine alleles of equal frequencies in the study population. Significantly negative lod scores ($\leq$-2.00, θ=0) for fully informative markers within the critical regions of BBS loci, for each of the 17 families, were used as criteria for exclusion. Positive lod scores were taken as support for linkage and lod scores $\geq$3.00 (θ=0) were considered statistically significant for linkage. Markers were selected for linkage analysis based on the parents being heterozygous with distinct genotypes. Families with recombinations in critical regions were assigned on the basis of the haplotype analysis. All marker and family data were imported into the FASTLINK (v4.0P) program via marker specific programs created by Visual dBase, which also contained the BBS database.

Previous to the genome scan on kindred B13, a simulation study to determine the estimated maximum lod score possible in this family was undertaken using SLINK (v2.65), an auxiliary program of LINKAGE (v5.21) (Ott, 1989; Weeks *et al.*, 1990). The three seeds for the random number generator were 25 006, 28 270 and 17 716. The number of replicates was 300, and they were simulated under the assumption of homogeneity. Simulation data were analyzed with the MSIM (v2.65) program using a disease penetrance of 0.95, eight equal marker alleles and a disease gene frequency of 0.008. Subsequently, analysis was run on all five families (B3, B4, B5, B6 and B13) unlinked to the five known BBS loci to determine a cumulative estimated lod score. Simulation and analysis conditions were the same as for the run using only family B13.

2.5 Mutational Analysis

Once the gene for McKusick-Kaufman syndrome was published, the cDNA sequence for *MKKS* was screened through the high-throughput genomic sequence database using the BLAST algorithm (HTGS; http://www.ncbi.nlm.nih.gov/BLAST/blast_databases.html). BAC clone RP11-368H14 (AL158197) exhibited >99.5% identity to the cDNA sequence. Although this clone was annotated to map to chromosome 22, electronic PCR (ePCR; http://www.ncbi.nlm.nih.gov/STS/) identified 28 chromosome 20p sequence tagged sites (STSs). It was concluded that this BAC contained the true *MKKS* locus. The BAC sequence was downloaded and aligned to the *MKKS* cDNA with programs from the GCG software package (Katsanis *et al.*, 1997). The sequence flanking all coding exons was identified and primers were designed to amplify both exons and intronic splice junctions with the Primer v3 program (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi).

Initial PCR on all individuals and controls were conducted in 25 µl reactions containing approximately 100-200 ng of template DNA; 2.5 µl each of 10x PCR Buffer and 2mM dNTPs; 0.1 µl of each forward and reverse 100pm/µl primer; 0.15 µl of Taq (0.75 units) and filled to volume with dH_2O (17.65 µl). Amplification was performed on a MWG Primus 96 Plus using a touchdown cycle as follows: 95°C for 7 min; 10 cycles of 95°C for 30 sec, a 1°C step-down/cycle from 65-55°C for 30 sec each, and 72°C for 45 sec; 30 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 45 sec; 72°C for 10 min;

and finally cool to 4°C. Samples were then run on a 1.2% agarose gel to ensure that the product was successfully amplified. Agarose gels were loaded dry with a mixture of 5 µl of PCR product and 2 µl of stop solution dye. Gels were run in buffer at 100 V for 1 hr. Successfully amplified PCR products from all available family members and controls were purified with the QIAquick Multiwell PCR Purification kit (Qiagen). Purified PCR products were then cycle sequenced on a PTC-225 DNA Engine Tetrad (MJ Research) using 1.2 µl of purified PCR product and 4 µl of either A,C,G, or T Big Dyes (Applied Biosystems) per individual sample. The following cycle sequencing protocol was used: 15 cycles of 95°C for 15 sec, 55°C for 1 sec and 72°C for 1 min; then 15 cycles of 95°C for 15 sec and 70°C for 1 min; and finally a cool down step to 4°C. Products were subsequently pooled by mixing 50 µl of 100% ethanol to each sample consisting of four ddNTP cycle sequencing products. Pooled products were chilled for 15 min at -20°C and spun down for 15 min at 3000 rpm in a vacuum sealed centrifuge. The ethanol was discarded and the pellet dried in vacuum sealed centrifuge and resuspended in 5 µl of ABI loading buffer. The mixture was then heated for 5 min on a 95°C heating block and immediately chilled on ice. A 1.25 µl volume of each sample was loaded into a 96 well comb on an ABI 377 automated sequencer (Applied Biosystems).

PCR products were also cloned using the Original TA Cloning Kit (Invitrogen) and sequenced to separate the different alleles. The competent cells used were E. coli DH5α, grown in LB broth containing ampicillin. Sequencing was performed using the T7 and exon 3 primers. Resulting sequences were aligned and mutations were evaluated

by the Sequencher sequence alignment program (ACGT Codes). *MKKS* exon amplification primers are listed in **Appendix I**.

Chapter 3 Results

3.1 Initial Genetic Survey of the Four Bardet-Biedl Syndrome Loci in Newfoundland BBS Families

To investigate the high incidence of BBS in the Newfoundland population, and in particular to determine if it is the result of a founder effect, members of 17 previously untested Newfoundland BBS families were genotyped using polymorphic microsatellite markers spanning the relevant critical regions of the four known BBS loci (Fig 3.1).

3.1.1 Families Assigned to *BBS1*

Three families (B8, B10 and B19) were assigned to the *BBS1* locus (Table 3.1) using the haplotype and linkage analyses criteria for inclusion (see **Chapter 2.3.2**). In the consanguineous family B8, the affected individual, personal identification 10 (PID 10), was HBD for the entire critical region of *BBS1* on chromosome 11 (Fig 3.2). A lod score of 1.29 ($\theta = 0$) at *D11S1883* further supported linkage of kindred B8 to the *BBS1* locus. This family was excluded from *BBS2* and *BBS3* because the affected individual shared

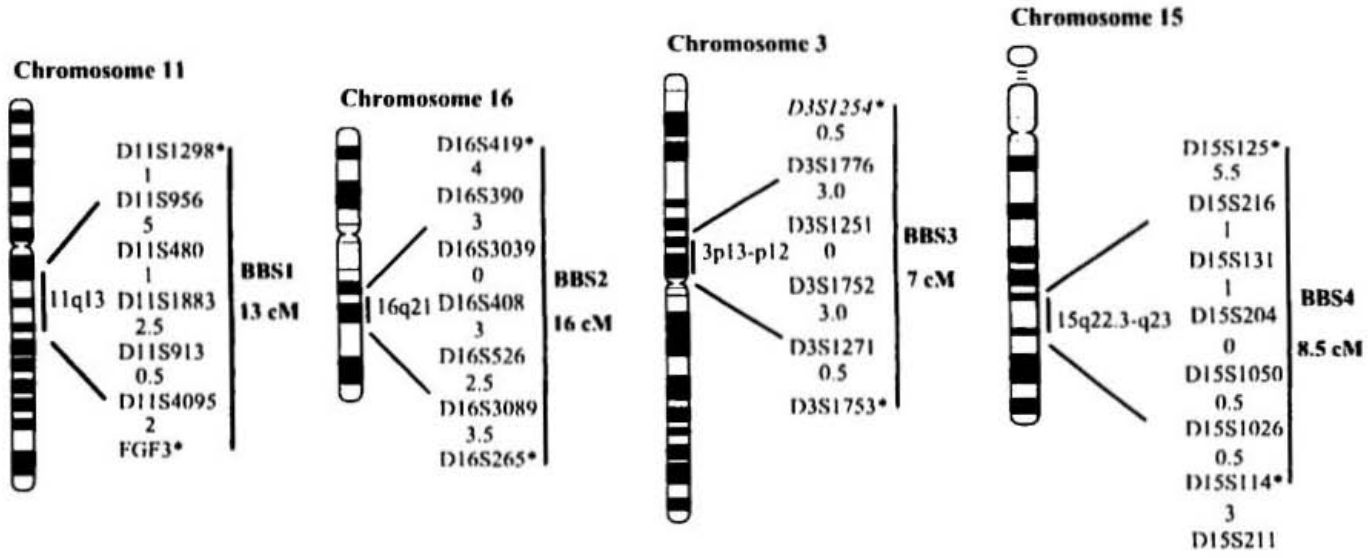


Fig 3.1 Microsatellites spanning the four known BBS critical intervals initially tested on the Newfoundland BBS families. *indicates flanking markers determined in the initial studies locating the four BBS loci (Kwiteck-Black *et al.*, 1993; Leppert *et al.*, 1994; Sheffield *et al.*, 1994; Carmi *et al.*, 1995). The flanking marker in italics was not used because of its low heterozygosity value. Distances between markers are from Genethon linkage maps, the Marsfield sex-averaged linkage maps (Genome Data Base) and GeneMap '99.

Table 3.1 Summary of linkage[a] and haplotype analyses in BBS families.

Kin No.	BBS1			BBS2			BBS3			BBS4			BBS1	BBS2	BBS3	BBS4
	STRP[b]	LOD	HAP[c]	STRP	LOD	HAP	STRP	LOD	HAP	STRP	LOD	HAP				
B1	913	-2.91	X	390	0.60	HS	1752	-2.91	X	131	0.60	HS	X	?	X	?
B2[d]	4095	-7.90	X	390	-15.10	X	1752	-2.86	HBS	131	-5.19	X	X	X	✓	X
B3[e]	1883	-1.75	X	265	-0.89	X	1271	-1.52	X	216	-1.34	X	X	X	X	X
B4	913	-5.47	X	390	-0.35	X	1752	-0.35	X	204	-0.35	X	X	X	X	X
B5	1883	-4.50	X	526	-1.90	X	1752	-0.60	X	131	-2.01	X	X	X	X	X
B6	1883	-4.93	X	408	-7.53	X	1752	- 0.11	X	131	-6.23	X	X	X	X	X
B7	1883	0.96	HS	265	1.07	HBS	1776	-1.53	X	204	-15.90	X	?	?	X	X
B8[e]	1883	1.29	HBD	408	-1.28	X	1752	-1.25	X	131	-1.27	NE	✓	X	X	?
B9[e]	1883	-8.29	X	408	-7.51	X	1251	-5.53	X	216	-11.70	X	X	X	X	X
B10[d]	4095	1.54	HS	408	-23.00	X	1752	-23.00	X	131	-7.87	X	✓	X	X	X
B11[d]	FGF3	-6.43	X	390	0.60	HS	1776	0.60	HS	204	0.60	HS	X	?	?	?
B12[d]	913	0.13	NE	265	0.13	NE	1752	-1.17	X	204	0.12	NE	?	?	X	?
B13[e]	FGF3	-4.44	X	526	-0.50	X	1752	-6.79	X	131	-4.04	X	X	X	X	X
B14[e]	FGF3	-1.19	X	408	-0.33	HBS	1752	-0.37	X	131	-1.26	X	X	✓	X	X
B15[d]	FGF3	0.94	HBS	419	0.82	NE	1271	-1.66	X	204	0.94	NE	?	?	X	?
B16[d]	913	-2.84	X	419	-6.19	X	1251	0.56	HS	114	-3.09	X	X	X	?	X
B19[e]	4095	0.87	HBS	408	0.75	NE	1251	$-\infty$	X	1026	0.87	NE	✓	?	X	?

[a] Lod scores are given at θ=0

[b] ID number of the short tandem repeat polymorphism

[c] Haplotype analysis: X, excluded; HS, haplotype sharing; HB(D/S), homozygosity by (descent/state); NE, not excluded.

[d] Suspected consanguineous relationship

[e] Confirmed consanguineous relationship

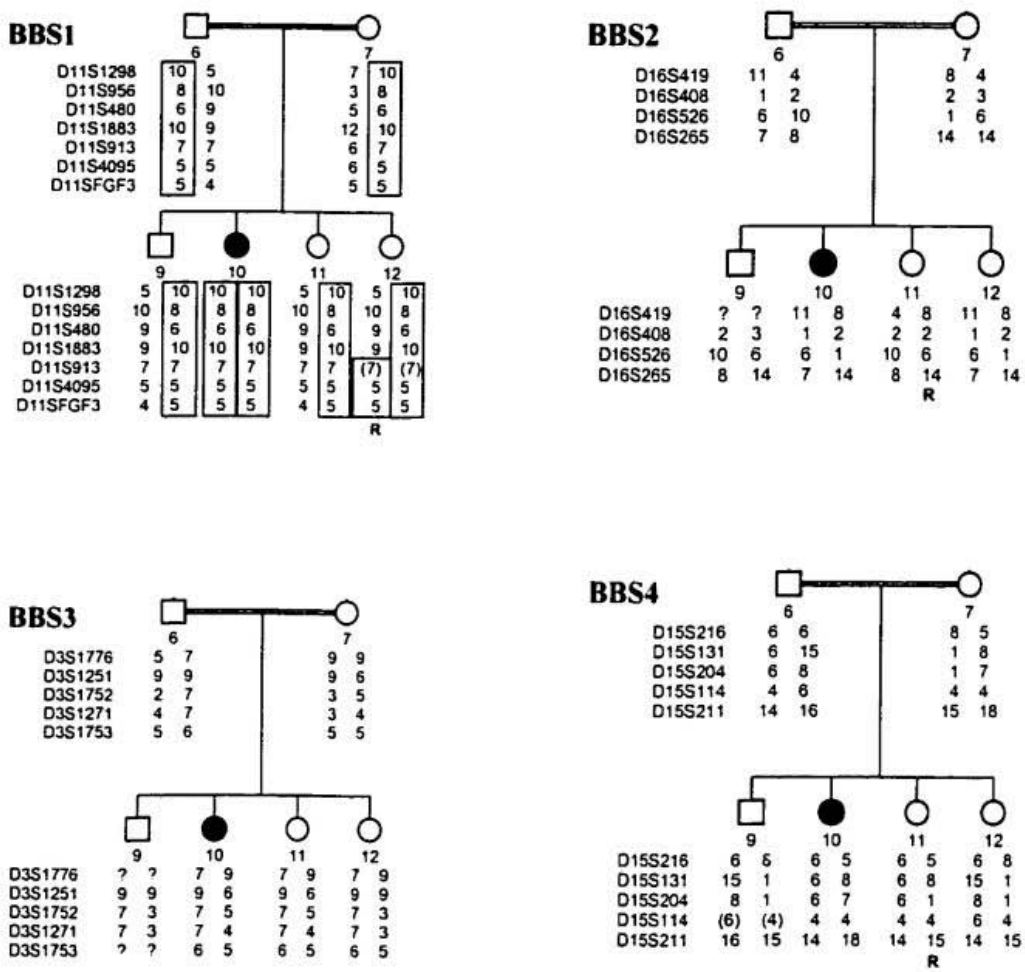


Fig 3.2 Markers spanning the four known BBS loci in the consanguineous family B8. Boxed haplotypes indicate DHs at the *BBS1* locus. Only the core pedigree is presented. 'R' indicates a recombinant haplotype. Alleles in parentheses were inferred. '?' indicates no information was obtained or inferred.

haplotypes with an unaffected sib, PID 12 and PID 11, respectively. Also, linkage analysis yielded negative lod scores at these loci (Table 3.1). For *BBS4*, the affected individual in family B8 had an identical paternal contribution as two unaffected siblings, but the qter portion of her maternal contribution was unique. Thus, this region, qter to *D15S131*, could not be excluded. Nonetheless, because the offspring of this second cousin union is homozygous by descent (HBD) for the entire *BBS1* critical region, family B8 was assigned to this locus.

Family B10 is a large kindred in which there is suspected consanguinity. Haplotype sharing (HS) was observed at *BBS1* between the two affected individuals (PIDs 9 and 22), but not with any of eight unaffected siblings (Fig 3.3). The assignment of *BBS1* to this kindred was supported by a positive lod score of 1.54 ($\theta = 0$) for *D11S1883* (Table 3.1). This family was excluded from *BBS2*, *BBS3* and *BBS4* based on haplotypes and significantly negative lod scores. For *BBS2*, the affected individuals have different maternal contributions and PID 9 shared haplotypes with an unaffected sib (PID 14). For *BBS3*, both affected individuals had different maternal and paternal contributions. Also, PID 22 shared her haplotypes with unaffected sibs PIDs 17 and 19. Also, PID 9 shared her haplotypes with unaffected sib PID 13. For *BBS4*, the affected patients have different paternal contributions and PID 22 shared her haplotype with the unaffected sib PID 19.

In the consanguineous family B19, the affected individual (PID 13), exhibited HBD for all five markers typed at the *BBS1* loci, and none of his unaffected siblings had a

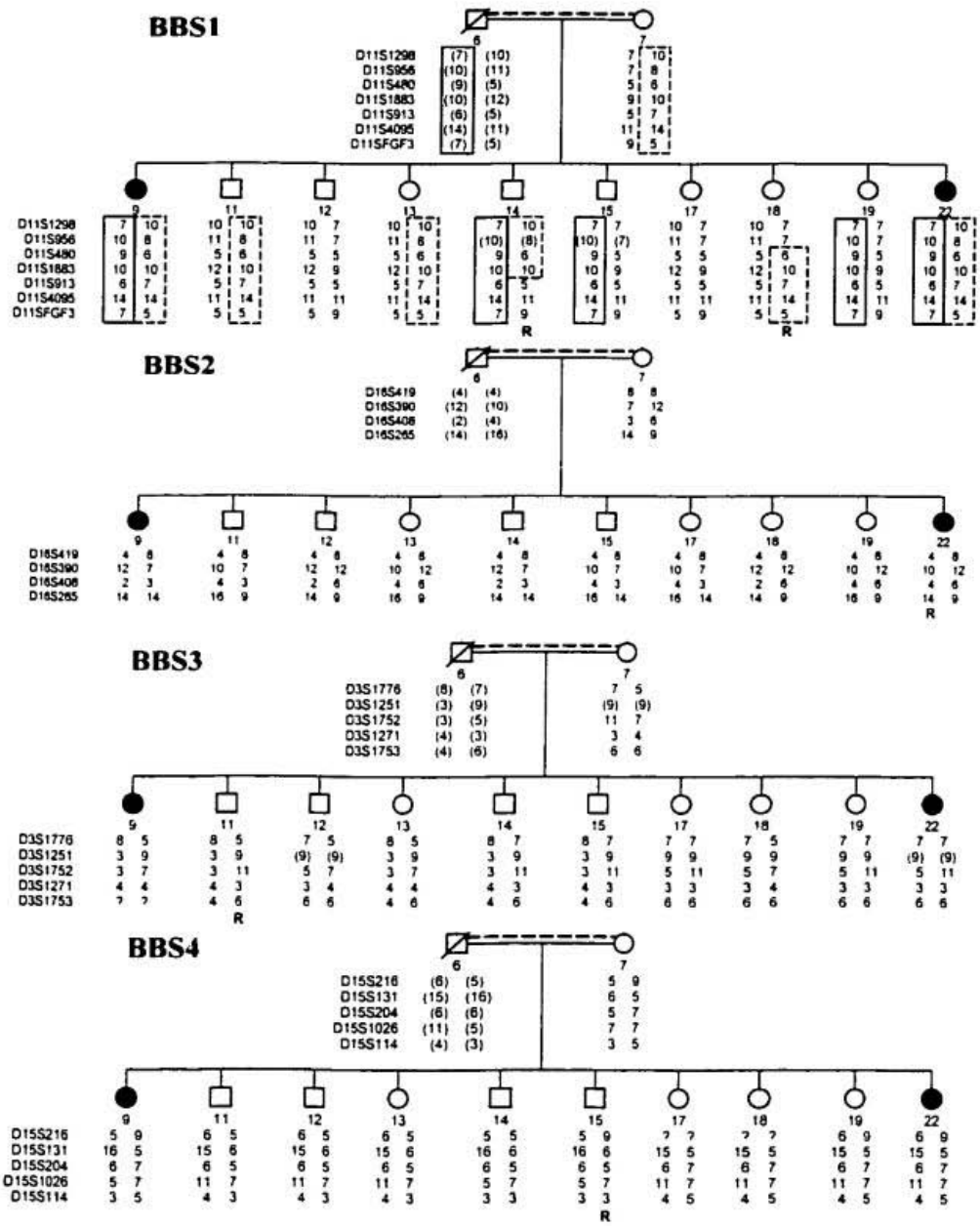


Fig 3.3 Markers spanning the four known BBS loci for the presumed consanguineous family B10. The disease haplotypes are boxed (solid and dotted lines) for the *BBS1* locus. Only the core pedigree is pictured. Alleles in brackets were inferred. 'R' indicates a recombinant haplotype and '?' indicated no information was obtained or inferred.

similar haplotype (Fig 3.4). Linkage was further supported by a positive lod score of 1.48 ($\theta = 0$) at *D11S4095* (Table 3.1). Family B19 could be excluded from *BBS3* since the affected individual had the same haplotypes as an unaffected sib (PID 10). As well, a statistically significant negative lod score was observed at *D3S1251*. Exclusion of this family from *BBS2* and *BBS4* was not possible because the only affected individual contained a unique non-homozygous haplotype. However, because there was observed homozygosity at 11q13 in the BBS patient of this known consanguineous family, the kindred was interpreted to be a BBS1 family.

3.1.2 Family Assigned to *BBS2*

One family, B14, was assigned to the *BBS2* locus. Haplotype analysis revealed that the affected individual was homozygous in the critical region of *BBS2* from *D16S3039* to *D16S265*, inclusive (Fig 3.5). Additional microsatellite markers were later typed between *D16S408* and *D16S526* (Fig 3.6), and this region was homozygosity by state (HBS) as well. However, this observed homozygosity is not explained by parental consanguinity, because the patient (PID 18) received one of his maternal grandmother's chromosomes, and she is not known to be part of the consanguinity loop (Fig. 3.6). Two-point linkage analysis yielded a negative lod score at the *BBS2* locus (-0.33 ($\theta = 0$) at *D16S408*) because the possible maternally inherited disease haplotype was derived from

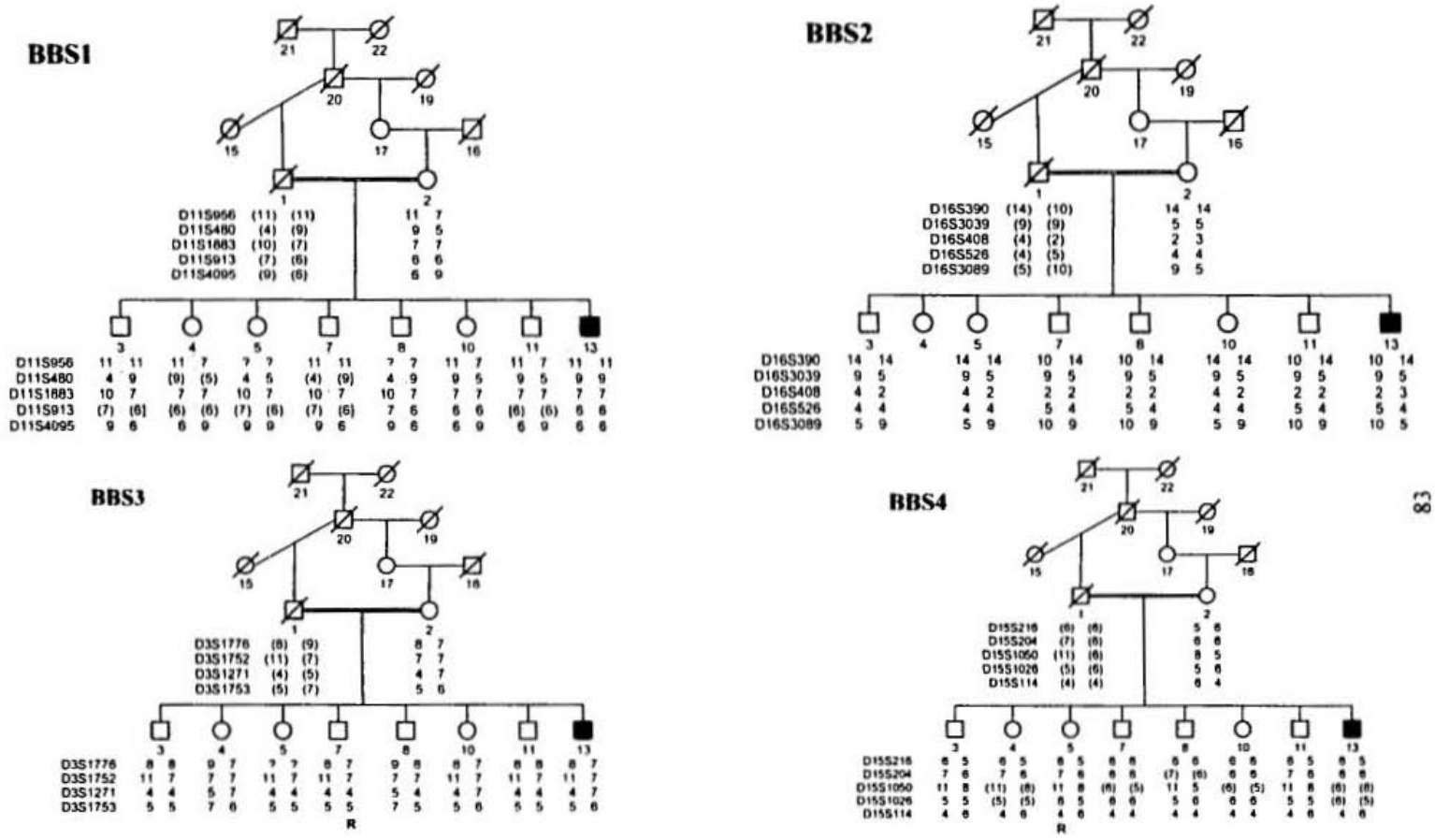


Fig 3.4 Markers spanning the four known BBS loci in the consanguineous family B19. A region of HBD (boxed) is present in the *BBS1* interval. Only the core pedigree is shown. Alleles in parentheses were inferred. '?' indicates no information was obtained or inferred.

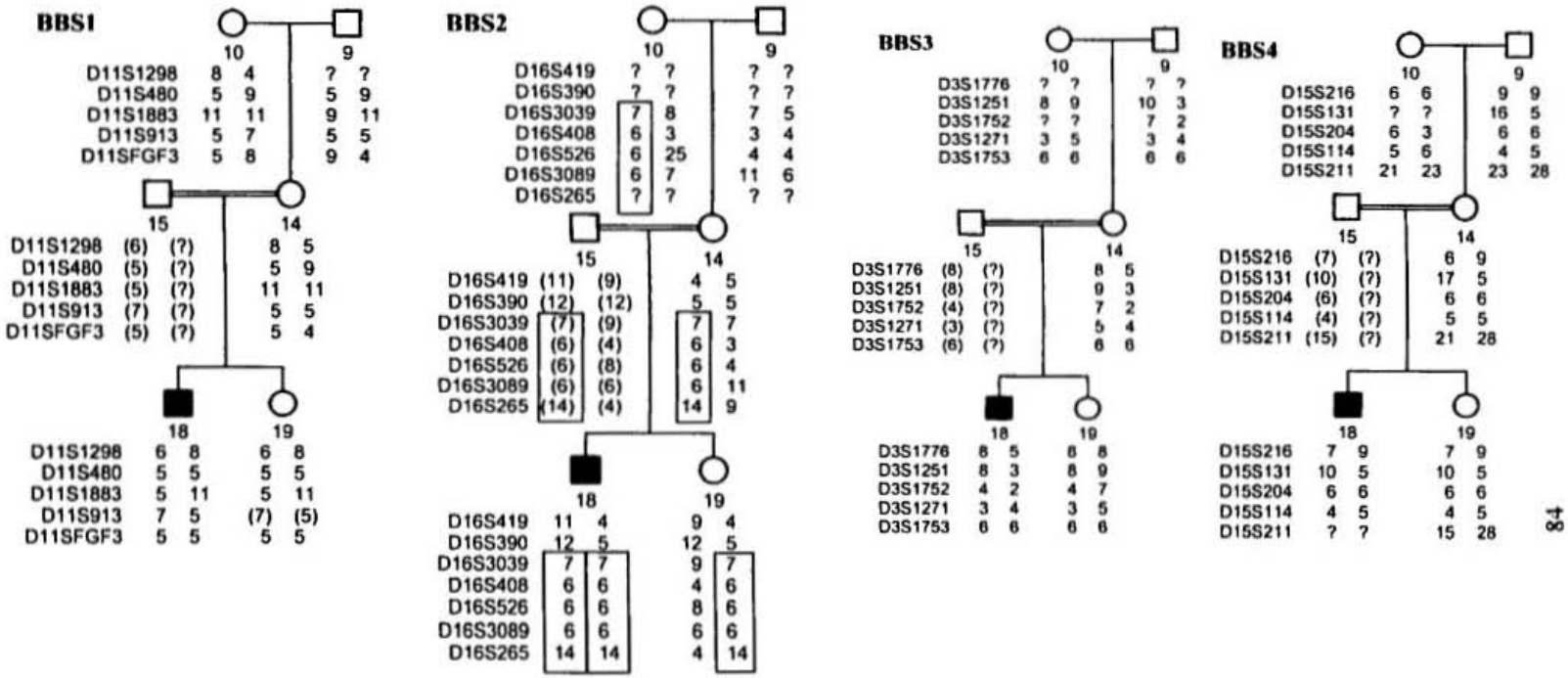


Fig 3.5 **Markers spanning the four known BBS loci in the consanguineous family B14. A region of HBD (boxed) is present in the *BBS2* interval and all other loci were excluded. Only the core pedigree is shown. Alleles in parentheses were inferred. '?' indicates no information was obtained or inferred.**

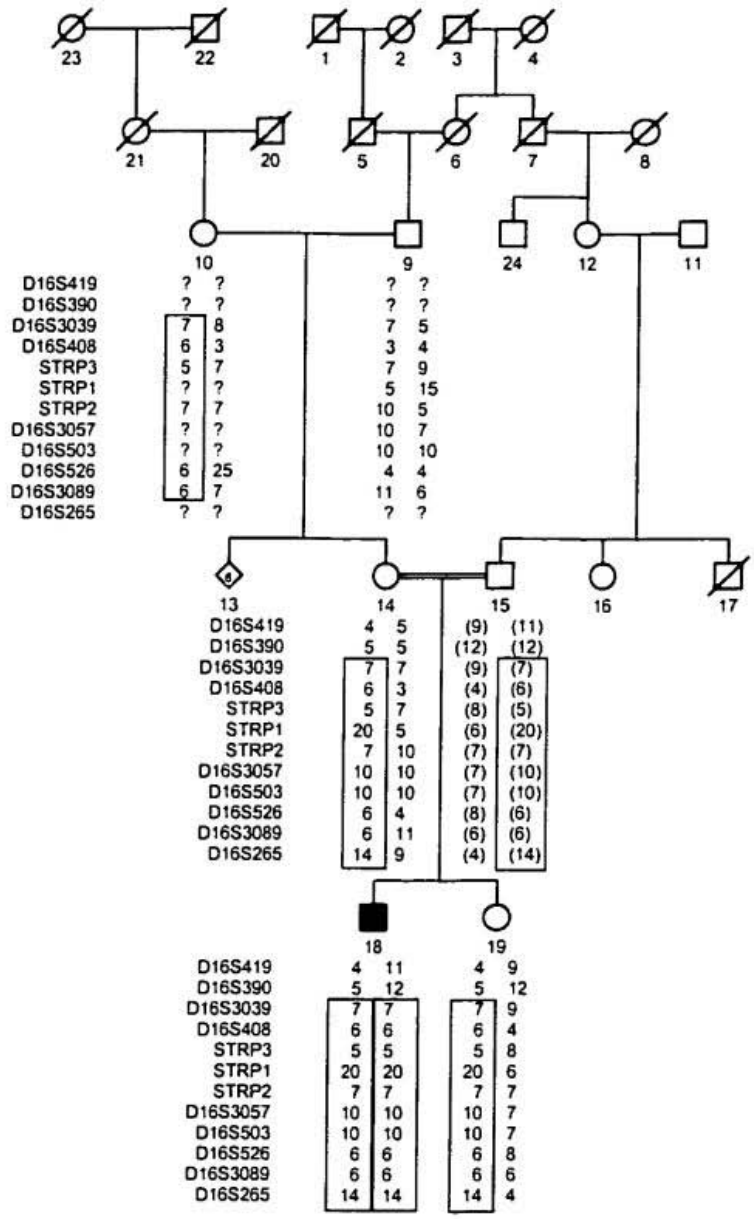


Fig 3.6 The affected person in family B14 showing HBS at the BBS2 critical interval. This region of HBS (boxed) spans ~ 12 cM between, and including, *D16S3039* to *D16S265*. Alleles in parentheses were inferred and '?' indicates no information was obtained or inferred.

outside the consanguineous loop. However, it is suspected that the affected individual received two copies of an ancestral chromosome. This family was excluded from *BBS1*, *BBS3* and *BBS4* on the basis of haplotype analysis. The affected person (PID 18) and unaffected sib (PID19) share haplotypes at *BBS1* and *BBS4*. If this kindred was a BBS3 kindred we would expect to observe HBD in this region; however, the affected individual was heterozygous within the *BBS3* critical region.

3.1.3 Family Assigned to *BBS3*

One large multiplex family, B2, with five affected individuals in two sibships, was assigned to the *BBS3* locus. All affected individuals (PIDs 14, 15, 22, 23 and 24) were HBS for a minimum of four consecutive markers at the *BBS3* locus (Fig 3.7), each with a lod score greater than 2.10 ($\theta = 0$). *BBS1*, *BBS2* and *BBS4* were all excluded on the basis of haplotype analysis and statistically significant lod scores (Table 3.1). At the *BBS1* locus, PIDs 14 and 15, in sibship A, had different contributions from one of the parents. Also, in sibship B, the affected individuals had different haplotypes. For *BBS2*, the affected sibs in sibship A had different maternal and paternal contributions, while one affected person in sibship B, PID 23, had different haplotypes than both other affected sibs. Although the affected persons in sibship A shared the same contribution from one parent, and some of the other parent's contribution at the *BBS4* critical interval (due to a

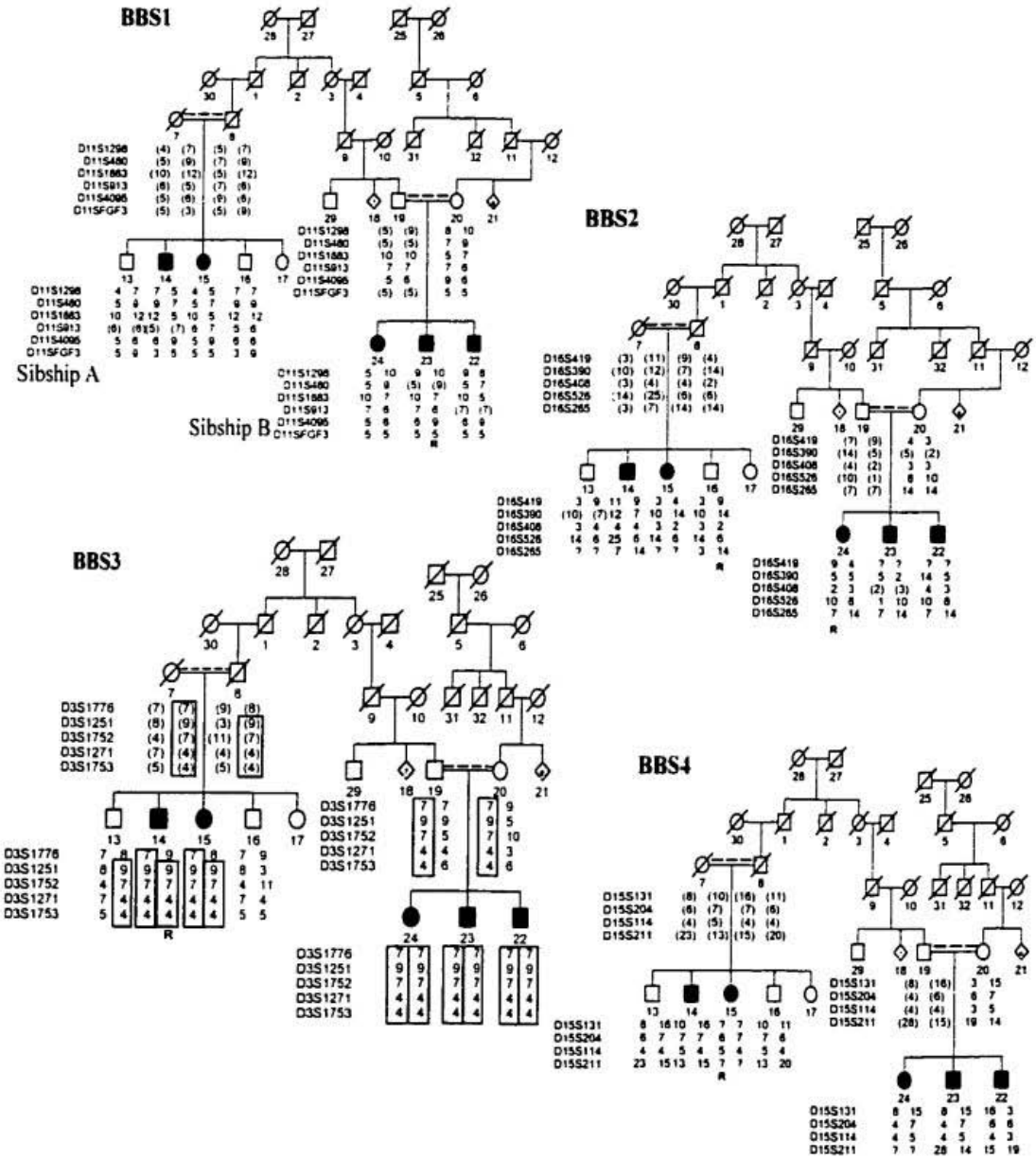


Fig 3.7 Family B2 with markers spanning the four known BBS loci. HBS (boxed) is observed at the *BBS3* locus and an ancestral haplotype is evident. All other loci were excluded. 'R' represents a recombination event. Alleles in parentheses were inferred. '?' indicates no information was obtained.

recombination in PID 15), there was no region of homozygosity. In addition, the haplotypes were different than those in the affected sibs in sibship B. Also, in sibship B, PID 22 had both maternal and paternal contributions which were different than in the other two affected siblings in the sibship. For these reasons kindred B2 was considered a BBS3 family.

3.1.4 Exclusion of Families to Known BBS Loci

Remarkably, 6 of the 17 families (B3, B4, B5, B6, B9 and B13) were excluded from all four known BBS loci, suggesting the presence of at least one other BBS locus (all haplotype data is shown in **Appendix A** for the following analyses). In the consanguineous kindred B3, markers at the four loci generated negative lod scores (Table 3.1), with none of the markers analyzed being homozygous (as would be expected in a second cousin marriage at a linked locus). Though there was no sharing of haplotypes between the affected sib and his unaffected brother at the four known BBS loci, all loci were excluded by haplotype analysis due to the lack of homozygosity in the affected person in this consanguineous family.

In kindred B4, at the *BBS1* locus, both affected individuals had different paternal contributions and one affected sib (PID 13) had the same haplotype as two unaffected siblings (PIDs 11 and 12). At the *BBS2* locus, both affected sibs had the same haplotypes

as one unaffected sib (PID 12) and the same was observed at *BBS3* and *BBS4*. However, at the *BBS3* locus, one affected sib (PID 10) had a recombination pter to the most centromeric marker, but she shared the remaining parental genetic contributions with PID 9. At the four BBS loci examined, negative lod scores ($\theta = 0$) for appropriate markers were obtained, and at all loci the haplotype analysis suggested exclusion (Table 3.1).

The affected individual (PID 14) in family B5 shared identical haplotypes with four unaffected siblings (PIDs 9, 10, 15 and 16) at the *BBS1* locus, thus excluding this locus. The *BBS3* locus was also ruled out because the affected person shared complete haplotypes with an unaffected sibling (PID 10). At the *BBS2* locus, the affected sib had a recombination at the pter portion of the maternally inherited chromosome. However, pter to this he shared his haplotypes with one unaffected sib (PID 13), and qter to the recombination he shared his haplotypes with two unaffected sibs (PIDs 9 and 15). A similar recombination had occurred at the *BBS4* locus, but the pter portion of the haplotypes were shared with two unaffected sibs (PIDs 13 and 16), and the qter portion with one unaffected sib (PID 9). These loci were excluded because of this compound haplotype sharing in combination with negative lod scores (Table 3.1). There is a remote possibility that these recombinations occurred in a critical area of the respective BBS genes, but such an occurrence seemed too remote to weigh heavily in the analyses.

Family B6, with two affected siblings (PIDs 10 and 11), was excluded from *BBS1* because both patients had different paternal contributions. Also, one affected person (PID 11) shared haplotypes with an unaffected sib (PID 12). Different paternal

contributions were inherited by the affected sibs at the *BBS2* locus, excluding this locus. Additionally, one affected individual (PID 10) had the same haplotypes as two unaffected sibs (PID 9 and 14). At the *BBS3* locus, both affected sibs shared identical haplotypes with an unaffected sib (PID 15). Paternal contributions were also different in the *BBS4* critical region in the affected sibs; one affected person (PID 10) shared haplotypes with an unaffected sib (PID 15); and the other affected sib (PID 11) shared haplotypes with two unaffected sibs (PIDs 9 and 14). Thus, the *BBS4* locus was excluded in this family. For three of the four loci examined, statistically significant negative lod scores ($\theta = 0$) for appropriate markers were obtained (Table 3.1).

In the large multiplex B9 kindred, *BBS1* was excluded because the two affected individuals in one sibship (PIDs 28 and 29) had different paternal contributions. Also, one affected person (PID 28) shared haplotypes with an unaffected sibling (PID 26), while the other unaffected person (PID 29) shared haplotypes with unaffected sibling PID 27. In the other sibship tested, the two affected sibs (PIDs 21 and 22) had different genetic contributions from their mother, and two of the three chromosomes segregating in these two patients were distinct from those in the affected sibs of the other sibship. For *BBS2*, only one sibship was typed. However, this locus was excluded because both BBS patients (PIDs 28 and 29) had different maternal contributions. Also, PID 28 shared haplotypes with an unaffected sib (PID 26). For *BBS3*, one affected person (PID 28) had a recombination in his maternally inherited chromosome, creating a combination of haplotypes that were unique to the two affected individuals in this sibship. However, the

other sibship, with two BBS patients, did not share any chromosomes with these patients. Also, both affected sibs, PIDs 21 and 22, had different maternal contributions. Also, PID 21 shared haplotypes with an unaffected sib (PID 34). For these reasons this locus was excluded based on haplotype analysis. At the *BBS4* locus, both affected siblings in one sibship (PIDs 28 and 29) had different genetic contributions from both parents. As well, PID 29 shared haplotypes with two unaffected sibs (PIDs 25 and 27). Using the limited results obtained in the other sibship, it seemed that the affected brothers (PIDs 21 and 22) have different paternal contributions. Regardless, this locus can be excluded solely on the analysis of the fully informative sibship. At all loci examined, statistically negative lod scores ($\theta = 0$) for appropriate markers were obtained (Table 3.1).

Finally, a consanguineous family, B13, provided negative lod scores and haplotype analysis results which indicated exclusion of the four known BBS loci (Table 3.1). At the *BBS1* locus, the two affected individuals (PIDs 12 and 13) received different paternal contributions and one (PID 13) shared haplotypes (except for the pter most marker - *D11S1298*) with an unaffected sib (PID 17). At the *BBS2* locus, both affected sibs shared haplotypes with an unaffected sibling (PID 18). At the *BBS3* locus, the BBS patients received different paternal contributions, and one affected sib (PID 13) had the same haplotype as three unaffected sibs (PIDs 15, 17 and 18). Also, at the *BBS4* locus, the affected sibs had different paternal contributions; one affected sib (PID 13) shared haplotypes with unaffected sibs (PIDs 15 and 18), and the other affected individual shared haplotypes with an unaffected sib (PID 17).

Three of these six kindreds (B3, B9 and B13) are known to be consanguineous, and family B9 has five affected individuals, in three lineages, that are interrelated by three consanguineous and two marriage loops. Both families B9 and B13 were considered good candidates to conduct a genome-wide screen on, to identify other BBS loci.

3.1.5 Families whose BBS Status could not be Assigned

It was not possible to assign six families (B1, B7, B11, B12, B15 and B16) with confidence to a particular locus or exclude them from the four known BBS loci (all haplotype data is shown in **Appendix A** for the following analyses). Families B1, B11 and B16 contain two affected individuals with no unaffected siblings. These families could only be excluded from a particular locus if the two affected individuals received different parental chromosomes. This was the case for: family B1 at the *BBS1* and *BBS3* loci; family B11 at the *BBS1* locus; and family B16 at the *BBS1*, *BBS2* and *BBS4* loci (Table 3.1).

In family B12, there are only two children, one affected and one unaffected (the other affected individual, in a previous generation, was unavailable). Due to the pedigree structure, only *BBS3* could be excluded, since the affected and unaffected individuals shared haplotypes.

Kindred B7 was excluded from both *BBS3* and *BBS4* on the basis of haplotype

analysis (Fig 3.8). At the *BBS3* locus, the affected siblings (PIDs 9 and 15) had different paternal contributions. As well, PID 9 shared haplotypes with one unaffected sib (PID 16), and PID 15 shared haplotypes with two unaffected sibs (PIDs 13 and 14). At the *BBS4* locus, both affected siblings had different paternal contributions. Also, PID 15 shared haplotypes with two unaffected sibs (PIDs 13 and 17). At the *BBS2* locus, the affected individuals in family B7 shared haplotypes and a region of homozygosity. Linkage at this locus was supported by a positive lod score of 1.07 ($\theta = 0$) at *D16S265*. In the region of *BBS1*, the two affected individuals shared the same non-homozygous haplotypes, which was not observed in four unaffected siblings. As a result, a positive lod score of 0.96 ($\theta = 0$) at *D11S1883* was generated. Thus, there was evidence to support the assignment of family B7 to both the *BBS1* and *BBS2* loci.

Family B15 is a suspected consanguineous kindred, and the only affected individual (PID 15) was HBS at the *BBS1* locus. The *BBS2* locus could not be excluded because the affected individual contained a unique pair of haplotypes among the offspring. Family B15 was excluded from the *BBS3* locus since the affected individual shared haplotypes with two unaffected siblings (PIDs 12 and 17). The *BBS4* locus was also not excluded, as the affected individual had a unique pair of haplotypes pter to *D15S114*. Thus, there was evidence to support the assignment of family B15 to *BBS1*, *BBS2* and *BBS4*. However, because HBS was observed at the *BBS1* locus in this suspected consanguineous kindred, *BBS1* was considered the most likely candidate to be causing the disease.

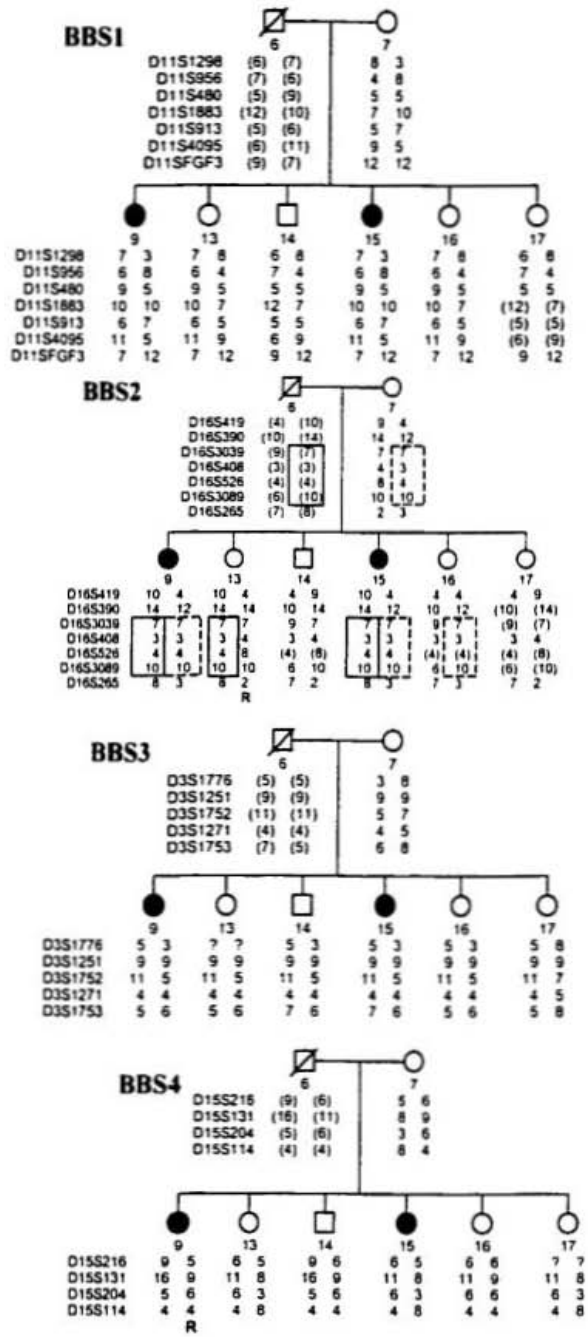


Fig 3.8 Markers for the four known BBS loci in family B7. There is a region of HBS (within boxed region) in the *BBS2* critical region, however *BBS1* could not be ruled out as there is HS between affected individuals.

Based on 11 families with unambiguous locus assignments to this point, it was calculated that 27% (3) of the families are linked to *BBS1*, 9% (1) to *BBS2* and 9% (1) to *BBS3*, and remarkably, 55% (6) of these families are linked to a yet unknown locus or loci.

3.2 The BBS3 Critical Region was Reduced with Family B3

In an attempt to reduce the critical interval of *BBS3*, which was originally 11 cM (Sheffield *et al.*, 1994), additional microsatellite markers were typed within this region using family B2, the only putative *BBS3* family in Newfoundland. Two-point linkage analysis provided strong support for linkage between BBS and chromosome 3 (*BBS3*) and the exclusion of linkage to *BBS1*, *BBS2* and *BBS4* (Table 3.1). Additional haplotype analysis with microsatellite markers spanning the *BBS3* critical region showed that all BBS patients were homozygous for a portion of the *BBS3* critical region (Fig 3.9). Three affected sibs (PIDs 22, 23 and 24) were homozygous for all typed markers. They inherited two copies of the *BBS3* haplotype, pter-7-5-19-7-9-7-4-4-qter. Affected persons PIDs 14 and 15 were homozygous for the same alleles as their affected cousins at marker loci in the qter portion of the *BBS3* critical region. The finding of identical, homozygous haplotypes in the affected relatives suggested that their parents share a common ancestry. The smallest region of homozygosity in an affected relative was found in person PID 14.

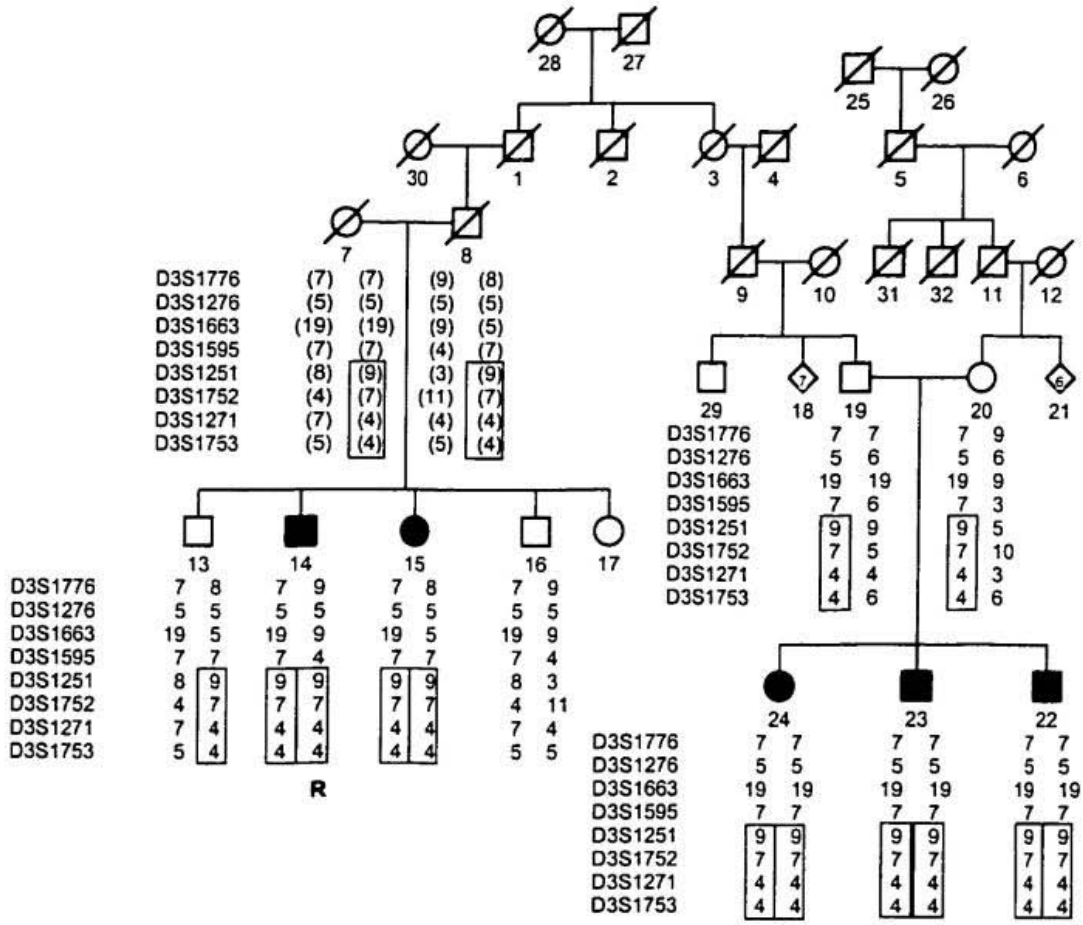


Fig 3.9 Family B2 with markers spanning the 7 cM *BBS3* critical interval. HBS is observed between *D3S1251* and *D3S1753* in all affected individuals (boxed), reducing the critical interval to 6 cM. Haplotypes for PIDs 7 and 8 were not distinguishable therefore were arbitrarily assigned. 'R' represents a recombination event. Alleles in parentheses were inferred. '?' indicates no information was obtained or inferred.

This region includes the microsatellite markers *D3S1251*, *D3S1752*, *D3S1271* and *D3S1753*, corresponding to the pter-9-7-4-4-qter haplotype. This interval is 6 cM in size (Fig 3.10). If we assume that the BBS patients in this family were HBD for the critical region, then the *BBS3* gene is located on the qter side of *D3S1595*, pter to *D3S1753*, the delimiting marker in the original *BBS3* linkage paper (Sheffield *et al.*, 1994). Two-point linkage analysis provided a lod score of 2.86 ($\theta = 0$) at *D3S1752* (Table 3.1). This was not a statistically significant result, but it did strongly suggest linkage of family B2 to the *BBS3* locus.

3.3 A Founder Effect Reduced the *BBS1* Critical Region to 1 cM

In an initial study by Leppert *et al.* (1994), the putative *BBS1* gene was tightly linked to two loci on chromosome 11q13: the gene for human muscle phosphorylase (*PYGM*) and *D11S913*. *BBS1* was localized to a 13 cM interval between *D11S1298* and *FGF3* (Fig 3.1). A more precise genetic and physical map of *BBS1* was required if this gene was to be positionally cloned.

Of the 17 Newfoundland families, three (B8, B10 and B19) were assigned to the *BBS1* locus and three were not excluded (B7, B12 and B15) because they yielded positive lod scores and haplotypes consistent with linkage to *BBS1*. However, only family B10 was excluded from all other known loci (Table 3.1). Of these six families, parental

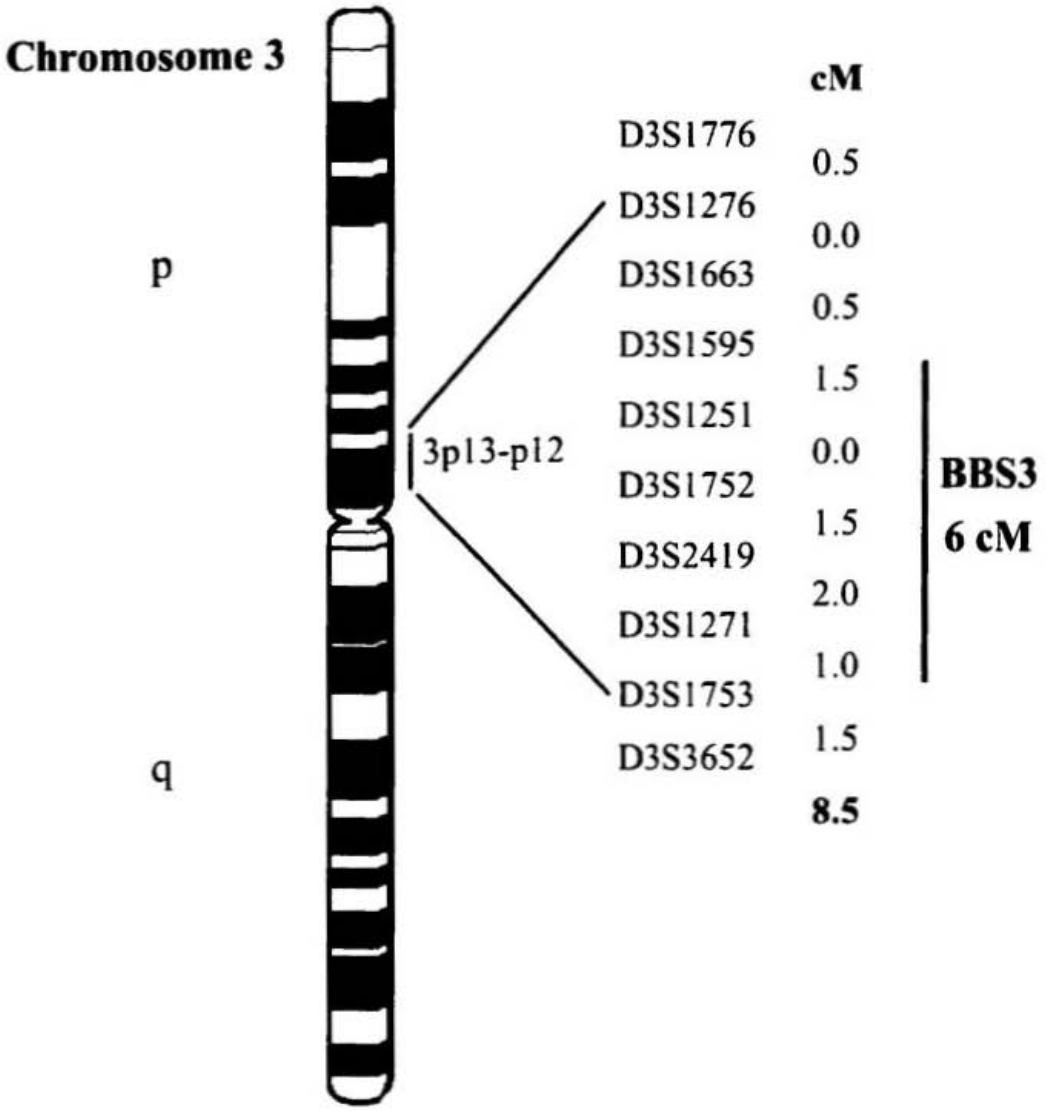


Fig 3.10 Location of microsatellite markers spanning the *BBS3* critical interval on chromsome 3. This region was narrowed from 9 cM to 6 cM using recombinants in kindred B2. Marker order and distances were obtained from the Marshfield sex-averaged linkage maps and Genethon linkage maps (GDB).

consanguinity was documented in families B8 and B19, and suspected in families B10, B12 and B15 on the basis of progenitors with the same surname originating from the same community (Fig 2.1). Extensive genotyping with markers mapped to the *BBS1* critical region was performed in the six families, representing eight BBS patients and 44 first- and second-degree relatives.

Five of the six possible BBS1 families lived along the south and southwest coasts of Newfoundland (Fig 3.11). Obligate recombinations involving the disease haplotypes (DHs) were detected in several families and used to refine the *BBS1* interval. In family B10, an unaffected individual, PID 14, inherited a non-recombinant DH from his father and a recombinant from his mother (Fig 3.12). The presence of two DHs for the centromeric portion of the *BBS1* critical region (*D11S1298* to *D11S1883*) in an unaffected individual suggested that marker *D11S1883* was the new centromeric boundary for *BBS1*. Similarly, a recombinant paternal haplotype inherited by an unaffected sib in family B8 (PID 12) suggested that *BBS1* was located centromeric to *FGF3*. Intrafamilial recombinations within DHs reduced the *BBS1* interval from a 13 cM region to approximately a 7.5 cM interval between *D11S1883* and *FGF3* (Fig 3.13).

Extensive genotyping at the *BBS1* locus focused on markers within the new *BBS1* interval, between *D11S1883* and *FGF3*. At this point the B7 kindred was taken out of the analysis because of the lack of template DNA available. The distribution of alleles at 14 polymorphic loci in disease and normal chromosomes is shown in Table 3.2. Linkage disequilibrium (LD) between marker alleles on DHs was observed across the families,

Fig 3.11 Locations of the BBS1 families on the island of Newfoundland.

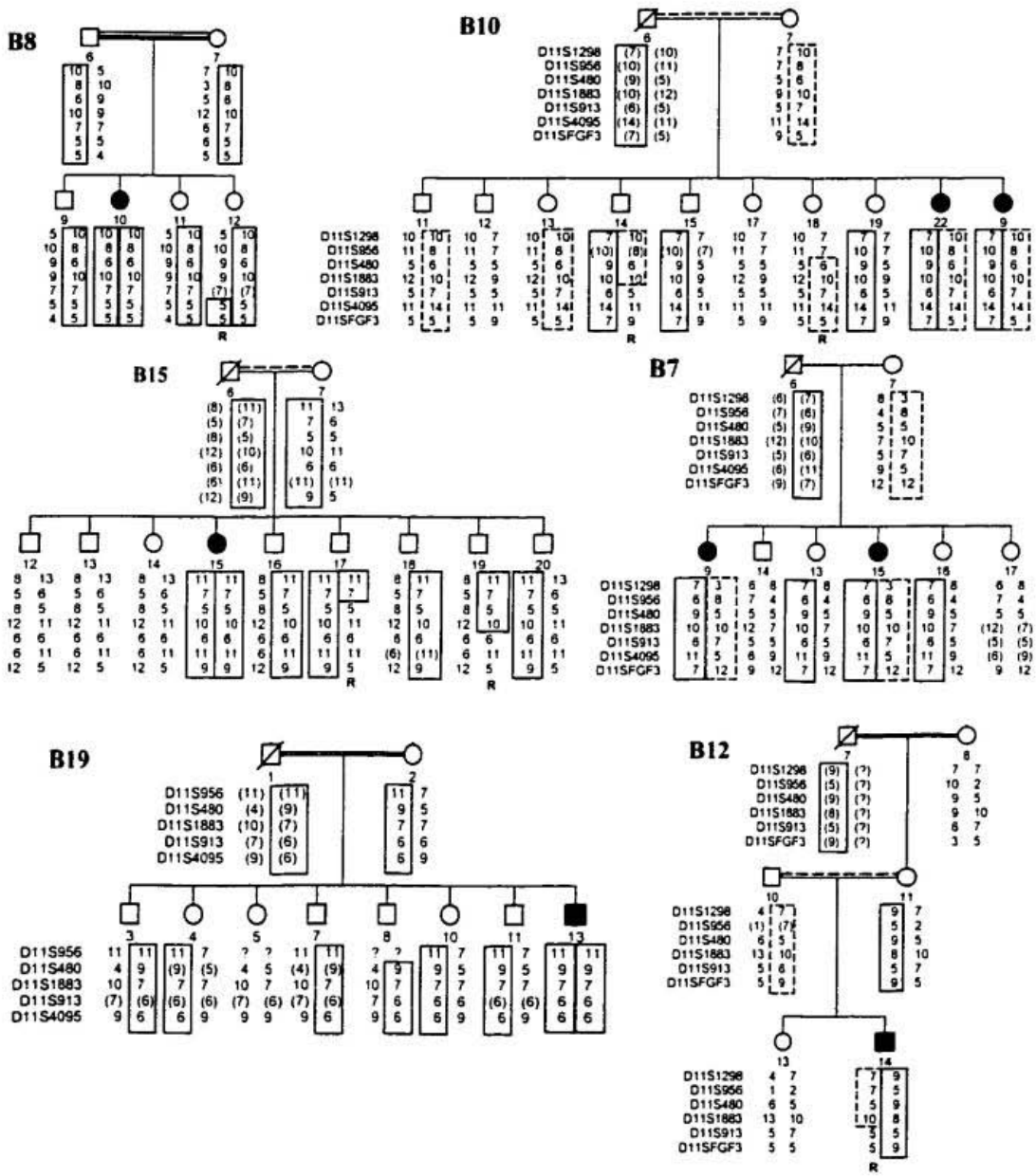


Fig 3.12 Three families with linkage to *BBS1* (B8, B19 and B10) and three unassigned families (B7, B12 and B15) haplotyped for six markers spanning the *BBS1* critical interval. Only core pedigrees shown. Boxed haplotypes (solid and dashed lines) indicate DHs. 'R' indicates haplotype is recombinant.

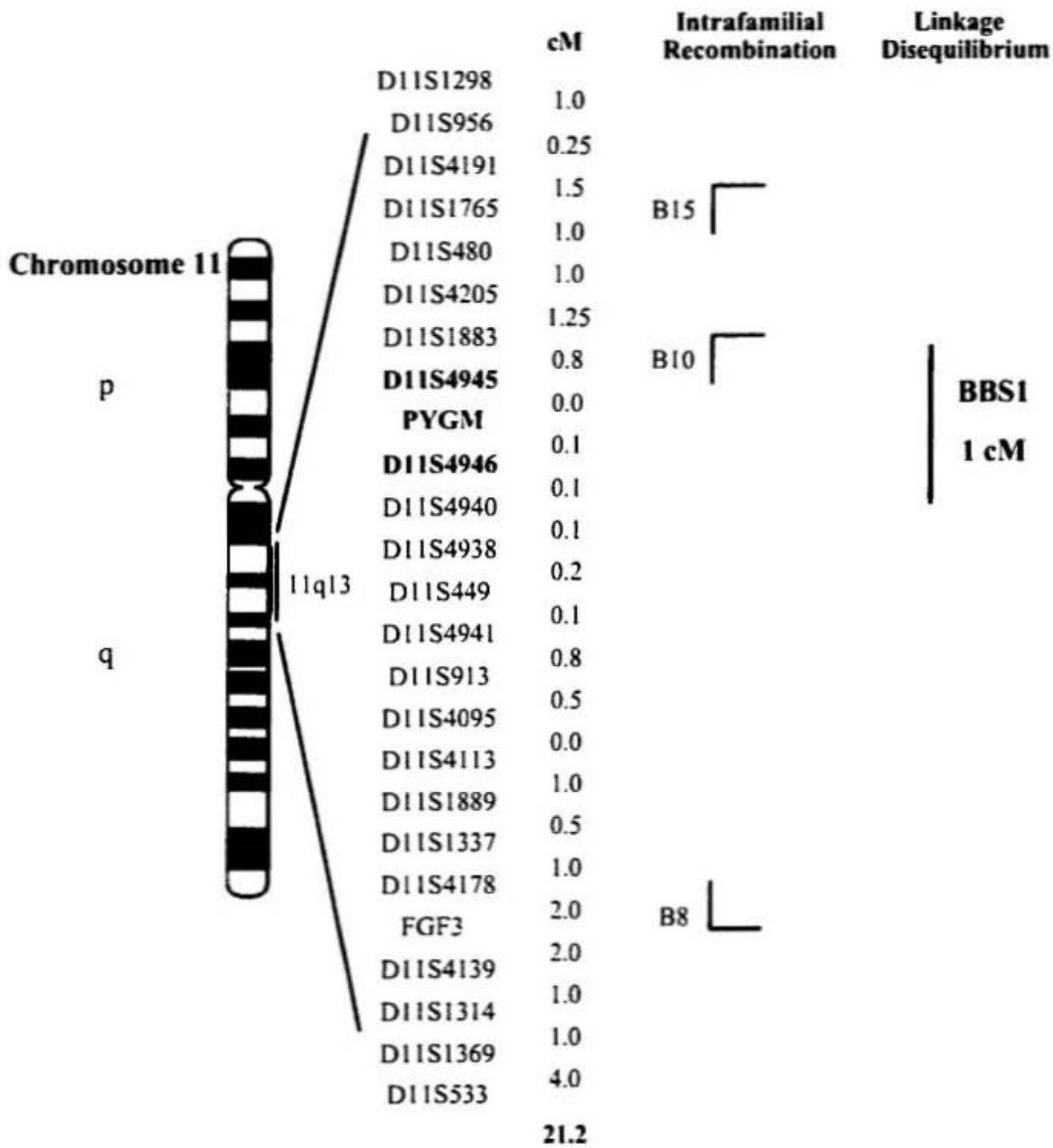


Fig 3.13 Refinement of the BBS1 interval by recombinational and LD mapping . The markers and their positions were selected from the map of the MEN1 region on 11q13 (Manickam et al., 1997; Sixth International SCW 11 Workshop 1998) and the Genethon and Marshfield sex-averaged linkage maps. Markers in bold were homozygous in all affected individuals.

Table 3.2 LD at the BBS1 locus on 11q13 among five BBS families.

Marker	DH for Kindred										Normal Haplotype for Kindred									
	B8		B10		B12		B15		B19		B8		B10		B12		B15		B19	
	p	m	p	m	p	m	p	m	p	m	p	m	p	m	p	m	p	m	p	m
D11S1298	10	10	7	10	7	9	11	11	—	—	5	7	10	7	4	7	8	13	—	—
D11S956	8	8	10	8	7	5	7	7	11	11	10	3	11	7	1	2	5	6	11	7
D11S480	6	6	9	6	5	9	5	5	9	9	9	5	5	5	6	5	8	5	4	5
D11S4205	4	4	3	4	4	4	4	4	4	4	3	4	4	5	4	4	3	4	4	4
D11S1883	10	10	10	10	10	8	10	10	7	7	9	12	12	9	13	10	12	11	10	7
D11S4945	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	4	10	10	9	9
PYGM	8	8	8	8	8	8	8	8	8	8	8	8	6	8	2	4	4	8	4	8
D11S4946	5	5	5	5	5	5	5	5	5	5	4	5	5	4	4	1	1	5	3	3
D11S4940	5	5	7	5	7	5	7	7	3	3	5	5	5	5	5	3	5	7	7	5
D11S4938	5	5	3	5	5	3	5	5	5	5	9	3	5	3	5	9	5	5	—	9
D11S449	7	7	3	7	3	6	5	5	3	3	5	3	4	3	4	2	5	5	3	4
D11S4941	9	9	11	9	2	2	4	4	4	4	8	3	6	11	2	4	4	6	4	4
D11S913	7	7	6	7	6	5	6	6	6	6	7	6	5	5	5	7	6	6	7	6
FGF3	5	5	7	5	9	9	9	9	—	—	4	5	5	9	5	5	12	5	—	—

Note - Haplotypes are arranged with the paternal haplotype (p) on the left and the maternal haplotype (m) on the right. Specific alleles associated with disease chromosomes are in red.

specifically at *D11S4205*, *D11S1883*, *D11S4945*, *PYGM* and *D11S4946*.

All disease chromosomes segregating in *BBS1* families contained the pter-9-8-5-qter subhaplotype at *D11S4945*, *PYGM* and *D11S4946*, respectively. The evidence suggested that the pter-9-8-5-qter subhaplotype represents either the remnants of a founder *BBS1* chromosome imported from England, or the background haplotype that sustained a *BBS1* mutation *de novo* in the germline of a single English settler.

LD mapping supported a position for the *BBS1* gene within a 1 cM region between markers *D11S1883* and *D11S4940*, surrounding the *PYGM* locus. The *D11S1883* boundary is also supported on the basis of intrafamilial recombination (Fig 3.13). This 1 cM genetic interval represents a physical distance of about 2 Mb (NCBI, Map Viewer; http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/maps.cgi?org=hum&chr=11) within a region of the genome that is gene rich.

3.4 A Fifth BBS Locus on Chromosome 2q31

In the initial genetic survey of BBS in Newfoundland (**Chapter 3.1.4**) there was evidence for at least one other BBS locus, as six kindreds were confidently excluded from the four known BBS loci. As well, there were two additional population surveys which found several unlinked BBS families, providing convincing evidence for at least a fifth BBS locus (Beales *et al.*,1997; Bruford *et al.*,1997).

Family B9, the largest of the six Newfoundland kindreds excluded from the four known BBS loci, had five affected members who were the products of three consanguineous unions interrelated through two founding couples. Employing this family, a genome-wide scan of pooled DNA samples was performed with microsatellite markers. Two control pools of DNA from four living parents and 11 unaffected siblings, as well as a test pool of DNA from the four surviving patients, were amplified. Of the first 322 markers successfully amplified, six showed a reduction in the number of alleles in the test pool, compared with the control pools. Subsequent genotyping of these markers on the extended family proved that they were not linked to BBS, as HBD was not evident, resulting in a false positive rate of 1.9%. However, the 323rd marker, *D2S1353*, showed a 4:1 allele shift, from the control pools to the test pool. Genotyping of *D2S1353* on the pedigree showed it to be exclusively homozygous in patients with BBS. At this time an archival sample of one affected individual (PID 24) became available and was added to the analysis. Two-point linkage analysis showed significant linkage [5.59 (θ = 0)] between BBS and *D2S1353*, with no recombination (Table 3.3). Genotyping of markers flanking *D2S1353* confirmed linkage to 2q31 and showed an ancestral haplotype that was HBD in all affected relatives (Fig 3.14). This extended haplotype is flanked by *D2S156/D2S1353* and *D2S1238*, a distance of approximately 13 cM (Fig 3.15), identified by the observation of two key recombinants in two unaffected parents (PIDs 15 and 12). A recombination occurred in PID 12 between *D2S335* and *D2S1238*, that was inherited by an affected individual (PID 24) who was HBD between and including *D2S2241* and

Table 3.3 Two-point lod scores between the BBS trait and 2q31 markers in family B9.

	Two-point lod scores at θ =					
Marker*	**0.00**	**0.01**	**0.05**	**0.10**	**0.20**	**0.30**
D2S442	-2.96	-1.40	-1.20	-0.69	-0.22	-0.05
D2S1399	-4.36	-0.49	0.91	1.26	1.31	0.67
D2S1353	5.59	5.48	5.00	4.40	3.17	1.94
D2S124	3.97	3.92	3.62	3.19	2.22	1.28
D2S2330	4.99	4.88	4.42	3.85	2.72	1.65
D2S1776	4.61	4.50	4.05	3.48	2.33	1.25
D2S1391	0.84	2.08	2.65	2.62	2.05	1.28

* Listed according to physical order (pter-qter) on chromosome 2q31.

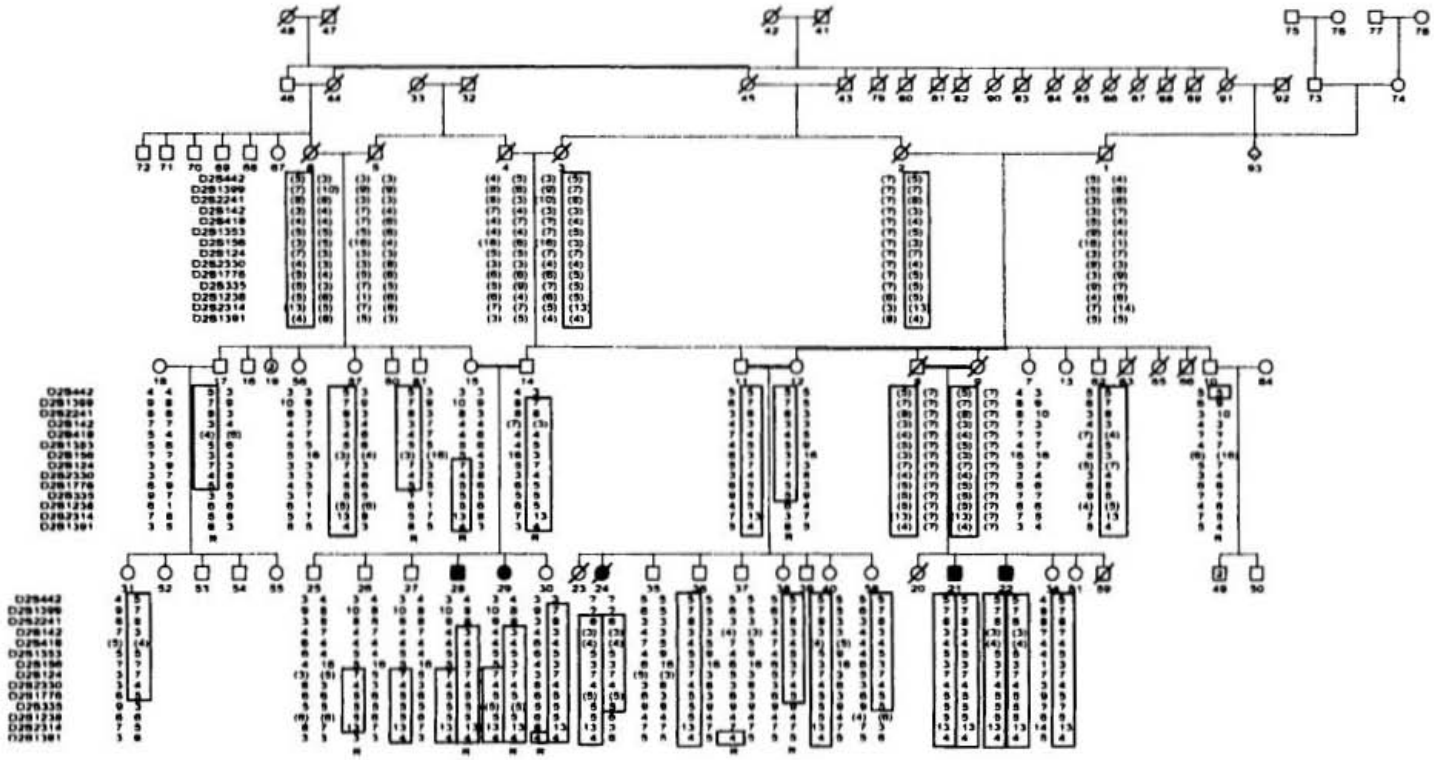


Fig 3.14 Cosegregation of BBS and an ancestral haplotype on chromosome 2q31 in kindred B9. The boxed haplotype is the DH. "R" indicates the haplotype was a recombinant. Alleles in parentheses were inferred. "?" indicates no information was obtained or inferred.

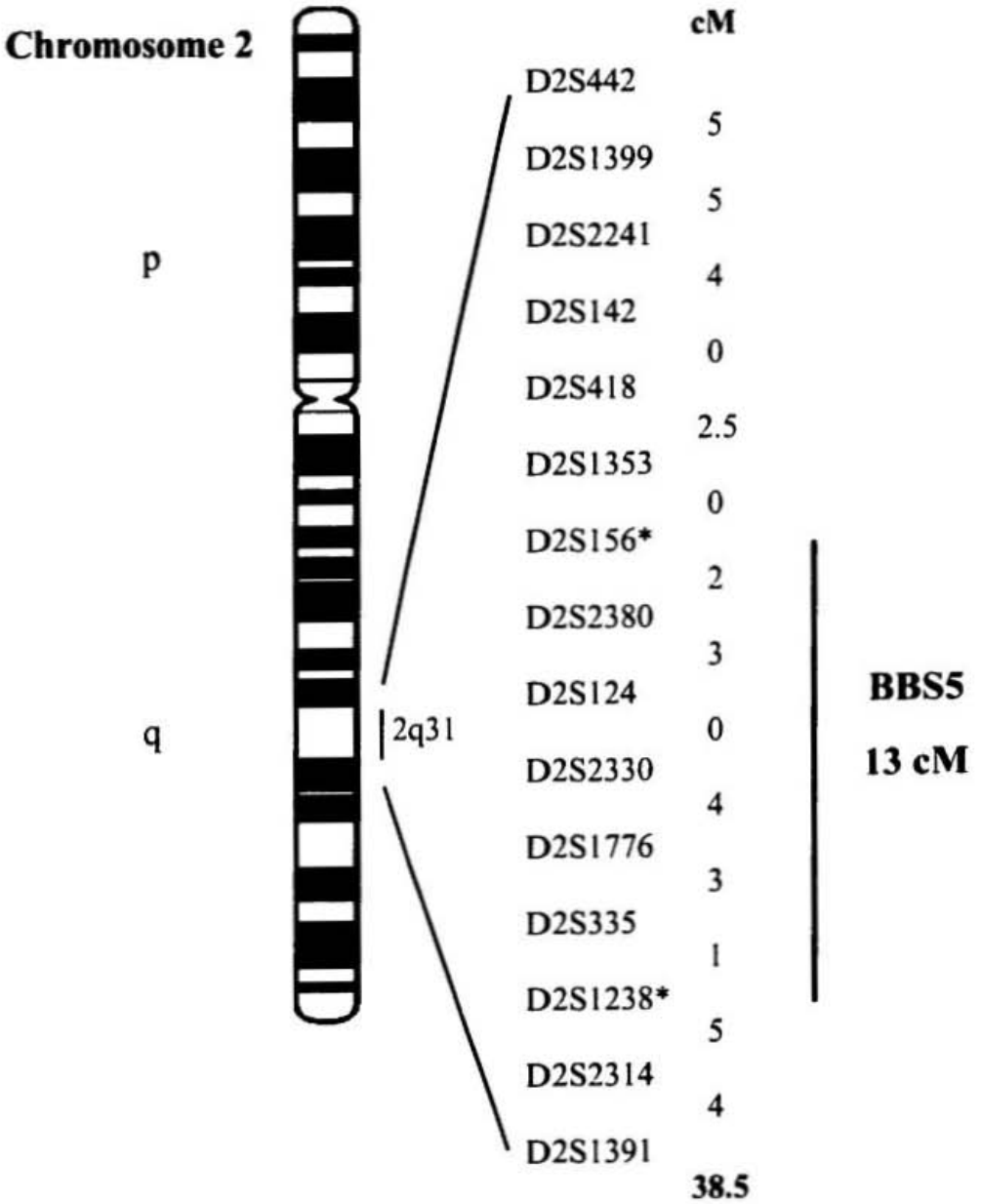


Fig 3.15 Location of markers spanning the *BBS5* interval on 2q31. By haplotype analysis on family B9 a critical region of 13 cM was evident. Marker distances and positions were obtained from Marshfield sex averaged and Genethon linkage maps.

D2S335. The other critical recombination, occurred in PID 15, between *D2S156/D2S1353* and *D2S124*, inherited by two affected offspring (PIDs 28 and 29), who had a region of HBD between and including *D2S124* and *D2S1391*. These results provided strong evidence for a fifth BBS locus (*BBS5*) on 2q31.

3.4.1 Analysis of the Remaining Unlinked BBS Families at the *BBS5* Locus

Although family B9 was geographically isolated from the remaining families excluded from *BBS1-4* (B3, B4, B5, B6 and B13; Fig 2.2), the *BBS5* locus was still considered a good candidate locus for these kindreds. Therefore, the flanking markers and those within the critical region of *BBS5* were tested on these families as well as on families which could not be assigned exclusively to any of the original BBS loci (B1, B7, B11, B16). Also, the putative *BBS2* kindred (B14) was tested to ensure it could be excluded from the *BBS5* locus, in order to provide greater support for its present categorization.

Both consanguineous families, B3 and B13, were excluded from *BBS5* by haplotype and linkage analyses (Table 3.4). The two affected individuals (PIDs 11 and 12) in family B3 inherited different genetic contributions from each parent within the *BBS5* critical region (Fig 3.16). Also, the affected sib (PID 12) shared haplotypes with his unaffected brother (PID 10). A lod score of -10.41 ($\theta = 0$) at *D2S2330* also supported

Table 3.4 Summary of linkage and haplotype analysis at the *BBS5* locus for families which could not be specifically assigned one of the four known BBS loci.

Kin No.	BBS5			BBS5
	Marker	LOD	HAP	
B1	D2S2330	0.60	HS	?
B3[a]	D2S2330	-10.41	X	**X**
B4	D2S2330	-9.98	X	**X**
B5	D2S2330	-1.90	NE	**NE**
B6	D2S2330	-0.11	X	**X**
B7	D2S156	-4.68	X	**X**
B11[b]	D2S2330	-2.92	X	**X**
B13[a]	D2S1353	-12.45	X	**X**
B14[a]	D2S2330	-0.33	X	**X**
B16[b]	D2S124	0.49	HS	?

[a] Confirmed consanguineous union.
[b] Suspected consanguineous relationship.

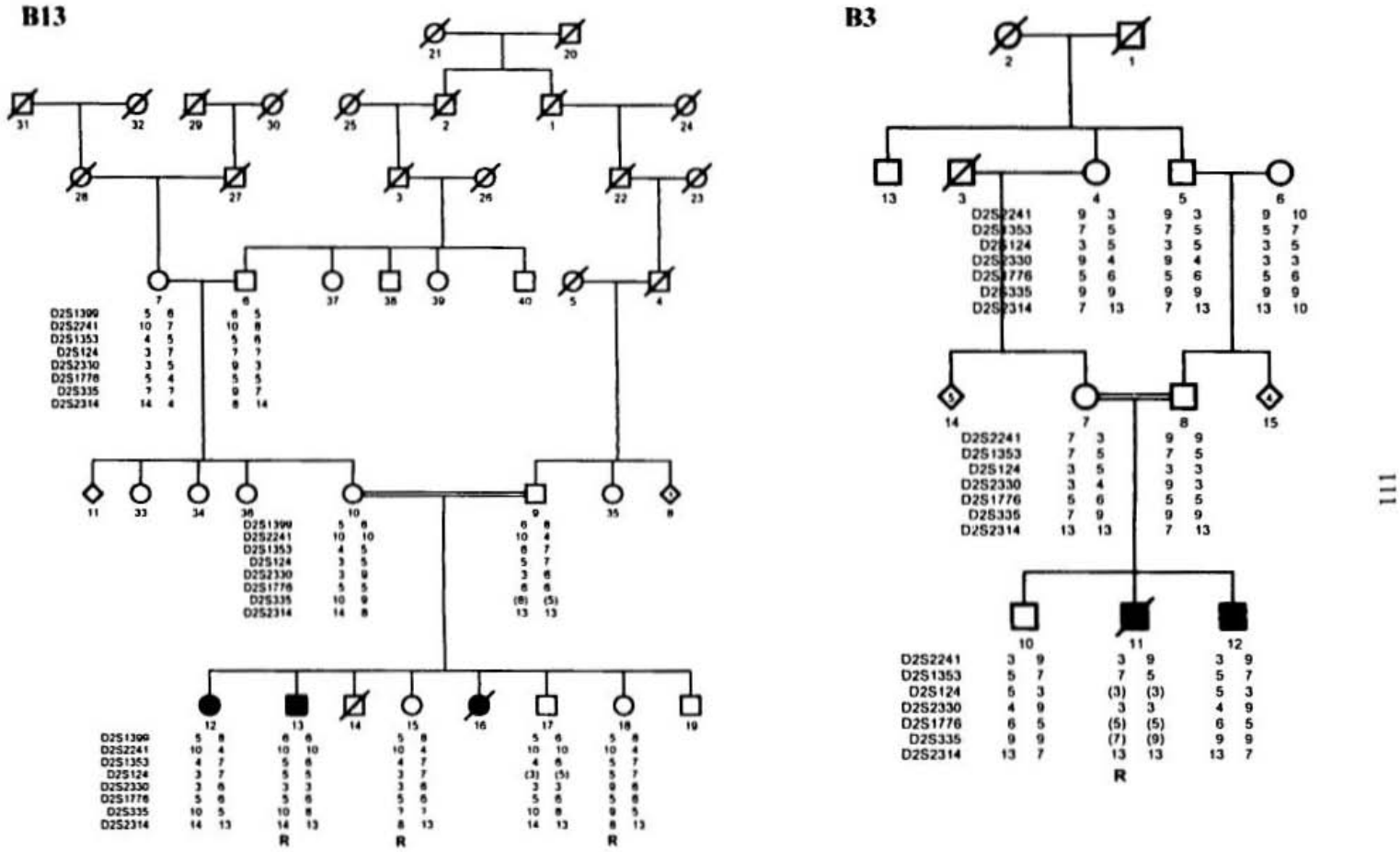


Fig 3.16 Chromosome 2q31 markers spanning the *BBS5* critical interval in families B3 and B13, which were excluded from the four known BBS loci. 'R' represents a recombination in the haplotype and alleles in parentheses were inferred. '?' indicates no information was obtained or inferred.

exclusion of family B3 from the *BBS5* locus. Two affected sibs in kindred B13, PIDs 12 and 13, inherited different chromosomal regions within the *BBS5* critical region from their father (Fig 3.16). PID 13 also shared his genetic contributions from both parents with an unaffected brother (PID 17). The lod score of -12.45 ($\theta = 0$) at *D2S2330* also supported exclusion of family B13 from *BBS5* (Table 3.4).

The non-consanguineous kindred, B4, contained two affected siblings (PIDs 10 and 13), each of which had inherited different genetic contributions from both parents (Fig 3.17). Also, PID 10 shared haplotypes with unaffected sibs, PIDs 11 and 12. This family provided a lod score of -9.98 ($\theta = 0$) at *D2S2330*, supporting the haplotype analysis (Table 3.4).

The affected individual in family B5 was recombinant for both parental chromosomes; however, the pter recombination in the maternally inherited chromosome was outside the *BBS5* critical interval (Fig 3.17). The recombination in the paternally inherited chromosome was within the critical region of *BBS5*, and the combination of parental genetic material in this region was unique in the affected individual. Therefore, this region of the *BBS5* critical interval (4 cM), qter to *D2S1776*, was not ruled out using haplotype analysis. However, the rest of this region was excluded because the affected person shared haplotypes with two unaffected sibs (PIDs 12 and 16).

Family B6, containing two affected siblings, was also excluded from the *BBS5* locus. Both affected individuals (PIDs 10 and 11) shared the same genetic contributions from the parents within the critical region; however, these haplotypes were also shared

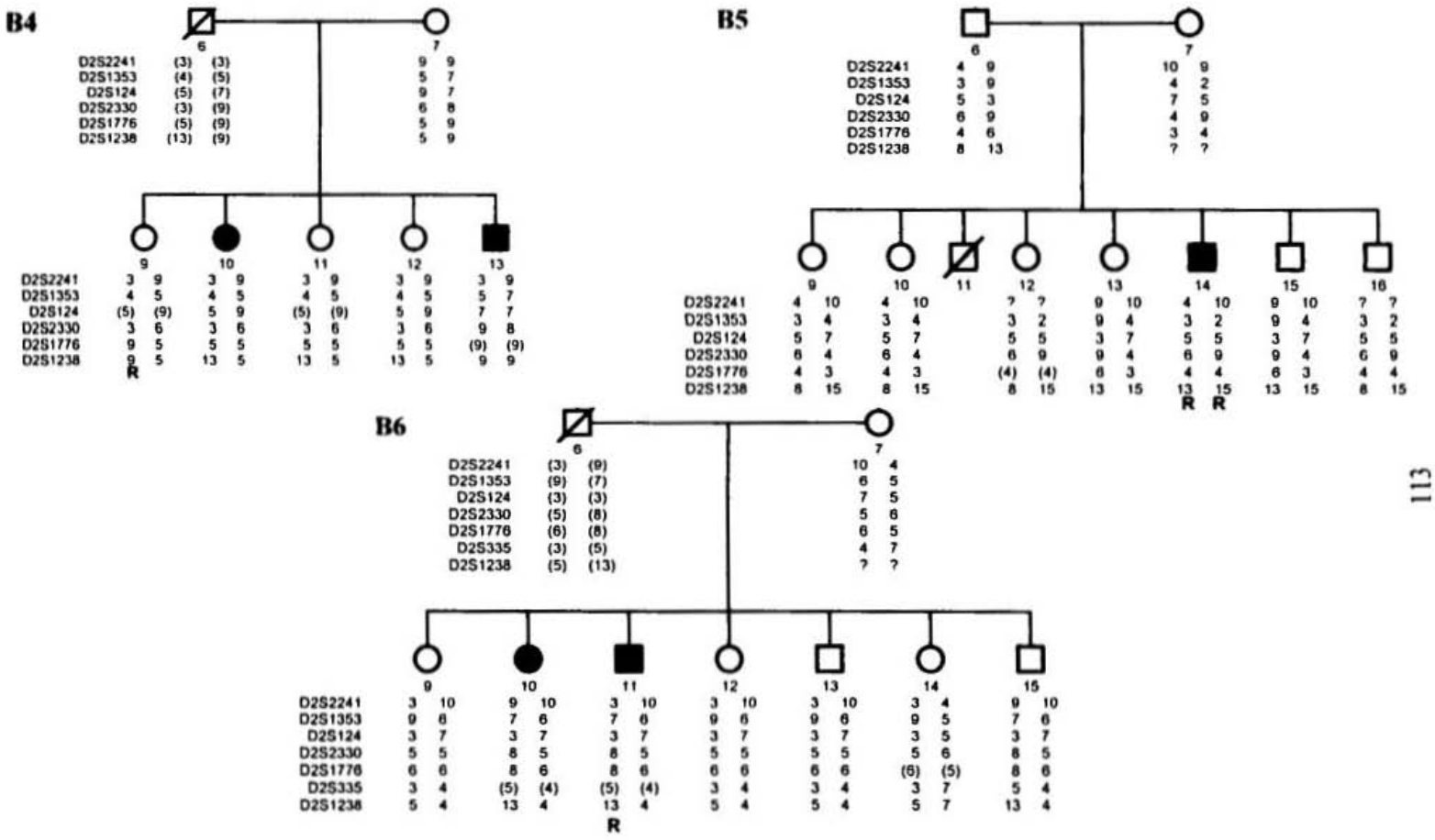


Fig 3.17 Chromosome 2q31 markers spanning the *BBS5* critical interval in families B4, B5 and B6, which were excluded from the four known BBS loci. Core pedigrees only are shown. 'R' represents a recombination in the haplotype and alleles in parentheses were inferred. '?' indicates no information was obtained or inferred.

with one unaffected sibling (PID 15; Fig 3.17). The lod score at the most informative marker in this region, *D2S2330*, was -0.11 ($\theta = 0$).

Three families (B1, B11 and B16), which were not assigned to any one of the first four BBS loci because of pedigree structure, were tested for linkage to the *BBS5* locus. For kindred B1, there was HS between the two affected brothers (PIDs 9 and 10) for the pter portion of the *BBS5* critical interval (Fig 3.18). However, a recombination in one of the patients resulted in the qter portion of the paternally inherited chromosomal region being different between the two brothers. Thus, the region distal to *D2S2330* was excluded. Within the region of HS, a lod score of 0.60 ($\theta = 0$) was calculated at *D2S2330*. Therefore, this locus could not be excluded in this family. However, in the similarly structured B11 kindred, exclusion of the *BBS5* locus was possible. Haplotype analysis demonstrated that the affected siblings (PIDs 8 and 9) inherited different paternal genetic contributions (Fig 3.18). Exclusion of the *BBS5* locus was further supported by a statistically significant lod score of -2.92 ($\theta = 0$) at *D2S2330*. Family B16, having only two affected sibs with no unaffected brothers or sisters, could not be excluded from *BBS5*, as both affected brothers seemed to have inherited the same genetic contributions from both their mother and father. Family B16 generated a lod score of 0.49 ($\theta = 0$) at *D2S124*.

Family B14, which was suggestive of linkage to *BBS2*, was tested for linkage at the *BBS5* locus. The B7 kindred was also examined at this locus. The consanguineous kindred B14 was excluded from *BBS5* due to the absence of homozygosity in this region,

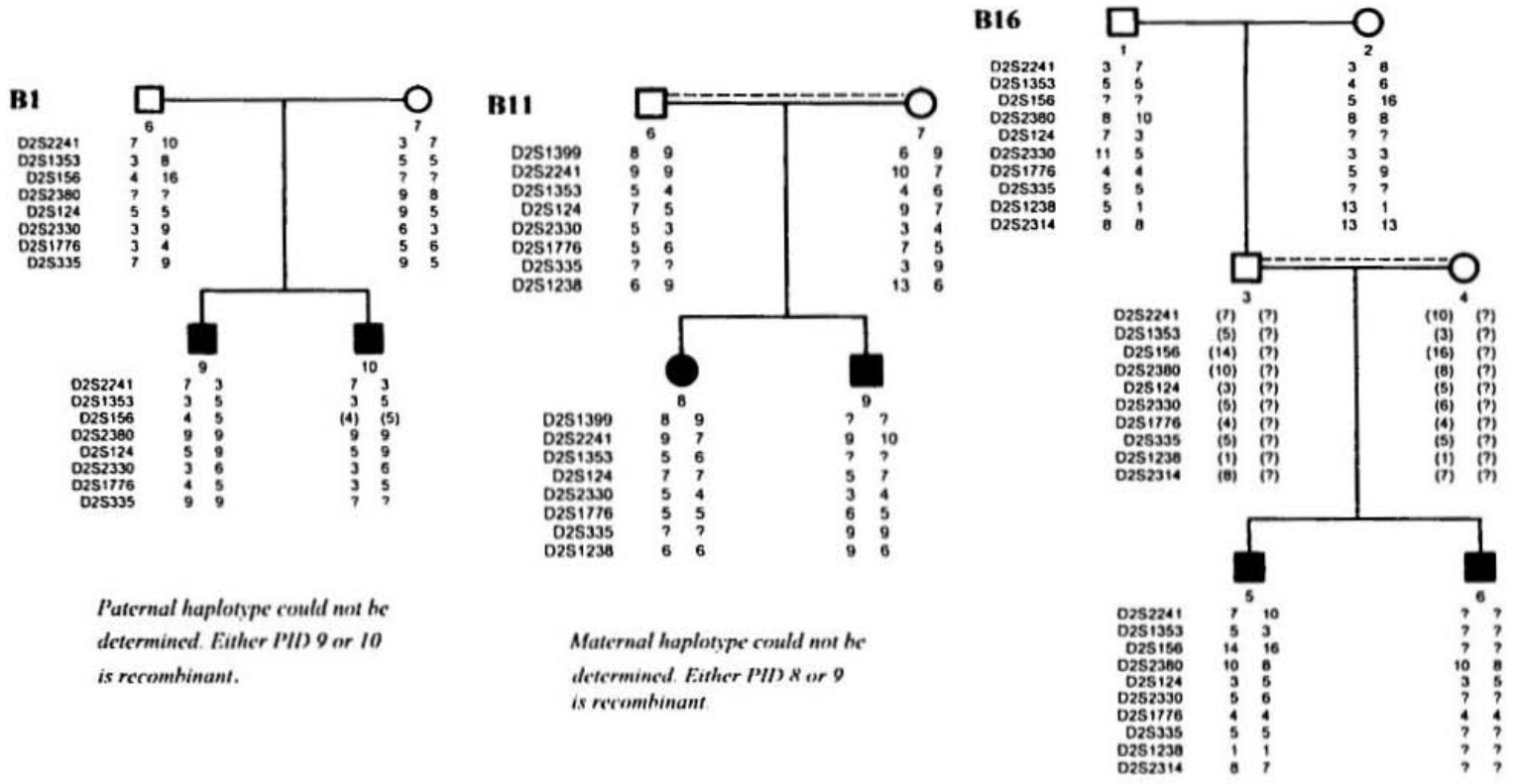


Fig 3.18 Chromosome 2q31 markers spanning the *BBS5* critical interval in families B1, B11 and B16, which could not be assigned to one specific disease locus. Only core pedigrees of B1 and B11 are shown. Alleles in parentheses were inferred and '?' indicates no information was obtained or inferred.

in the affected individual. Family B7 was excluded from this locus since both affected sisters (PIDs 9 and 15) had inherited different maternal genetic contributions (see **Appendix A** for haplotypes). Linkage analysis generated a lod score of -4.68 ($\theta = 0$) at *D2S156*, indicative of exclusion at this locus (Table 3.4). The exclusion of family B14 from *BBS5* increased the likelihood that this was a BBS2 kindred.

Surprisingly, all of the families excluded from the first four BBS loci (except B9) were also excluded from *BBS5*, with the exception of the qter 4 cM of the critical interval in family B5. This indicated that there was yet another BBS gene segregating in the presumed homogenous population of Newfoundland.

3.5 Analysis of Chromosome 18p11.31-p11.2 for a Possible BBS Gene

On the suggestion of a close colleague (Dr. P.L. Beales, personal communication), the region of chromosome 18p11.31-p11.2 was analyzed by microsatellite markers for linkage to BBS in families which could not be assigned to a single known BBS locus (B1, B3, B4, B5, B6, B11, B13 and B16). While performing a genome wide scan with a large consanguineous Indian BBS kindred, Dr. Beales had observed a region of HBD with markers *D18S458* and *D18S62* in three affected patients, but not in the fourth unaffected sib. In addition, there were two small Kurdish families which were HBD in this region. These and other microsatellites were typed in the above mentioned Newfoundland BBS

kindreds (Fig. 3.19).

Families B3, B4, B5, B6 and B13 were investigated for linkage to BBS on chromosome 18p. Interestingly, in family B3, the parents had the same haplotype for four consecutive markers in this region, which was likely inherited from a common grandparent (Fig 3.20). This haplotype had at least been partially inherited by each of the affected individuals (PIDs 11 and 12) from each parent, but not by the unaffected sib (PID 10). Each affected sib was homozygous for the two fully informative pter markers, *D18S481* and *D18S63*, while the lone unaffected sib was not. However, in one affected person (PID 12), there was a recombination in the paternally inherited chromosome proximal to *D18S63*, but no more data were obtained for the other affected sib (PID 11). Unfortunately, the tissue sample from which DNA was extracted for this deceased individual was of poor quality. The lod score at *D18S63* [1.80 ($\theta = 0$)] reflected the HBD in PIDs 11 and 12 (Table 3.5). If this chromosomal region contained a BBS locus, it would reside qter to *D18S52*, according to the haplotype analysis in this kindred.

Family B13 was excluded from the possible chromosome 18 BBS locus by haplotype analysis. Both affected patients analyzed (PIDs 12 and 13) shared common parental haplotypes (except for the most distal marker) which they shared with an unaffected sib (PID 17). Also, haplotypes were shared with the unaffected sib PID 15 pter to *D18S62* (Fig 3.20).

Kindred B4 was excluded from a possible BBS locus in this region since the affected individuals (PIDs 10 and 13) had different paternal contributions (Fig 3.21).

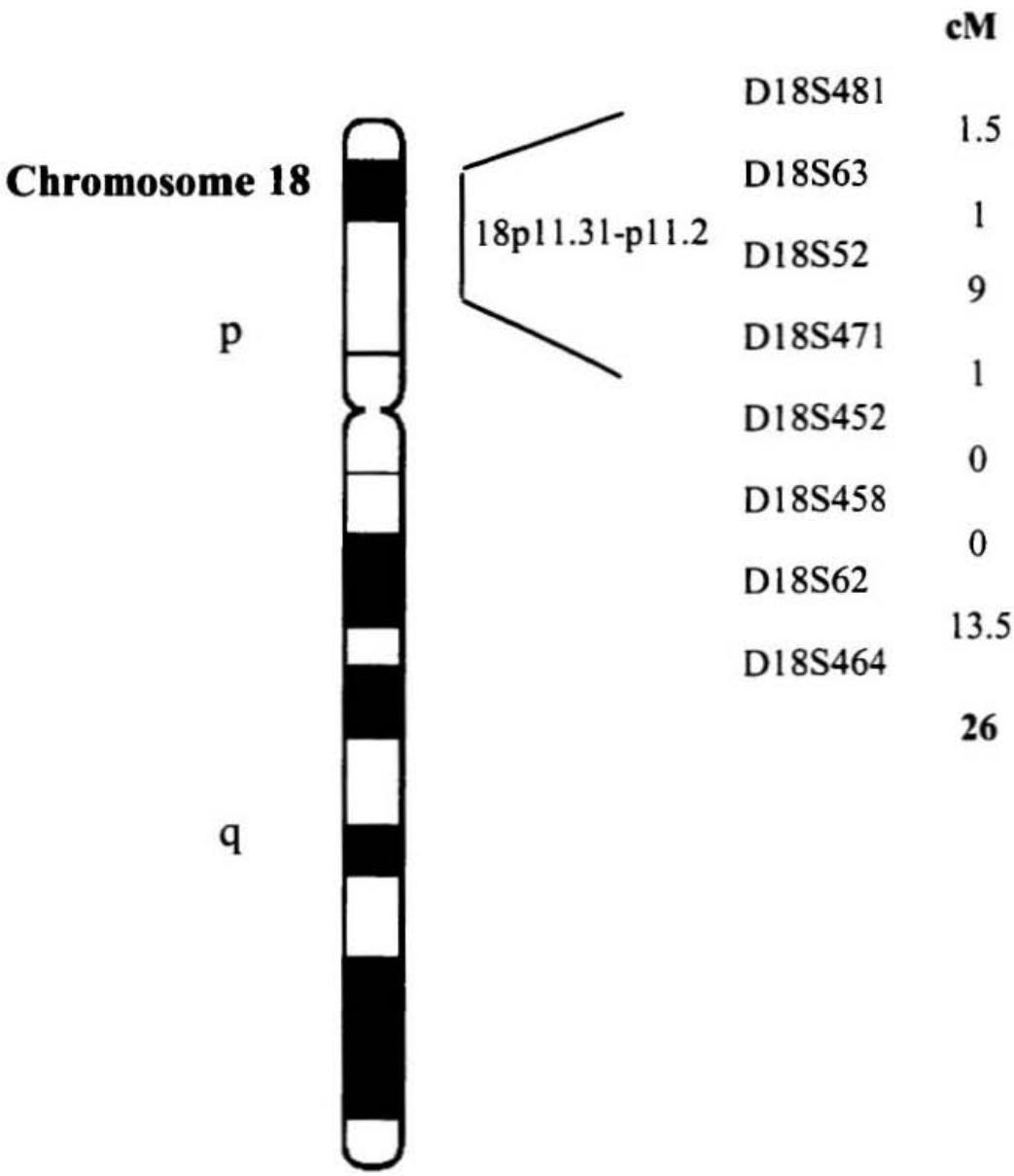


Fig 3.19 Location of markers spanning a region of HBD found in a large consanguineous Indian kindred with BBS excluded from the five known BBS loci (Dr. P.L. Beales, personal communication). Marker order and distances were obtained from the Marshfield sex-average and Genethon linkage maps.

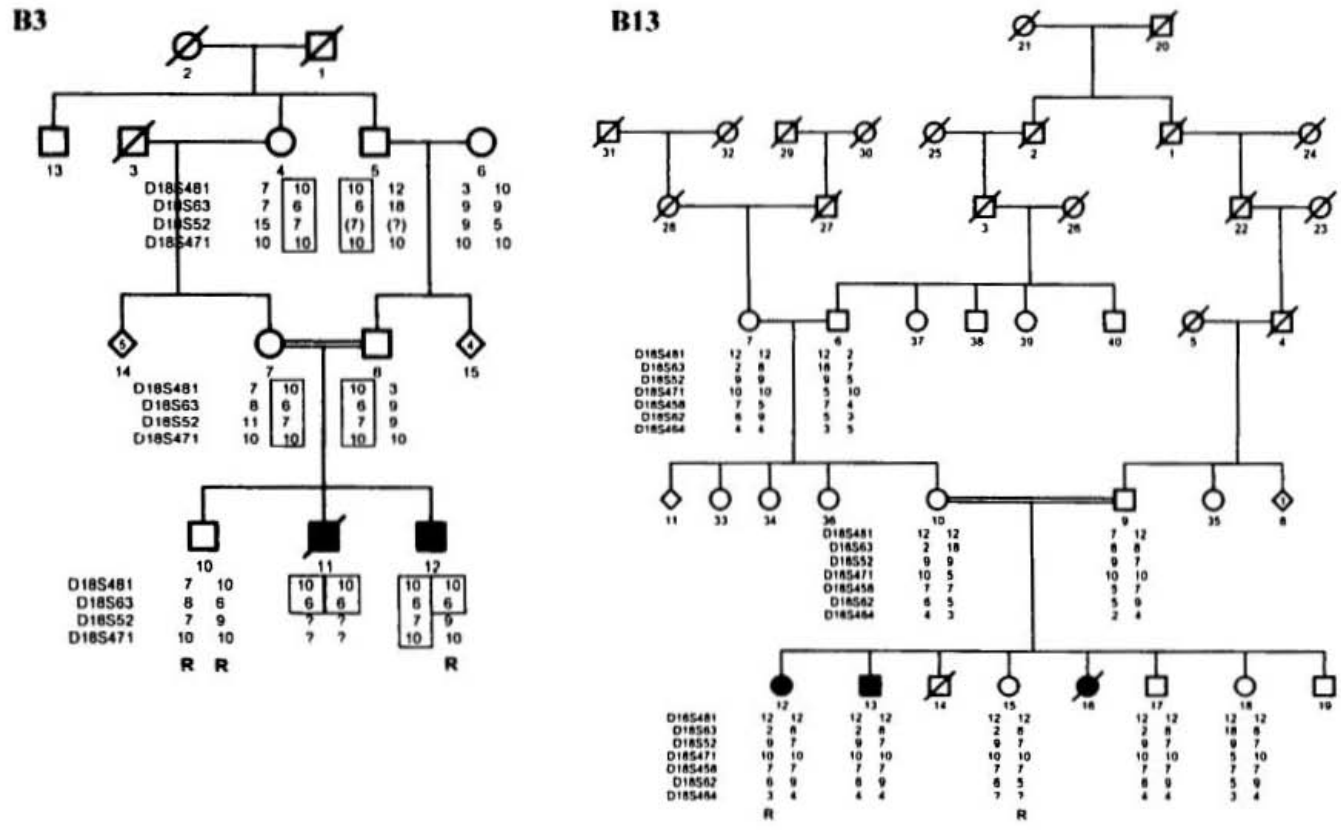


Fig 3.20 Chromosome 18 p11.31-p11.2 markers on families B3 and B13, which had been excluded from all five known BBS loci. Only the core pedigrees are shown. Alleles in parentheses were inferred. '?' denotes no information was obtained or inferred. 'R' indicates a recombination has occurred in that haplotype.

Table 3.5 Summary of linkage and haplotype analysis for chromosome 18p11.31- p11.2 for families which could not be specifically assigned one of the five known BBS loci.

Kin No.	Chromosome 18p11			Chr 18
	Marker	LOD	HAP	
B1	D18S62	-6.43	NE	?
B3[a]	D18S63	1.80	HBD	?
B4	D18S471	-6.77	X	**X**
B5	D18S63	-3.20	X	**X**
B6	D18S62	-0.11	X	**X**
B11[b]	D18S458	0.60	HS	?
B13[a]	D18S62	-0.52	X	**X**

[a] Confirmed consanguineous union.

[b] Suspected consanguineous relationship.

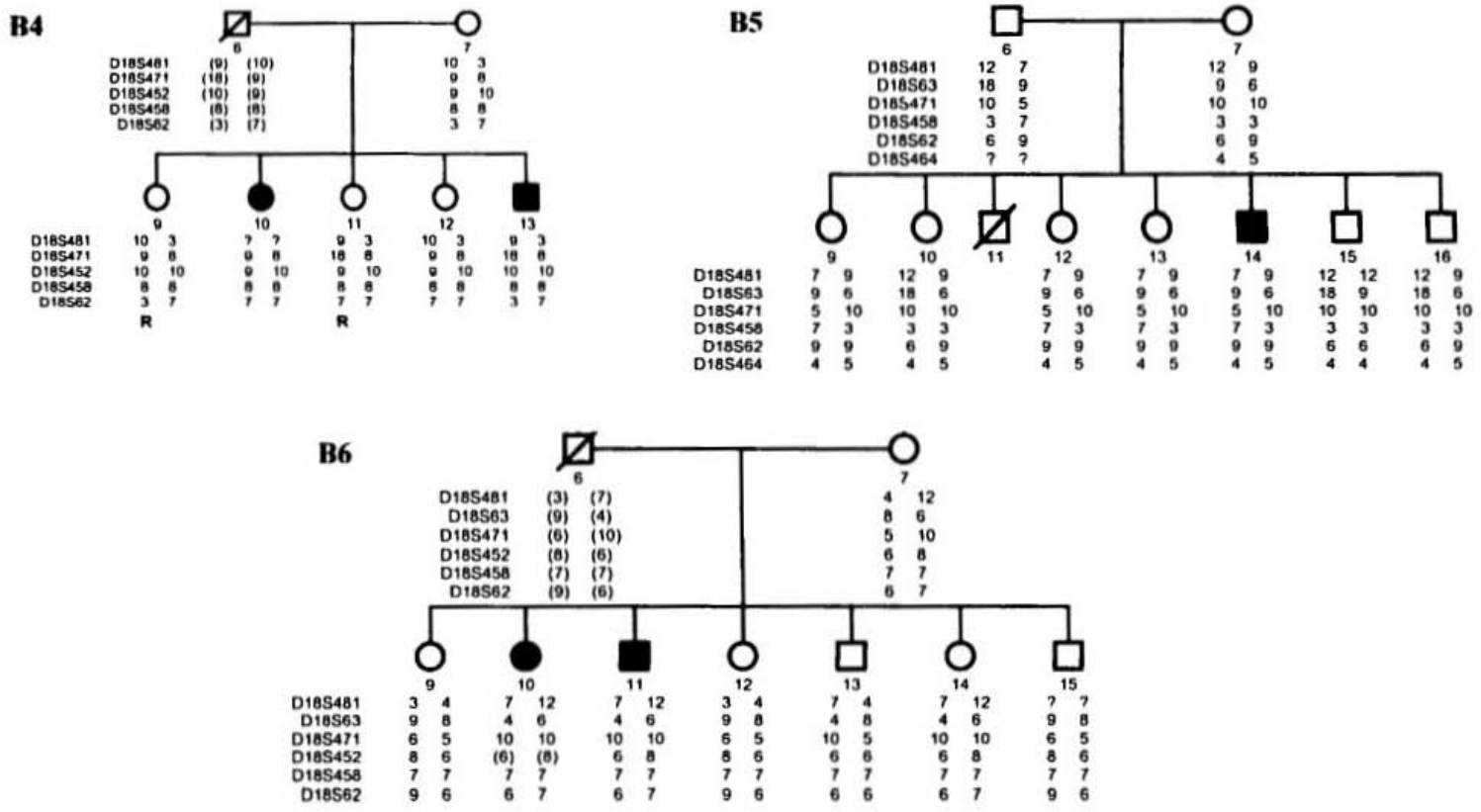


Fig 3.21 Chromosome 18p11.31-p11.2 markers on families B4, B5 and B6. Only the core pedigrees are shown. 'R' indicates a recombination has occurred in that haplotype. Alleles in parantheses were inferred. '?' denotes no information was obtained or inferred.

Also, an unaffected sib (PID 12) shared haplotypes with an affected sib (PID 10). A significantly negative lod score also supported exclusion of this putative BBS locus in this family (Table 3.5).

Family B5 had a single affected male (PID14) who shared his maternal and paternal genetic contributions within this region with three unaffected sibs (PIDs 9, 12 and 13; Fig 3.21). A statistically significant lod score of -3.20 ($\theta = 0$) at the fully informative marker, *D18S63*, further supported the exclusion of this region as a candidate BBS locus in this kindred (Table 3.5).

Two affected patients (PIDs 10 and 11) in kindred B6 shared both parental haplotypes within the region studied; however, they also shared these haplotypes with an unaffected sib (PID14), excluding this region by haplotype analysis (Fig 3.21).

For the three families (B1, B11 and B16) that could neither be categorized to one specific BBS locus, nor excluded from all BBS loci, haplotype and linkage analyses were performed with chromosome 18p marker data (Table 3.5). Unfortunately, in family B1, due to the uninformative nature of the markers analyzed and the problem of marker amplification in PID 10 (Fig 3.22), this family was not excluded nor assigned to this putative BBS locus (Table 3.5). In PID 10, it was uncertain which paternal haplotype was inherited, except at a single marker, *D18S62* (Fig 3.22). This marker provided a lod score of -6.43 ($\theta = 0$), indicating exclusion. However, the maternal haplotype could not be determined. Thus, B1 was uninformative at this putative BBS locus.

Like kindred B1, kindred B11 was also uninformative for most markers in this

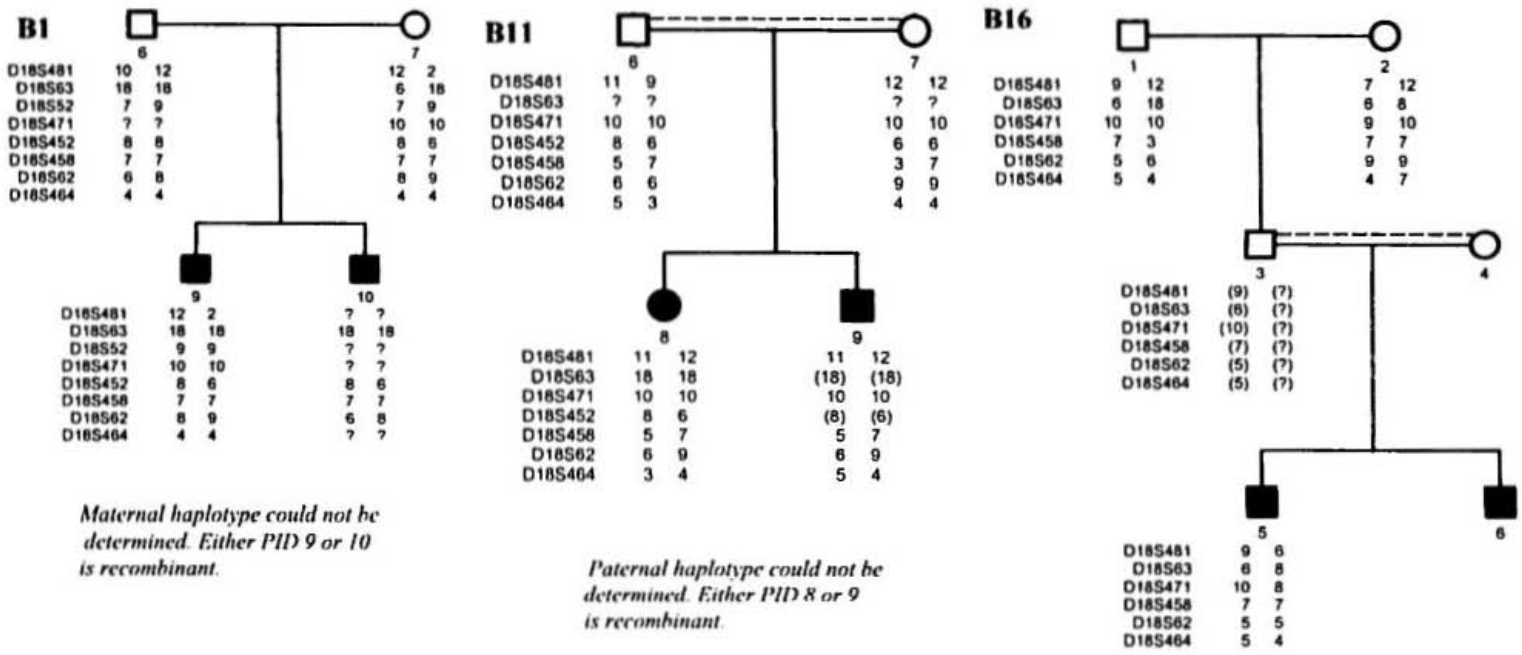


Fig 3.22 Chromosome 18p11.31-p11.2 markers on families B1, B11 and B16, which had not been assigned a BBS locus. Only the core pedigrees of families B1 and B11 are shown. Alleles in parentheses were inferred. '?' indicates no information was obtained or inferred. DNA for PID 6 in B16 was depleted previous to this analysis.

region (Fig 3.22). However, if we assume the least number of cross-overs, none, then the only two sibs, both of whom are affected, shared parental haplotypes. At the only fully informative marker (*D18S458*), the family generated a lod score of 0.60 ($\theta = 0$), suggesting linkage.

Finally, kindred B16 was not assessed because DNA from one of the two affected sibs (PID 6) did not amplify for any of the chromosome 18 markers tested, and there were no unaffected siblings to analyze (Fig 3.22).

In conclusion, it was possible to exclude families B4, B5, B6 and B13 from a putative BBS locus on chromosome 18p. However, it was not possible to categorize families B1 and B16, primarily due to the pedigree structure of these two families. Interestingly, there was some evidence for the assignment of families B3 and B11 to this putative BBS locus.

3.6 Evidence for a Sixth BBS Locus (*BBS6*) on Chromosome 20p12 in B13

With five BBS families excluded from all identified and putative BBS loci, there was significant evidence for a sixth BBS gene. Therefore, family B13 was used for a genome-wide scan implementing DNA pooling and homozygosity mapping. Unfortunately, family B13 was not as large a family as B9, but it was composed of a third cousin marriage and two affected individuals with four unaffected sibs. When a

simulated linkage analysis was performed, using SLINK (v2.65) and MSIM (v2.65), the maximum estimated lod score was calculated as 1.85 ($\theta = 0$). Although this simulation analysis indicated that a significant result could not be obtained by linkage analysis at a truly linked locus to a BBS gene, it was hypothesized that all remaining families which were unlinked to all known BBS loci would be linked to the sixth BBS locus. Therefore, a simulation study was performed on the five unlinked families to determine if a statistically significant lod score could be obtained. The estimated maximum cumulative lod score was calculated at 6.46 ($\theta = 0$). Therefore, a genome-wide screen was performed on kindred B13 to locate a sixth BBS locus, with the intent of typing the remaining four families with any candidate markers which provided a positive lod score and exhibited an extensive region of HBD in affected individuals in family B13.

The genome scan was begun on chromosome 22. Ten of the first 153 markers tested exhibited a reduction to one in the number of alleles in the test pool. These markers were examined more closely by extensive typing of additional adjacent markers on all available family members in family B13. Additional markers were obtained if the marker was homozygous in the affected individuals and not in the unaffected sibs. These additional microsatellites were tested on family B13 to determine if an extensive region of homozygosity was detectable in the affected sibs [as would be expected in a third cousin consanguineous marriage at a locus associated with the disease (Genin *et al.*, 1998)]. These markers were also tested on the other unlinked families to determine if linkage was suggestive at these loci. Also, any possible allele sharing between families

was examined, since a founder effect may be observed, as was the case for *BBS1*.

The 154th marker typed was *D20S189*, on chromosome 20p12, which displayed a reduction of alleles from three to one, from the control pools to the test pool. When this marker was typed for each family member individually, it was fully informative and homozygous in affected persons, but not in the four unaffected sibs (Fig 3.23). Therefore, additional microsatellite markers were obtained and tested in this region. Subsequently, an extensive region of homozygosity was observed between *D20S192* and *D20S189*, a distance of approximately 8 cM (Fig 3.24). A lod score of 1.98 ($\theta = 0$) was generated with *D20S851*.

3.6.1 Analysis of the Remaining Unassigned BBS Families at the Putative *BBS6* Locus

To further investigate this putative BBS locus, *D20S189* and adjacent markers were typed on kindreds B3, B4, B5 and B6, and used for linkage analysis. These markers were also typed on families B1, B11 and B16, which had not been categorized as yet. The results of haplotype and linkage analyses in these families (except B11) are shown in Table 3.6.

The consanguineous kindred B3, in which only one affected member was available for haplotype analysis, generated a lod score of 0.95 ($\theta = 0$) at *D20S917*, indicating support for linkage at this locus. More significantly however, the affected

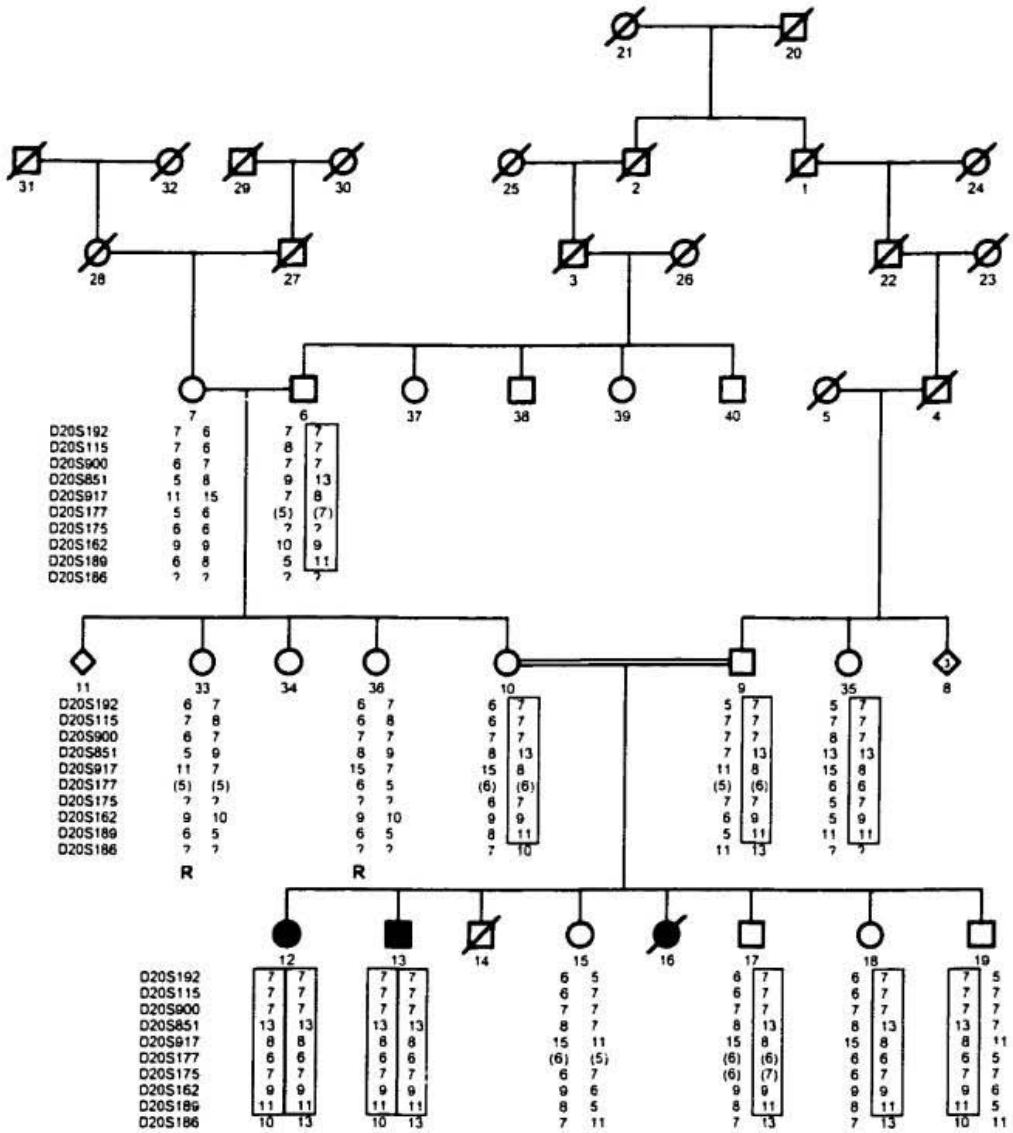


Fig 3.23 Chromosome 20p12 markers spanning an ~ 8.5 cM region containing the *BBS6* locus in family B13. Boxed haplotype indicates DH. 'R' indicates the haplotype is a recombinant. Genotypes in parentheses were inferred. Alleles in parentheses are inferred and '?' indicates no information was obtained or inferred.

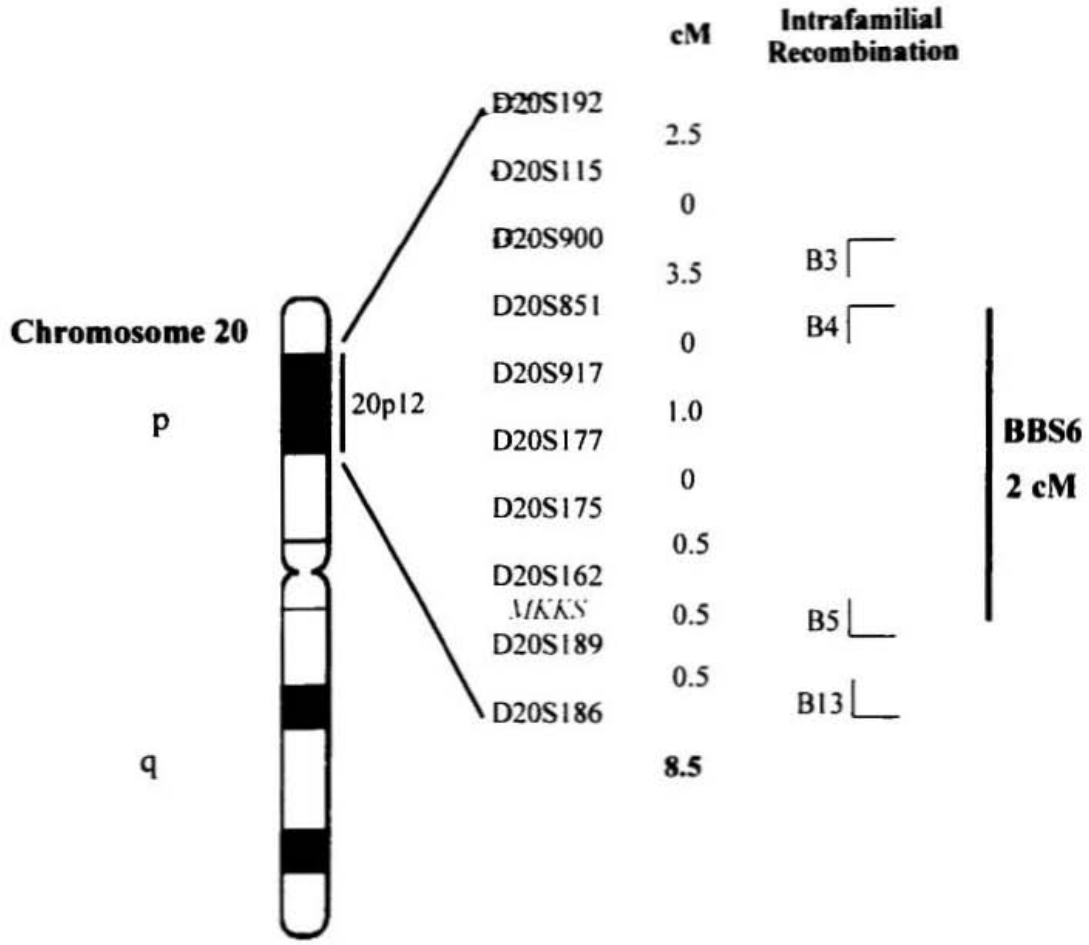


Fig 3.24 Location of markers flanking the *BBS6* gene, *MKKS*, on 20p12. Distances and marker positions obtained from the Alagile syndrome candidate region (GDB) and the Marshfield sex-average linkage maps.

Table 3.6 Summary of linkage and haplotype analysis for chromosome 20p12 markers in families which could not be assigned to one of the five known BBS loci.

Kin No.	BBS6			BBS6
	Marker	LOD	HAP	
B1	D20S917	0.57	HS	✓
B3[a]	D20S917	0.95	HBD	✓
B4	D20S917	0.96	HBS	✓
B5	D20S851	0.71	UH[c]	✓
B6	D20S917	-4.93	X	X
B11[b]	D20S115	-6.43	X	X
B13[a]	D20S851	1.98	HBS	✓
B16[b]	D20S162	0.56	HS	✓

[a] Confirmed consanguineous union.
[b] Suspected consanguineous relationship.
[c] Unique Haplotype - Different from six unaffected sibs.

person was HBD for the markers qter to *D20S900*, but the unaffected sibling was not (Fig 3.25). Also, the disease carrying chromosome was shown to have originated from one of the founders, PID 1 or 2, since the affected person's parents inherited their copies of this chromosome from their parents, who were siblings.

Kindred B4, with two affected sibs, also gave a positive lod score at *D20S917* [0.96 ($\theta = 0$)]. The haplotype analysis was also supportive of linkage of BBS to this locus. Both affected individuals shared haplotypes at the three most proximal markers (*D20S917*, *D20S189* and *D20S186*), which were not shared with the three unaffected sibs (Fig 3.25). Interestingly, one affected individual, PID 13, was homozygous for all markers typed in this region, indicating he may have received two copies of part of an ancestral chromosome. However, the other affected person, PID 10, had a recombination in her paternal chromosome, between *D20S851* and *D20S917*. Therefore, she was only homozygous for markers proximal to *D20S851*.

There was one affected individual and six unaffected siblings available for analyses in family B5, which provided a positive lod score of 0.71 ($\theta = 0$) at *D20S851*. PID 14, the affected son of PIDs 6 and 7, had unique parental haplotypes between *D20S192* and *D20S162* when compared to his unaffected siblings (Fig 3.25). However, both PIDs 13 and 16, who share full paternal, but not maternal contributions with PID 14, had recombinations between *D20S162* and *D20S189* on the maternally inherited chromosome. Therefore, they shared haplotypes proximal to these cross-overs with PID 14.

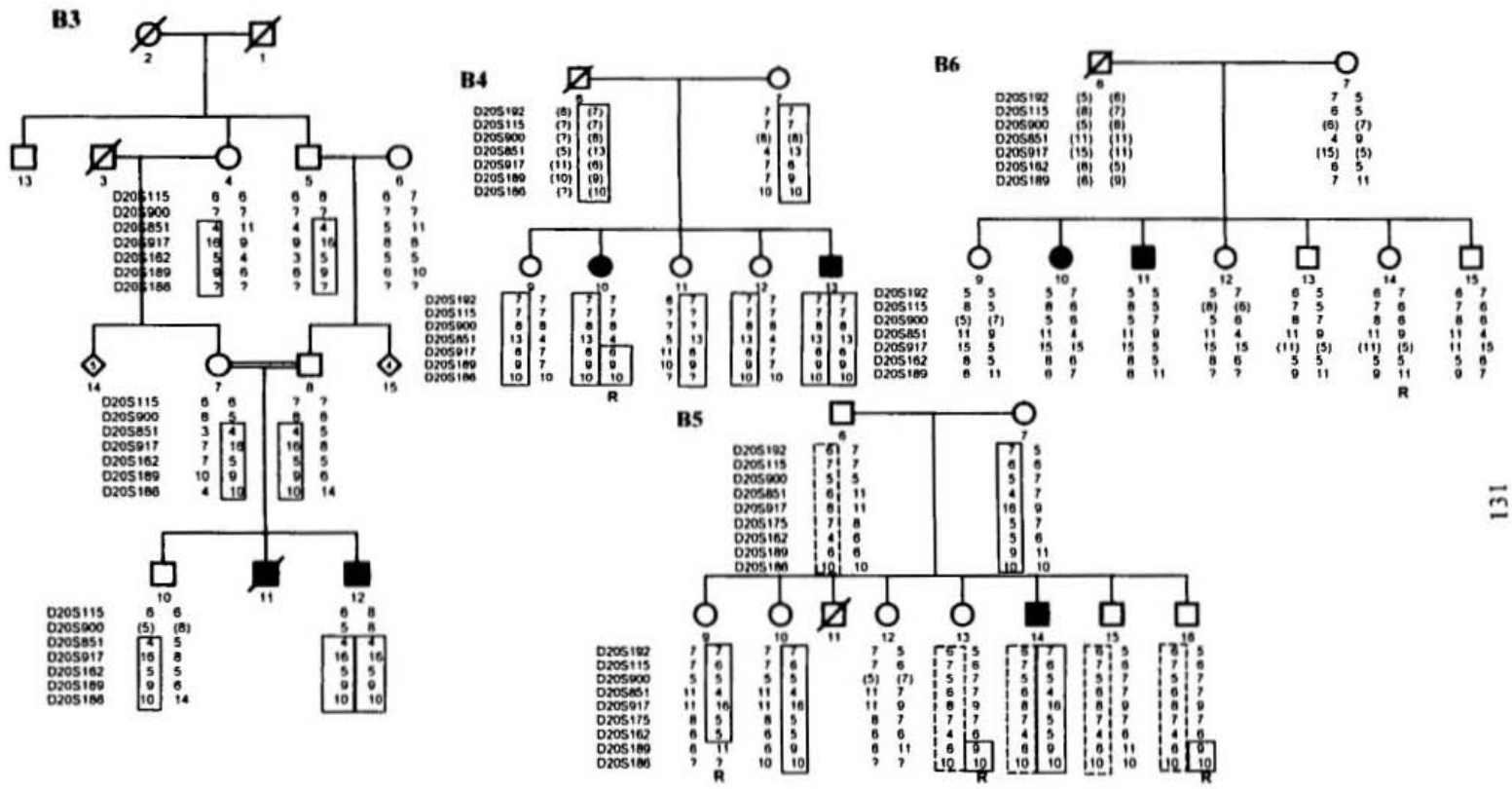


Fig 3.25 Chromosome 20p12 markers spanning the *BBS6* critical interval in families B3, B4, B5 and B6. Intrafamilial recombinations narrowed the *BBS6* interval to ~ 2cM. Haplotypes which are boxed (with solid or dashed lines) are DHs and 'R' represents a recombination in the haplotype. Non-consanguineous families have only core pedigrees presented. Alleles in parentheses were inferred. '?' indicates no information was obtained or inferred.

Unlike the other four families excluded from *BBS1-5*, kindred B6 did not show evidence of linkage at the putative BBS locus on chromosome 20p. At *D20S917* the lod score was -4.93 ($\theta = 0$). The haplotype analysis also did not support linkage at this locus, since the two affected persons, PIDs 10 and 11, have different maternal contributions in this region. Also, PID 10 shared both parental contributions with an unaffected sib, PID 12. Additionally, the other affected sib (PID 11), shared his parental contributions with an unaffected sib (PID 9). Thus, this locus was excluded in family B6. Remarkably, this family can now be excluded from all six known BBS loci, indicating evidence for a another BBS locus.

Of the three other families not yet assigned to a locus, two, families B1 and B16, were not excluded from this BBS locus. Family B1 was not excluded from this locus because the only two sibs, which are both affected, share the same haplotypes (Fig 3.26). Linkage analysis generated a lod score of 0.57 ($\theta = 0$) at *D20S917*. A similar result was obtained for family B16, where there are two affected brothers but no unaffected individuals in the sibship. Haplotype analysis of kindred B16 (Fig 3.26; Table 3.6) indicated that both affected sibs shared paternal and maternal contributions in this region. However, proximal to *D20S162*, one of the brothers had a recombination in his maternally inherited chromosome. The third family in this category, B11, could be excluded from this locus since the only two sibs, both affected, have different contributions from their father, as well as from their mother between *D20S115* and *D20S162* (Fig 3.26). A lod score of -6.43 ($\theta = 0$) at *D20S115* further supported exclusion

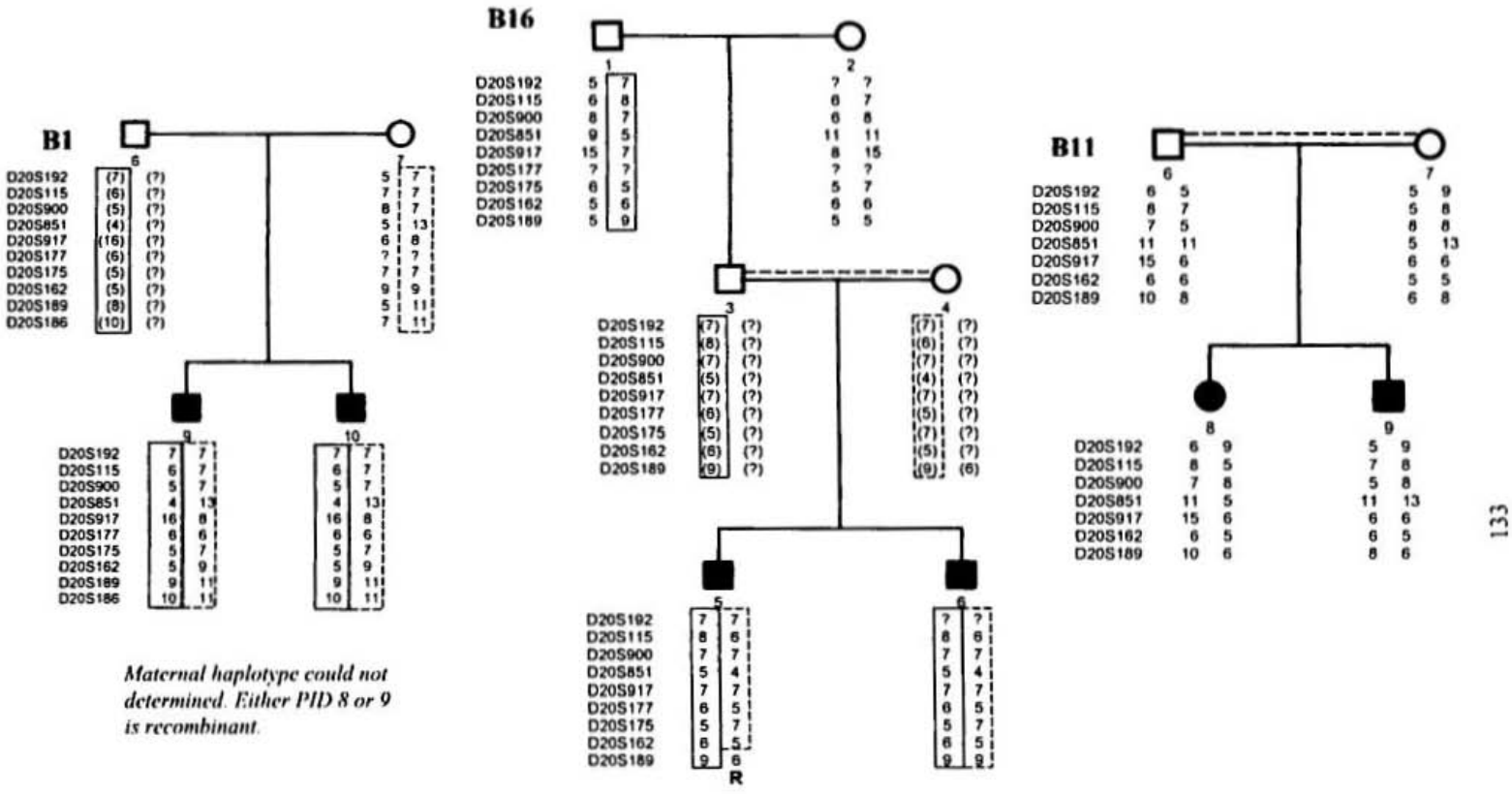


Fig 3.26 Chromosome 20p12 markers spanning the *BBS6* critical interval in families B1, B11 and B16, which had not been assigned a BBS locus. Haplotypes which are boxed are (with solid or dashed lines) are DHs and 'R' represents a recombination in the haplotype. Only the core pedigrees of families B1 and B11 are shown. Alleles in parentheses were inferred. '?' indicates no information was obtained or inferred.

of a BBS gene at this locus in family B11.

Through haplotype and linkage analyses, the disease in four of five families excluded from all five known BBS loci, and two of three families that could not be exclusively categorized, was highly suggestive of being linked to a BBS gene on chromosome 20p12 (*BBS6*).

3.6.2 Analyzes of a Candidate Gene (*MKKS*) in the Delimited Critical Region of *BBS6*

To delineate the *BBS6* critical interval, haplotypes of families B1, B3, B4, B5 and B13 were examined (Table 3.7). Haplotype analysis identified a potential 2 cM critical interval between *D20S851* and *D20S189* supported by several lines of evidence. The distal boundary was determined to be *D20S851* on the basis of a recombination in individual PID 10 of family B4 between *D20S851* and *D20S917* (Fig 3.25). Also, in families B3 and B4, all affected patients shared a pter-9-10-qter haplotype for *D20S189-D20S186*, but not for the distal markers. The proximal boundary was delineated by a recombination in two unaffected sibs, PIDs 13 and 16, in family B5 (Fig 3.25). These individuals had the same paternal contribution as their affected sib and the same maternal contribution proximal to *D20S189*. Therefore, the causative gene had to be distal to *D20S189* if this family was linked to a BBS locus in this region. This boundary was supported by the loss of HBD of markers proximal to *D20S189* in both affected

Table 3.7 DHs on chromosome 20p12 segregating in six BBS families

Marker	B1		B3		B4		B5		B13		B16	
	p	m	p	m	p	m	p	m	p	m	p	m
D20S115	6	7	8	6	7	7	7	6	7	7	8	6
D20S900	5	7	8	5	8	8	5	5	7	7	7	7
D20S851	4	13	4	4	13	13	6	4	13	13	5	4
D20S917	16	8	16	16	6	6	8	16	8	8	7	7
D20S162	5	9	5	5	—	—	4	5	9	9	6	5
D20S189	9	11	9	9	9	9	6	9	11	11	9	9*
D20S186	10	11	10	10	10	10	10	10	13	10	—	—

Note - Haplotypes are arranged with the paternal haplotype (p) on the left and the maternal haplotype (m) on the right. Common ancestral haplotypes are matched by color. Recombinations occurred in the maternal DHs of B4 and B16, however, only one haplotype is shown.
* The other affected person in B16 was a recombinant at this locus (maternal allele was 6).

individuals in family B13 (Fig 3.23).

There appeared to be two separate founder effects in these families (see Figs 3.23, 3.25, 3.26 and Table 3.7). One of the disease haplotypes, in families B1 and B5, the pter-7-6-5-4-5-5-**9-10**-qter (*D20S192-115-900-851-175-162-**189-186***), was partially the same as in family B3, pter-4-5-**9-10**-qter (*D20S851-162-**189-186***), and family B4, pter-**9-10**-qter (*D20S**189-186***). In addition, the DH found in family B13, the pter-7-7-7-13-8-9-11-qter (*D20S192-900-115-851-917-162-189*), was identical to one of the DHs in family B1. These data suggested that *BBS6* maps to an approximately 2 cM region between *D20S851* and *D20S189* (Fig 3.24). Also, it indicates there are multiple mutations in this one gene segregating in Newfoundland.

Previous to the above analyses, a gene for McKusick-Kaufman syndrome, *MKKS*, was identified in the same region of 20p12 (Stone *et al.*, 1999; see **Chapters 4.3** and **4.4.1** for more detail concerning *MKKS* and its putative protein function). Given the overlapping clinical phenotype of the two syndromes (**Chapter 1.3.2**) and their concordant mapping position, it was hypothesized that *MKKS* was a candidate for *BBS6*. To investigate this hypothesis, primers were designed (**Appendix I**) to amplify all the coding exons of *MKKS*. Sequencing of these amplicons, which included the exons and splice junctions, identified several coding region alterations (Table 3.8). All affected individuals from pedigrees B3, B4 and B16 were homozygous for the deletion 281ΔT (numbering begins with the first base of the start codon), which resulted in a frameshift after amino acid F94, terminating the protein at amino acid 103 (F94fsX103; Fig 3.27).

Table 3.8 Mutations found in *MKKS* in seven Newfoundland BBS pedigrees.

Mutations	Families with Mutations	Number of Disease Chromosomes	Newfoundland Controls	North American Controls
fs1 -F94fsX103 (281ΔT)	B1, B3, B4, B5 and B16	8	76	172
fs2 -D143fsX157 (429ΔCT/433ΔAG)	B1 and B13	3	84	168
L277P (T829C)	B5	1	80	154
A242S	B14	1	79	166
Total	7	13		

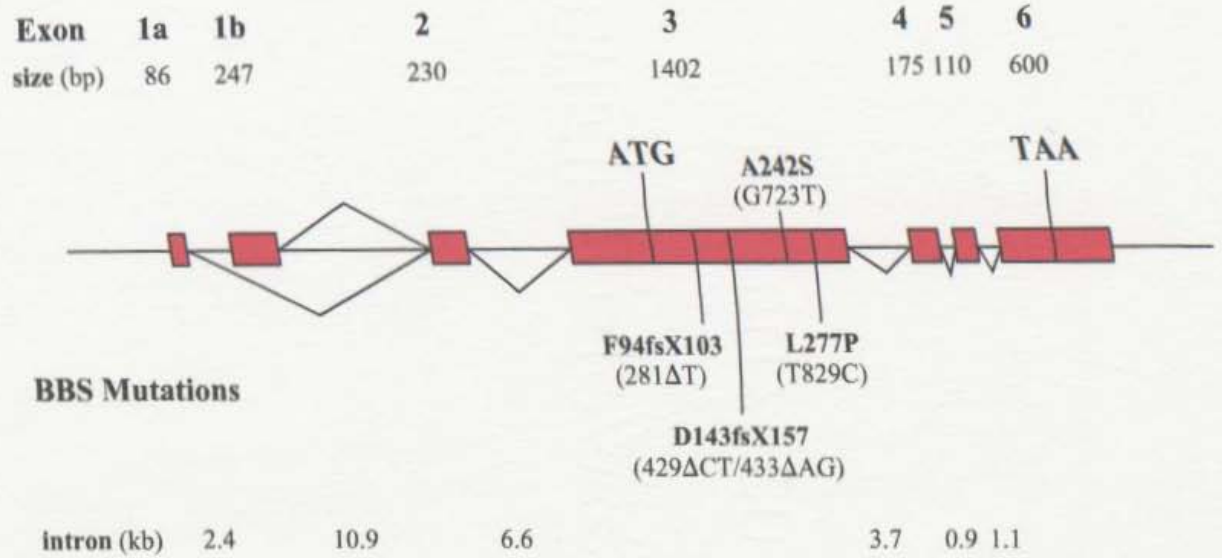


Fig 3.27 Genomic structure of *MKKS* with *BBS6* mutations identified in Newfoundland. Exons are shown as rectangles with alternate splicing of exons 1a and 1b as indicated.

The same alteration was also found in the heterozygous state in patients PIDs 9 and 10 in family B1, and in PID 14 of kindred B5. The 281ΔT alteration segregated with the haplotype-inferred prediction of a common ancestral chromosome in four families (B1, B3, B4, B5). This alteration was not found in 172 European or 76 Newfoundland unrelated control chromosomes (Table 3.8). The second disease associated allele in family B5 was a T→C transition at the 829th base, that was not found in 234 control chromosomes, resulting in the missense substitution L277P (Fig 3.27; Table 3.8). A complex 429ΔCT/433ΔAG allele (Fig 3.27) was also detected that co-segregated with the disease in pedigrees B1 and B13, resulting in a frameshift (D143fsX57; Table 3.8). Once again the alteration segregated with the haplotype-inferred prediction of a common ancestral chromosome in these two families. Cloning and sequencing of the PCR product from subject PID 12 in family B13 indicated that both deletions were on the same strand.

Additionally, a single alteration was identified in family B14, which had previously been linked to the *BBS2* locus. A G→T transversion at the 723rd base resulted in an A242S missense mutation. This alteration was reported previously in an MKS patient as part of a complex allele, segregating with an H84Y alteration (Stone *et al.*, 2000). Both the affected and the unaffected individuals in the Newfoundland kindred inherited the alteration from their mother, and she from her mother. In family B14, no other alterations were identified.

Finally, after eight years and the discovery of six BBS loci, the first gene to cause Bardet-Biedl syndrome was identified.

Chapter 4 Discussion

4.1 Genetic Heterogeneity of Bardet-Biedl Syndrome in Newfoundland

The colonization of the island of Newfoundland in the 18th and 19th centuries resulted in many small isolated communities arising around its coast. These were often founded by a few families, originally from the same part of the West Country of England or southeast Ireland. The stability of the communities, combined with large families that often intermarried, provide an increased opportunity for otherwise rare autosomal recessive diseases to manifest themselves (Davidson, 2000). In these cases, it is expected that the disease allele came from a single ancestral founder and has been duplicated in the offspring of consanguineous marriages of his/her descendants. The disease in question is usually localized to a specific geographical area (Bear *et al.*, 1987 and 1988). However, the lack of clustering of BBS in Newfoundland is striking (Fig 4.1), suggesting that a single founder event did not occur in this instance unless the mutation was very old and had spread more recently. The results of the current research reveal that there have been at least eight founders who have brought about the disease on the island. Furthermore, despite non-clustering of cases, the prevalence of BBS is very high.

Fig 4.1 Distribution of BBS families in Newfoundland indicating BBS locus assignment.

4.1.1 Genetic Categorizations of Newfoundland BBS Families

It was possible to identify mutations in a causative gene for six BBS6 families (B1, B3, B4, B5, B13, B16) and to categorize another eight of 17 Newfoundland families to a BBS locus through linkage and haplotype analyses (B2, B8, B9, B10, B12, B14, B15, B19). One family (B7) suggested linkage to two BBS loci, and a small non-consanguineous family (B11) was excluded from three loci only. Remarkably, one kindred (B6) was excluded from all six known BBS loci and the possible BBS locus on chromosome 18.

In the initial genetic survey of Newfoundland BBS patients, three families (B8, B10 and B19) were considered BBS1 kindreds and three others (B7, B12 and B15) were not excluded from this locus (**Chapter 3.1.1**; Woods *et al.*, 1999). Using additional microsatellite markers within the *BBS1* critical interval, five of these six families with DNA available (all but B7) exhibited a founder effect. Five of these six families (all but B15) originated on the south and southwest coasts of the island (Fig 4.1), a region that was settled predominantly by the spread of settlers by sea in an east-west direction (Mannion, 1986). In the absence of mutation analysis, the identification of a single founder effect required that all patients were homozygous for specific alleles at marker loci tightly linked to *BBS1*. It was shown that all BBS1 patients, available for fine mapping, were homozygous for a relatively rare haplotype spanning a 1 cM region centered around the *PYGM* gene on chromosome 11q13 (**Chapter 3.3**; Young *et al.*,

1999b).

Confirmation of this region as the *BBS1* critical interval was made by Katsanis *et al.* (1999) by the use of a large cohort of BBS patients. The localization of *BBS1* between *D11S1883* and *D11S4940* was small enough to allow positional cloning to be attempted. Unfortunately, however, although this is not a large region for a candidate gene approach, it is a transcript rich interval (>30 transcripts) (Katsanis *et al.*, 1999). Despite extensive searching, the *BBS1* gene has not been identified (Dr. Nicholas Katsanis, personal communication).

One family was linked to the *BBS2* locus (B14) and one to the *BBS3* (B2) locus (**Chapters 3.1.2** and **3.1.3**; Woods *et al.*, 1999). Family B14, a small consanguineous kindred, exhibited HBS in the *BBS2* critical region but it did not delimit this interval.

The discovery of a *BBS3* family (B2) was rather surprising, because this form of the disease had not been observed in previous surveys of BBS kindreds of northern European descent (Beales *et al.*, 1997; Bruford *et al.*, 1997), and indeed, previously it had only been described in a single Bedouin family (Sheffield *et al.*, 1994). The initial linkage of BBS to chromosome region 3p13-p12 localized the putative *BBS3* gene to a 11 cM region (Sheffield *et al.*, 1994). Haplotype analysis with the B2 family showed homozygosity, presumably HBD, in all affected members for markers in the *BBS3* critical region. One affected individual inherited one copy of a recombined ancestral haplotype that retained the qter side of the *BBS3* critical region. Because all the parents originated from a small fishing village on the Avalon Peninsula of the island, it is probable that they

shared the same ancestry. This was apparent from the haplotypes of the affected children in this extended family (Fig 3.7). On the basis of homozygosity mapping in this kindred and the *BBS3* Bedouin pedigree (Sheffield *et al.*, 1994), it was predicted that the *BBS3* gene is located within a 6 cM interval on chromosome 3p (**Chapter 3.1.3**; Young *et al.*, 1998). Additional confirmations of the *BBS3* locus were published recently from families of Iranian and European descent (Ghadami *et al.*, 2000; Beales *et al.*, 2001). Also, the critical region was refined to a 2 cM region between *D3S1603* and *D3S1251* (Beales *et al.*, 2001), which is within the *BBS3* critical interval delimited by the B2 family.

Six families (B3, B4, B5, B6, B9, B13) were excluded from the four heretofore identified BBS loci. Family B9 was a large kindred with five affected members who are the result of three consanguineous unions interrelated through two founding couples. This pedigree structure lent itself to a genome scan of pooled DNA samples and homozygosity mapping. Consequently, a fifth BBS locus was identified on 2q31 between markers *D2S156* and *D2S1238* (**Chapter 3.4**; Young *et al.*, 1999). Subsequently, the other five unlinked families were tested to determine if they too were linked to the *BBS5* locus. Two small families (B1 and B16), each of which contained two affected offspring, with no unaffected siblings, exhibited haplotype sharing at this locus (**Chapter 3.4.1**). Thus, linkage analysis resulted in small positive lod scores. These kindreds, therefore, suggested linkage to *BBS5*. The eight other families tested (B3, B4, B5, B6, B7, B11, B13, B14) were excluded by a combination of haplotype and linkage analyses. Recently, there has been a report of three North American/European BBS families associated with

the *BBS5* locus (Beales *et al.*, 2001). However, these kindreds did not narrow the *BBS5* critical interval.

Surprisingly, five families (B3, B4, B5, B6, B13) were excluded from all identified BBS loci. One of these kindreds, a consanguineous family (B13), was utilized in a genome-wide screen using homozygosity mapping of pooled DNA samples. By itself, family B13 would not yield a statistically significant lod score at a true BBS locus. However, in conjunction with the other unlinked families, a putative BBS locus was determined through haplotype and linkage analyses. The microsatellite marker *D20S189* suggested a sixth BBS locus on 20p12. Subsequent testing of this marker on three additional unlinked families (B3, B4, B5) and two unassignable families (B1 and B16), further suggested a BBS gene at this locus. The affected individuals in kindreds B3 and B4 (and one of the affected siblings in B16) were homozygous at *D20S189*. Surprisingly, however, a different allele was observed in the BBS patients of the B13 family. Additional microsatellite marker typing showed an extended region of HBD in family B13 and in kindreds B3 and B4. Once again, however, the alleles at the homozygous markers closest to *D20S189* were different in the latter two families than in kindred B13. Interestingly, family B1 had one haplotype identical to kindred B13 and one identical to the B3 kindred. As well, family B5 shared a haplotype at 20p12 with family B3, but the affected person in family B5 also had one unique haplotype. By comparing haplotypes between families and using the observation of a few key recombinations within families, the *BBS6* critical interval was delimited to a 2 cM interval on 20p12. Interestingly,

previous to the identification of a sixth BBS locus, the *MKKS* gene had been isolated within this region (Stone *et al.*, 2000). Since mutations in this gene cause McKusick-Kaufman syndrome (MKS), a disorder which has phenotypic overlap with BBS, it was considered a good candidate gene to screen for mutations in BBS patients. Shortly thereafter, mutations were identified in *MKKS* causing BBS in six Newfoundland families (B1, B3, B4, B5, B13, B16). This was the first gene identified which has proven to be responsible for Bardet-Biedl syndrome. Three different mutations, all in exon 3, were identified in the six Newfoundland BBS6 families (**Chapter 3.6.2**; Katsanis *et al.*, 2000). Two were alterations causing frameshift mutations, resulting in a premature stop codon in exon 3 (F94fsX103 and D143fsX157) and the other a missense mutation (L277P). This indicates that multiple founder mutations were present. Affected individuals in families B3, B4 and B16 were homozygous for the same frameshift mutation (F94fsX103), and the affected person in kindred B5 was heterozygous for this frameshift. The latter kindred also segregated the missense alteration. These observations agree with the initial haplotype analysis. These families were located on the Conception Bay coast, within relatively short distances by sea from one another (Fig 4.1). Using haplotype analysis it was possible to approximate how these families were related to one another (Fig 4.2). For instance, it can be assumed that the parents of the affected individuals in family B4 are related, although this was not presumed previously, and both were related to the parents of families B3 and B16, since all had the same disease-causing mutation (F94fsX103). In addition, the affected person in kindred B5 also inherited this mutation

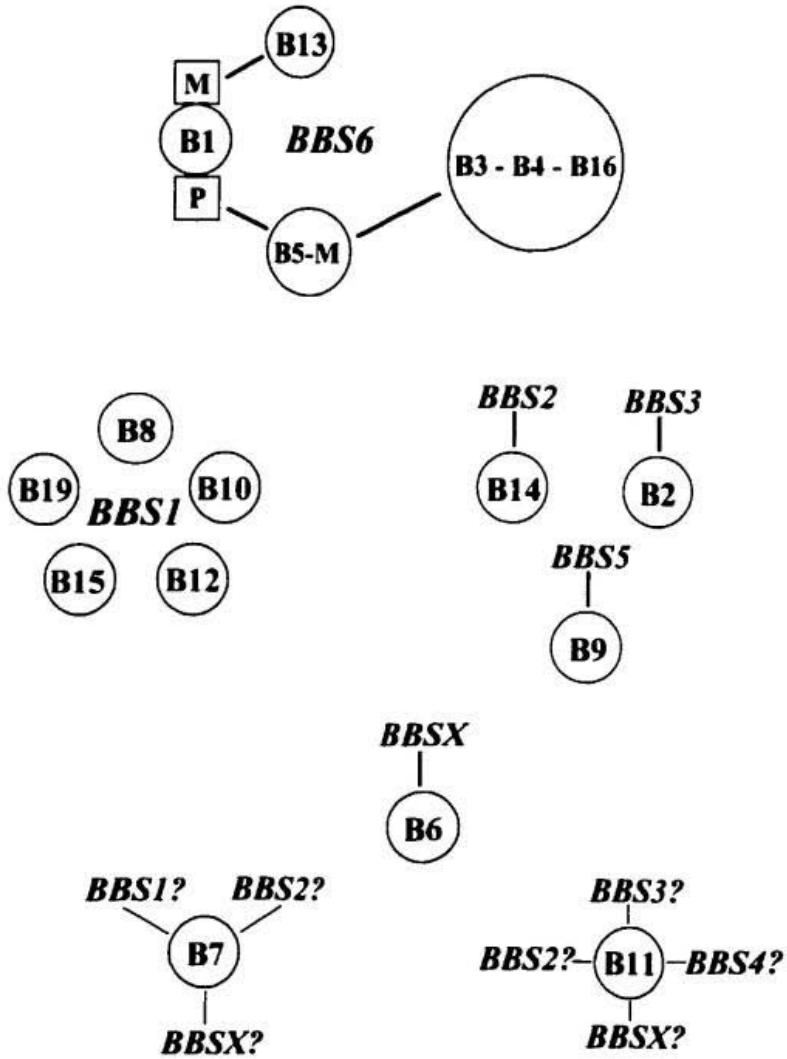


Fig 4.2 Genetic distribution and ancestral relationships of BBS pedigrees in Newfoundland.
M= Maternal Ancestry and P = Paternal Ancestry.
BBSX is a yet to be identified disease locus.

in a heterozygous state. This affected individual inherited the frameshift alteration from his mother. Therefore, it was the mother who was presumably distantly related to the parents of the affected persons in families B3, B4 and B16. The other affected persons heterozygous for the F94fsX103 mutation were in family B1. The two affected individuals in family B1 received this frameshift mutation from their father. Thus, their father is likely related to the mother of the affected person in family B5 and both parents of the patients in families B3, B4 and B16.

The other founder effect was observed with families B1 and B13 which have the other identified frameshift mutation in *MKKS/BBS6* (D143fsX157). The affected individuals in kindred B1 were heterozygous for the D143fsX157 mutation. They received this frameshift from their mother, who, therefore, is probably related to the parents of the affected persons in B13. Through these complex relationships all BBS6 families were linearly linked to one another (Fig 4.2).

Evidence for a seventh BBS locus (*BBS7*) was demonstrated in family B6 where all known BBS loci were ruled out, as well as a possible locus on chromosome 18. This implied there were at least six different BBS genes segregating in the Newfoundland population (*BBS1*, *BBS2*, *BBS3*, *BBS5*, *BBS6* and *BBS7*) - five more than originally hypothesized. In family B6 there are two affected siblings who had five unaffected sibs. This kindred was not known or presumed to be a consanguineous family. Performing linkage analysis on this family alone, at a fully informative locus linked to a BBS gene, would not provide a statistically significant lod score. However, Beales *et al.* (2001)

noted that 14% (most of which were of Turkish and Pakistani origin) of their 92 pedigrees studied did not show linkage of the disorder to any of the six known loci, and one kindred could be statistically excluded from *BBS1-6* . In the future, a genome wide scan using family B6 may suggest a possible BBS locus which could be confirmed in a linkage study using multiple pedigrees excluded from *BBS1-6.*

Unambiguous assignment of a single BBS locus or gene to a family was not possible in two instances (B7 and B11). BBS in family B7 was initially considered to be caused by mutations in *BBS1* because of the similarities in haplotypes with the other *BBS1* families (**Chapter 3.3**; Young *et al.*, 1999). Unfortunately, the DNA collected from this non-consanguineous kindred was exhausted, and the additional markers utilized in the LD study were not studied. However, on close inspection of the *BBS2* locus in this family an extensive region of homozygosity in the affected patients was noticed, between *D16S3039* and *D16S3089*, inclusive (Fig 3.8). This ~ 6 cM interval contains the recently identified *BBS2* gene (Nishimura *et al.*, 2001). Once again, because of the lack of available DNA, the additional markers tested on the putative BBS2 family, B14 (all of which were within the *BBS2* critical interval), were not amplified in family B7. When compared with kindred B14, there was no sharing of haplotypes around or within the *BBS2* locus. Nonetheless, this does not exclude this locus. As was observed in the BBS6 families, there may be more than one disease haplotype at a single BBS locus in Newfoundland. For these reasons, it was difficult to categorize family B7 to just one BBS locus. It is possible that the affected member of family B7 had inherited mutations

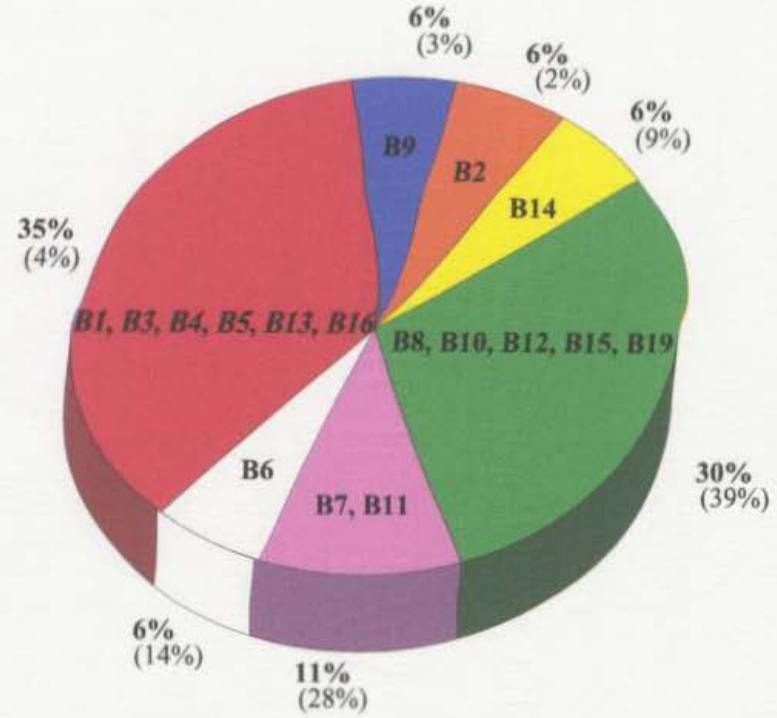
in both *BBS1* and *BBS2*. Now that *BBS2* has been identified, it should be possible to categorize this family more specifically.

Kindred B11, presumed to be consanguineous and containing two affected individuals with no unaffected siblings, could not be excluded from *BBS2-4* by haplotype or linkage analyses, since both affected sibs shared haplotypes at these loci (**Chapter 3.1.5**; Woods *et al.*, 1999). Therefore, haplotypes of the *BBS2* and *BBS3* markers in family B11 were compared with haplotypes in families B14 (*BBS2*) and B2 (*BBS3*). Additionally, family B11 was compared with family B7, a possible BBS2 kindred, at this locus. Adjacent to *BBS2*, there was only a single allele in common between families B11 and B14 - the *10* allele at *D16S3057* which is homozygous in B14 but heterozygous in B7. Also, a single allele was identical in the possible BBS2 family, B7, and B11 families at *D16S408* (allele *3*). Again, the allele was heterozygous in the B11 kindred. This similarity was on the opposite chromosome than the *10* allele of *D16S3057* in B11. Theoretically, this could indicate B11 shares different disease chromosomes with both B7 and B14 at *BBS2* if this locus is responsible for the disease in these two families. Haplotype comparisons between families B11 and B2 at the *BBS3* locus are even less convincing, as there is only one allele in common between them (allele *4* at *D3S1753*), and this is in the heterozygous state in family B11. Since there were no putative *BBS4* families identified on the island, the haplotypes at this locus were not compared. Evidence that the disease in kindred B11 is caused by *BBS2-4* is relatively unconvincing, which may mean that this kindred could be associated with a yet unidentified BBS locus.

However, since *BBS2* and *BBS4* have recently been identified (Nishimura *et al.*, 2001; Mykytyn *et al.*, 2001) they can now be screened for mutations in kindred B11. Additionally, when markers at the putative BBS locus on chromosome 18p were tested in this family, haplotype sharing was evident. If a BBS gene is categorized on 18p, then it could also be screened for mutations in kindred B11.

4.1.2 Distribution of BBS Loci in Newfoundland

The distribution of BBS loci in the 17 Newfoundland families is different than that observed in a recent large North American and north European survey of BBS families (Beales *et al.*, 2001). The most notable difference was the contribution of *BBS6* to the total proportion of BBS families. In Newfoundland, 35% of BBS families were categorized as BBS6 by mutation analysis of *MKKS/BBS6* (Fig 4.3). The Beales *et al.* (2001) study showed that in only 4% of 92 BBS families could the disease be attributed to mutations in *BBS6*. Less striking differences in frequencies of the other BBS loci were observed between the two studies. *BBS1* contributed to approximately 30% of the disease in Newfoundland and 39% in the larger study. The frequencies of *BBS3*, *BBS4* and *BBS5* are small in both this and the Beales *et al.* (2001) investigations. In Newfoundland, one (6%) of the 17 BBS families was unlinked to any of the six known loci and 14% of Beales *et al.* (2001) families were unlinked to any of the known loci. A large proportion



Note: BBS4 contributed 1% to the total in Beales *et al.* (2001) study.

Fig 4.3 Summary of the relative distribution of each BBS locus in Newfoundland compared with those calculated in the Beales *et al.* (2001) study of 92 North American/northern European BBS families (in italics).

(28%) of the North American/northern European survey BBS families were unassignable. This latter proportion was larger than identified in the Newfoundland studies (11%), probably because the pedigree structures (e.g. the large size and frequency of consanguinity) of Newfoundland kindreds made them more amenable to haplotype and linkage analyses.

However, the astonishing facts about BBS in Newfoundland were the high frequency and the genetic heterogeneity of BBS segregating in this small population. Newfoundland was settled by a relatively small number of migrants from the West Country of England and southeast Ireland. Interestingly, all BBS families in Newfoundland are Protestants, indicating their ancestors were English. Because of the lack of admixture between Protestants and Catholics, religious segregation organized the BBS families into an even more delineated population. Thus, it was originally suspected that all BBS patients in the province originated from a common founder. The first clue that this was not the case was the lack of clustering of BBS families all along the coast. However, it was not until genetic analyses were performed that the true heterogeneity of the syndrome was realized.

Since a single founder haplotype was evident in *BBS1* Newfoundland families (presumably corresponding to a single founder mutation), the population structure of the small, isolated communities along the south coast, from where these families originated, may contribute to the high frequency of BBS in Newfoundland. However, there were three mutations in *MKKS/BBS6*, presumably one each for *BBS1*, *BBS3* and *BBS5*, and at

least one for *BBS2*. Moreover, the likelihood of a seventh BBS locus has been raised. This suggests that the Newfoundland population is not a homogeneous genetic isolate, but comprised of multiple genetic isolates.

From a genetic perspective, the surprising number of BBS genes segregating in Newfoundland could be explained by a high mutation rate at these genes due to environmental circumstances. Namely, a mutagen was present in the environment causing DNA at BBS genes to become altered. Alternatively, BBS heterozygotes may have a survival advantage. Both hypotheses are highly speculative and not mutually exclusive. Although there was a report that suggested BBS heterozygotes were more obese than non-carriers (Croft *et al.*, 1995), there have been few studies of BBS heterozygotes. Not until an in-depth investigation is undertaken, comparing BBS heterozygotes with their non-carrying siblings, can a heterozygote phenotype, if present, be established. Only then could one postulate a possible advantage for this genotype.

The phenomenon of multiple founders of a rare recessive disorder, in a small isolated population, is not unique to Newfoundland. The island of Reunion, located in the Indian Ocean off the east coast of Madagascar, has approximately 700 000 inhabitants. Limb-girdle muscular dystrophy type 2A (LGMD2A), an autosomal recessive disease characterized by progressive symmetrical atrophy and weakness, has an estimated global prevalence of 1:100 000. But, on the small island of Reunion, its prevalence is over four fold higher. All LGMD2A patients on La Reunion belong to a small genetic isolate, presumed to have derived from a single ancestor who arrived in the

1670s (Richard *et al.*, 1995). However, when microsatellite marker analysis was performed on LGMD2A families within the putative *LGMD2A* critical interval, haplotype analysis demonstrated six different haplotypes segregating in the population. When *CALP3* was identified as the LGMD2A causing gene, mutation analysis showed six different mutations segregating in affected families (Richard *et al.*, 1995). Each mutation segregated with a different haplotype. This apparent contradiction of a disorder with a relatively low prevalence rate and multiple mutations in a single disease-causing gene, occurring in a small genetic isolate, was termed the 'Reunion paradox' (Richard *et al.*, 1995).

Several models to explain the 'Reunion paradox' have been put forward. As mentioned above, a high mutation rate at the disease gene may be the cause of this phenomenon (Zlotogora *et al.*, 1996). Two autosomal recessive disease genes are known to have multiple mutations, segregating in a small population in the region of the Galilee in Israel (Bach *et al.*, 1993; Heinisch *et al.*, 1995). In 14 families affected with either Hurler syndrome or metachromatic leukodystrophy, the parents of the affected were related. However, there was no known interfamilial relationship. Multiple homozygous mutations were demonstrated for each disease gene. These large consanguineous families originated from 13 villages that include a total of 150 000 inhabitants. Due to this population structure, Zlotogora *et al.* (1996) calculated that some of the disease mutations were recent and some more ancient. However, the etiology of the high mutation rate and of the mutation diversity is unknown. The authors suggest there could be a selective

advantage for heterozygotes.

An alternate model is that digenic inheritance accounts for the 'Reunion paradox' (Richard *et al.*, 1995; Beckmann, 1996). In this model, mutations at two unlinked genes are required to produce a phenotype. Specifically, a 'suppressor gene' is required that is active in the wider population but is inactivated by mutation in the isolated population. This hypothesis predicts that the population frequency of mutant alleles should be higher than the frequency estimated on the basis of disease prevalence alone. Second, several independent mutations are expected and there should be some families which have individuals with pathogenic mutation(s) but no clinical symptoms (Beckmann, 1996). For BBS, additional mutation analyses on controls in the Newfoundland and other populations would need to be performed to determine if unaffected individuals are carriers of BBS mutations. For the case of *MKKS/BBS6* in Newfoundland, independent mutations have been identified. Also, in the original study identifying *MKKS*, there were three individuals who were homozygous for the H84Y/A242S allele who did not present with the disease phenotype (Stone *et al.*, 2000).

4.2 Possible Digenic Inheritance of BBS in Newfoundland?

True digenic inheritance, defined as the inheritance of heterozygous mutations at two unlinked loci resulting in a human disease, is thought to be uncommon. This

phenomenon has been illustrated by mutational analysis in retinitis pigmentosa (RP)(Kajiwara *et al.*, 1994; Jacobson *et al.*, 1995). However, in the literature, the term digenic inheritance is often used to describe circumstances whereby two independent mutations at one locus and an additional mutation at an unlinked locus causes an autosomal recessive disease. This type of inheritance has been shown, by mutational analysis, to occur in Waardenburg syndrome type 2 with autosomal recessive ocular albinism (Morell *et al.*, 1997) and in junctional epidermolysis bullosa (JEB)(Floeth and Bruckner-Tuderman, 1999). Additionally, this latter definition of digenic inheritance is suspected to occur in non-syndromic hereditary hearing loss (Balciuniene *et al.*, 1998); Usher syndrome (Adato *et al.*, 1999); and Antley-Bixler syndrome (Reardon *et al.*, 2000). JEB is an autosomal recessive disease usually caused by mutations in *COL17A1* or *LAMB3*. The authors above described a proband with JEB who was a compound heterozygote for two *COL17A1* mutations and heterozygous for a *LAMB3* mutation. The Adato *et al.* (1999) investigation of digenic inheritance of Usher syndrome, also a recessive condition, indicated that one affected individual who had a heterozygous mutation in *MYO7* (which causes Usher syndrome when two independent mutations are inherited), also had haplotypes suggesting two defective *USH3* alleles. Kajiwara *et al.* (1994) described RP patients who had heterozygous mutations in two autosomal recessive RP genes (*peripherin/RDS* and *ROM1*). Interestingly, a study of a large multiplex Newfoundland family with autosomal dominant polycystic kidney disease (ADPKD) demonstrated bilineal inheritance of *PKD1* and *PKD2* in affected family

members (Pei *et al.*, 2001). This indicated that segregation of two unlinked mutant genes, causing a similar phenotype, in a single kindred has occurred in Newfoundland.

Interestingly, there is one Newfoundland BBS family, B14, in which the affected individual has a single *MKKS/BBS6* alteration (A242S) and was also HBS at the *BBS2* locus. Both genetic and mutational data suggest that the A242S alteration may not conform to a Mendelian model of disease transmission. First, a second mutant allele at this locus was not identified. Second, and of greater importance, both affected and unaffected sibs have the same chromosome 20 haplotypes and *MKKS/BBS6* alteration. One explanation for this might be that the A242S allele (and possibly other missense mutations) acts in conjunction with mutations at another locus, which would explain the family B14 haplotypes around *BBS2*, the inability of researchers to identify a substantial proportion of the second disease allele in patients with *MKKS/BBS6* mutations (Beales *et al.*, 2001), and pedigrees that cannot be assigned to any locus (family B6; Beales *et al.*, 2001). This model would not constitute true digenic inheritance - since there must be two mutations in *BBS2*, which, by themselves, are expected to cause the disease - but rather a modifier effect for some *MKKS/BBS6* mutations. Family B14 does exhibit a severe phenotype, since the patient presented with classic BBS features with an early age of onset of obesity and retinal disease. However, given the substantial heterogeneity of the syndrome, the interpretation of these data is circumspect until additional BBS genes and a substantial number of pedigrees exhibiting this complex pattern of inheritance are recognized. Interestingly, there are two published BBS families in which only a single

MKKS/BBS6 mutation was identified; both families demonstrated linkage to the *BBS4* locus (Beales *et al.*, 2001).

Another explanation of this observation is that the A242S allele does not cause BBS but is a rare neutral polymorphism. In the original study isolating *MKKS/BBS6*, the A242S alteration was identified as part of a complex mutation in an Amish MKS patient in which the other alteration was H84Y (Stone *et al.*, 2000). The authors concluded that the H84Y component of the complex allele in *MKKS* is also required for the phenotype.

To determine if family B6, which was excluded from all six BBS loci, possibly exhibits digenic inheritance due to the inheritance of a single mutation at two different BBS loci, disease haplotypes were examined and compared between BBS loci. Two scenarios were identified in which digenic inheritance of BBS was possible in the B6 kindred (Table 4.1). One possible scenario was that the affected siblings inherited a mutant maternal *BBS1* allele and a mutant paternal *BBS5* allele. However, when the complete haplotypes at these loci in family B6 were compared with those of the BBS1 and BBS5 families, little similarity was observed, although one allele in each haplotype within the critical regions was identical (Table 4.1). This could have occurred by chance, or perhaps because these markers were closest to the BBS genes, and these alleles were in linkage disequilibrium with the BBS gene (e.g. if family B6 was very distantly related to all BBS1 families and the BBS5 family on the island).

A second digenic scenario was that the affected individuals in family B6 inherited a mutant maternal *BBS3* allele and a mutant paternal *BBS6* allele. For the *BBS3* locus,

Table 4.1 Comparisons of haplotypes of affected siblings in family B6 with DHs from the *BBS1* and *BBS5* loci, and the *BBS3* and *BBS6* loci.

Marker	B6 m[a]	BBS1 HAP[b]	Marker	B6 p	BBS5 HAP	Marker	B6 m	BBS3 HAP	Marker	B6 p	BBS6 HAP (B5p)
D11S480	9	5/6/9	D2S1353	7	5	D3S1776	8	7/8/9	D20S192	5	6
D11S4205	4	3/4	D2S156	-	3/5	D3S1595	-	4/7	D20S115	5	7
D11S1883	9	7/8/10	D2S124	3	**7**	D3S1251	4	**9**	D20S900	8	5
D11S4945	-	**9**	D2S2330	8	**4**	D3S1752	6	**7**	D20S851	11	6
PYGM	8	**8**	D2S1776	8	**5**	D3S2419	-	**7**	D20S917	15	-
D11S4946	-	**5**	D2S335	5	**5**	D3S1271	3	**4**	D20S162	8	4
D11S913	7	5/6/7	D2S1238	13	5	D3S1753	4	**4**	D20S189	6	6

[a] m, indicates maternal haplotype; p, indicates paternal haplotype

[b] alleles in bold indicate all affected individuals in families linked to this BBS locus are homozygous at that marker

there was only a single allele in the affected sibs of family B6 identical to an allele in the BBS3 kindred (B2), and this allele was a marker outside the newly defined *BBS3* critical region (Table 4.1). Again, one allele was identical between the paternal *BBS6* haplotype of the affected sibs in family B6 and the affected individuals in the B5 family. This allele was at a marker within 0.5 cM of *BBS6*. Unfortunately, it will not be possible to test for digenic inheritance until all BBS genes have been identified. Moreover, proving digenic inheritance will require knowledge about how the BBS gene products interact, if indeed they do, and how observed alterations affect the gene products. Like the B14 kindred, the B6 kindred may have alterations in more than one BBS gene, but still suggest linkage to an unidentified BBS locus (e.g. *BBS7*).

4.3 The *MKKS/BBS6* Gene Product - A Putative Chaperonin

The role of molecular chaperones in the cytoplasm is vital for the proper folding of various proteins. Correct folding of a protein inhibits aggregation which can cause proteins to become non-functional. However, this is a difficult process because the cytosolic environment is fraught with potential impediments to proper folding. For example, the folding of a protein is coupled with its synthesis, thus the amino acid sequence becomes available sequentially. This may not be a problem for secondary structure fabrication, but it is inhibitory to tertiary structure formation, as only domains

that rely on contiguous segments of sequence can be folded. A topological restraint is also present, since there is a span of ~ 30 C-terminal amino acids within the ribosomal machinery waiting to exit at any one time. These cannot participate in folding (Agashe and Hartl, 2000). It is during the synthesis of proteins that chaperones may protect polypeptides from mis-folding and aggregating with other cytosolic proteins. The highly crowded cytoplasm also has an inhibitory effect on proper folding. Because the cytoplasm is dense with proteins, those which escape mis-folding during synthesis may still aggregate with other non-native proteins if they do not rapidly fold. Proteins which cannot fold quickly and independently are probably the ones which rely heavily on chaperonins.

The chaperonins, a family of sequence related chaperones of ~ 60 kDa, form double-ring complexes that enclose a central cavity in each ring. They are cylindrical, oligomeric complexes which have been shown to promote protein folding in conjunction with ATP hydrolysis (Braig, 1998; Wickner *et al.*, 1999). To promote proper folding, the chaperonin captures the substrate by hydrophobic contacts with multiple subunits in a ring. Then it displaces the protein into the inner central cavity, protecting it from the hostile environment outside (Agashe and Hartl, 2000). It has also been suggested that necessary forced unfolding, before proper folding, of the non-native polypeptide occurs (Shtilerman *et al.*, 1999).

There are two families of chaperonins: type I, including the bacterial GroEL, the mitochondrial Hsp60 (heat shock protein 60 kDa) and Hsp70, and the RuBisCO

(ribulose-bisphosphate carboxy/oxygenase) subunit binding protein from chloroplasts; and type II, consisting of the thermosomes from archaea [e.g. TF55 (thermophilic factor 55)] and the chaperonins found in eukaryotic cytosol [e.g. CCT (chaperonin containing *t*-complex polypeptide 1) also named c-cpn (cytosolic chaperonin) or TriC (TCP1-ring complex)]. The extensively studied GroE group (type I) have 7-fold symmetry and provide an enclosed chamber for protein subunit folding (Schoehn *et al.*, 2000). The central cavity is closed by a co-protein called GroES (Langer *et al.*, 1992). The type II chaperonins lack this cap-like co-protein, instead having a long insertion in the substrate binding domain which acts as a built-in lid. Recently, a hetero-oligomeric protein complex called prefoldin (or GimC, for genes involved in microtubule biogenesis complex) has been found to participate in type II chaperonin-mediated folding (Vainberg *et al.*, 1998; Leroux *et al.*, 1999). Type I chaperonins are homo-oligomeric with seven subunits per ring, while type II chaperonins in the eukaryotic cytosol are hetero-oligomeric, containing eight distinct, but related, subunits.

The human MKKS/BBS6 protein (570 amino acids), which is 76% identical to a homologous mouse protein (Stone *et al.*, 2000), shows closest similarity to type II chaperonins. A search of the protein sequence databases using the BLASTP algorithm (v2.1.2)(Altschul *et al.*, 1997) indicated that, apart from the mouse homolog of MKKS (Mkks; AF254074; score = 358, E value = 9e-98), the most similar sequence to the MKKS protein (AAF73872) was the thermosome chaperone protein thsA of *Pyrodictium occultum* (AJ006549) (score = 77, E value = 6e-13). The same query identified

sequences corresponding to 39 species of eukaryota, archaea and bacteria including human, chicken, goldfish, torafugu, fruit fly, round worm, yeast and numerous thermophilic bacteria. As well, the Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) identified a 493 amino acid consensus sequence, cpn60/TCP1 (pfam00118), contained in the chaperonin type II CCT proteins. This conserved domain was found in 17 species of fungi, archaea and bacteria. Also, the putative folding pattern of MKKS/BBS6 was most similar to the thermosome from *Thermoplasma acidophilum* (Stone *et al.*, 2000).

Unfortunately, little is known about the mechanism and substrates of the type II chaperonins. However, the *E. coli* GroEL structure has been widely studied and is considered the archetypical chaperonin. It is made of two stacked back-to-back heptameric rings (not octameric as in type II chaperonins) which create two cavities. Each of the two subunits contains three domains: (1) an ATP-binding equatorial domain; (2) a hinge-like domain in contact with both the ATP-binding and apical domains; (3) an apical domain which has an opening to the environment and exposes some hydrophobic residues towards the cavity for the binding of a non-native polypeptide (Agashe and Hartl, 2000). Unlike the case for the type II chaperonins, a second structure, GroES, attaches to the apical opening after the addition of ATP, forming a cage-like structure which traps the non-native polypeptide within the ring cavity. Folding occurs when the ATP hydrolyzes. With additional ATP binding to the adjacent inoperative ring, the GroES "cap" is released and the "cage" is opened. The folded protein can now leave.

Presumably, the cycle can start again in the adjacent ring structure once a non-native polypeptide attaches (Agashe and Hartl, 2000).

In *E.coli*, approximately 10% of new polypeptides are folded by the GroEL-GroES complex, and over 50 of these have been identified thus far. They include proteins involved in transcription, translation and metabolism. These proteins may initially be bound by chaperones co-translationally and then relayed to a subset of chaperonins where folding takes place, in what is called the 'pathway model' of assisted protein folding (Thulasiraman *et al.*, 1999). In this model, some chaperonins could be specific for relatively few substrates. This would result in the minimization of non-native polypeptides entering the cytosol, which would reduce aggregation. Alternatively, the 'network model' contends there is a competition for the substrate at all times, implying that non-native polypeptides are often released and captured again for additional folding (Farr *et al.*, 1997).

Recently, more information has been gathered on type II chaperonins. Eight subunits (α, β, λ, δ, ϵ, η, θ and ζ) with 30% identity to one another make up the TriC chaperone. One subunit, ζ, has a slightly different composition in mammalian testes than in other tissues, perhaps indicating an interaction with tissue specific substrates (Kubota *et al.*, 1997). In general, TriC is abundant in the testes and leukocytes, and weakly expressed in most other tissues (Gutsche *et al.*, 1999).

The structural features of the thermosome, the archetypical type II chaperonin, is similar to GroEL in many respects but there are documented differences. For example,

the inter-ring contacts differ between the two types, with each ring having different secondary structures in communication (Gutsche *et al.*, 1999). Also, as mentioned above, the GroES subunit associates with GroEL; however, there is no type II homolog. Instead, the latter protein complex contains a long protrusion which may act as a lid-like structure - holding the sequestered protein inside the ring cavity. Additionally, it has been suggested that GroEL only recognizes the overall hydrophobicity of its targets, but for TriC the pattern of hydrophobicity may be important (Rommelaere *et al.*, 1999).

Though there could be many possible substrates for the type II chaperonins, as approximately 10-15% of all newly made proteins interact with TriC (Thulasiraman *et al.*, 1999), the cytoskeletal proteins and the actins and tubulins are by far the most prominent substrates identified thus far (Kubota *et al.*, 1997; Lewis *et al.*, 1996). Other substrates include cyclin E (Won *et al.*, 1998) and G_{α}-transducin (Farr *et al.*, 1997) and up to 70 different polypeptides were found to transiently interact with TriC during their biogenesis in mammalian cells (Thulasiraman *et al.*, 1999). Because type II chaperonins may fold a wide range of polypeptides, spatial conformation is likely to be critical to these molecules, and disruptions may reduce the efficiency or abolish the ability to fold target proteins.

4.3.1 Results of Mutations in *MKKS/BBS6* on Putative Protein Function

The frameshift mutations, discovered in *MKKS/BBS6* in Newfoundland BBS families, result in the truncation of the polypeptide at amino acids 103 and 157. Therefore, only 18% and 28% of the protein would be produced, respectively. It is predicted that a non-functioning product is formed in each case. The pathogenic effect of the missense mutation, L277P, is more difficult to interpret in the absence of a functional assay. But, any point mutations may potentially cause a structural abnormality that could result in a functionally null protein, especially if that amino acid position is essential for proper function. According to one three-dimensional model of the protein (Stone *et al.*, 2000), this mutation would disrupt an α-helix potentially altering its structure. It is this α-helix along with two β-sheets in the *T. acidophilum* thermosome which protrude outward from the end of the subunit, blocking the entrance to the central cavity by forming a lid domain. Normally, this residue is part of a hydrophobic core which fixes the apical domain to the thermosome (Ditzel *et al.*, 1998). The additional kink at this position in the polypeptide could destabilize the lid structure, interfering with substrate binding and/or compartmentalization of the substrate. Additionally, this position is conserved within the conserved domain consensus sequence, cpn60/TCP1. These observations strongly suggest that this missense mutation causes the protein function to be abolished, or, at least, severely impairs the performance of the MKKS/BBS6 protein.

The result of the A242S alteration on the protein function, if any, is much less

obvious. As stated previously, A242S was originally identified in an MKS patient with a complex allele composed of the H84Y/A242S alterations (Stone *et al.*, 2000). The authors contended that the A242S "variant did not appear to alter the intermolecular interactions within the molecule". However, the alanine at this position is identical in *T. acidophilum* and in the cpn60/TCP1 consensus sequence, indicating that it perhaps is an important amino acid regarding the protein structure and function. This residue is part of a β-sheet in the α-subunit of the *T. acidophilum* thermosome, very near to the apical domain; however, there has been no report of the importance of this secondary structure in the function of this protein (Ditzel *et al.*, 1998). Due to its proximity to the lid area, it could play a role in substrate contact or lid mobility, but this is uncertain at this time.

Initially, there was a suggestion that all mutations causing BBS would be severe, causing obvious deleterious effects on the protein (e.g. frameshifts) (Katsanis *et al.*, 2000; Slavotinek *et al.*, 2000b), while those causing MKS would be less catastrophic (e.g. missense) (Stone *et al.*, 2000). However, more recently, missense mutations have been associated with BBS (Table 4.2). The effect of these mutations is more difficult to predict, although they likely affect either local polarity or the secondary or tertiary structure of the protein (Beales *et al.*, 2001). Notably, three of these mutations lie in exon 6 of *MKKS/BBS6*, which contradicts the hypothesis that exon 3 mutations are more severe and thus cause BBS rather than MKS (Katsanis *et al.*, 2000; Slavotinek *et al.*, 2000b). Polymorphisms within *MKKS/BBS6*, or variations in the promoter region, may influence the phenotype. Polymorphisms or mutations at other loci could also be causing the

Table 4.2 ***MKKS/BBS6*** **mutations identified thus far causing BBS and MKS.**

Alteration	Exon	Disorder	Reference
F94fsX103	3	BBS	**Chapter 3.6.2**; Katsanis *et al.*, 2000; Slavotinek *et al.*, 2000
D143fsX157	3	BBS	**Chapter 3.6.2**; Katsanis *et al.*, 2000; Slavotinek *et al.*, 2000
L277P	3	BBS	**Chapter 3.6.2**, Katsanis *et al.*, 2000,
Y37C	3	BBS/MKS[1]	Katsanis *et al.*, 2000; Stone *et al.*, 2000
T57A	3	BBS	Katsanis *et al.*, 2000
I32M	3	BBS	Beales *et al.*, 2001
Q147X	3	BBS	Beales *et al.*, 2001
S235P	3	BBS	Beales *et al.*, 2001
D285A	3	BBS	Beales *et al.*, 2001
G52D	3	BBS	Slavotinek *et al.*, 2000
Y264X	3	BBS	Slavotinek *et al.*, 2000
H84Y[2]	3	MKS	Stone *et al.*, 2000
1223-1224 delGG	5	MKS	Stone *et al.*, 2000
C499S	6	BBS	Beales *et al.*, 2001
S511A	6	BBS	Beales *et al.*, 2001
R518H	6	BBS	Beales *et al.*, 2001
A242S	3	BBS/MKS	**Chapter 3.6.2**; Stone *et al.*, 2000; Beales *et al.*, 2001

[1] Homozygous in BBS patient and heterozygous in MKS patient;[2] Identified as a part of a complex allele with A242S in MKS patients

differences in phenotype between MKS and BBS patients. Once the function of the MKKS/BBS6 protein has been elucidated, it may be possible to identify genotype-phenotype correlations. Additionally, novel mutations in a single BBS gene may account for the variation of the disease in general.

4.4 Candidate Genes for the *BBS1*, *BBS3* and *BBS5* Loci

The identification of *MKKS/BBS6* as a cause of BBS should expedite the isolation of other BBS genes. Based on the sequence of *MKKS/BBS6* and the putative structure and function of the MKKS/BBS6 protein, other candidate genes may be ascertained. Some understanding about when and where the BBS gene products are expressed in the developing fetus may also aid in identifying possible candidates.

4.4.1 Chaperonin-related BBS Candidate Genes

With the availability of sophisticated databases containing enormous amounts of continuously updated genetic data, it is possible to perform a significant amount of *in silico* research. The nucleotide and protein sequences of *MKKS/BBS6* were analyzed for sequence similarities in order to search for possible BBS candidate genes.

First, the cDNA sequences of the two splice isoforms of *MKKS/BBS6* were used to perform a search of the non-redundant nucleotide databases at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/BLAST/) using the BLASTN algorithm (v2.1.2) (Altschul *et al.*, 1997). The 1a splice isoform of *MKKS/BBS6* (AF221992) was queried, resulting in 74 BLASTN hits consisting of small sequence identities of less than 40 bp (scores $\leq$46, E values $\geq$0.05), other than the human and mouse *MKKS* genes. One hit of interest (score = 46, E value = 0.095) was a sequence contained in the filamin gene's (*FLNB*) exons 15, 16 and 17 (AF191606). An *MKKS/BBS6* splice isoform 1b (AF221993) BLASTN query returned the same results and two additional hits, consisting of a mouse sequence and a 21 base human sequence on chromosome 16 (both score = 44, E-value = 0.40). When this 21 base human sequence was queried, 59 sequences of little similarity resulted.

Because filamin is an actin-binding protein, and actin is one of the principle substrates of cytosolic chaperonins, it was considered a reasonable candidate. This filamin isoform is found in the cytoplasm of all non-muscle tissues cells (Chiang *et al.*, 2000). *FLNB* was mapped to chromosome 3; however, it was recently localized to the 3p14.3 band (Brocker *et al.*, 1999), terminal to the *BBS3* gene on 3p13-p12. Therefore, it was ruled out as one of the known BBS genes on the basis of chromosomal position.

No other nucleotide sequence results of significance were identified using BLASTN and, interestingly, only the mouse had a homolog of the human gene in the databases. Thus, the MKKS/BBS6 nucleotide sequence was partitioned into smaller

segments and queried. When overlapping sequences of 100, 200 or 500 bases were queried the results were the same as with the entire sequence - only small sequence identities of less than 40 bp (scores ≤46, E values ≥0.10) were identified, other than the human and mouse *MKKS/BBS6* genes.

A BLASTP search was also performed to determine if the amino acid sequence of MKKS/BBS6 was similar to any other known proteins. As mentioned above, the most similar sequence was the chaperone protein, thsA, from the thermophilic *Pyrodictium occultum*. Other chaperonin proteins from an array of organisms were also identified. These organisms had a conserved domain called cpn60/TCP1 unique to the type II chaperonins. Unfortunately, no other human proteins were similar to the MKKS/BBS6 protein. The cpn60/TCP1 conserved domain spanned about 470 of the 570 N-terminal amino acids of the MKKS/BBS6 protein. The 100 C-terminal amino acids were not part of the recognized conserved domain. Therefore, another BLASTP query was performed with this sequence, but no similarities with other sequences were identified. Additionally, when the MKKS/BBS6 protein sequence was queried in the Cluster of Orthologous Groups (COGs) database (http://www.ncbi.nlm.nih.gov/COG/), COG0459 was identified. COG0459 [Chaperonin GroEL (HSP60 family)] represents 80 proteins in 40 prokaryotic species.

Since the MKKS amino acid sequence was most similar to type II chaperonins, all chaperonin-like genes in the human genome were possible candidates. Therefore, all genes reported to have chaperonin function or those associated with chaperonins were

investigated to determine if their chromosomal positions mapped within the critical intervals of any BBS loci.

The most obvious candidates were the CCT family of genes, which are the eukaryotic equivalents of the thermosomes of the type II chaperonins. There are eight *CCT* genes, corresponding to the eight subunits of the functional chaperonin. None of these genes are located in any of the known BBS critical intervals. As well, the six subunits of the prefoldin protein complex, which escorts non-native polypeptides to the type II chaperonin, were considered putative candidate genes for BBS. However, again, none of these genes mapped to the six BBS loci.

Homologs of the heat shock type I chaperonins are also present in the human genome. Over 75 human heat shock related genes have been identified and these were also considered possible candidates, due to their function as chaperones. One Hsp40 homolog, *DNAJC4*, maps to 11q13 and was examined for mutations in *BBS1* families. However, no alterations were found in splice sites or exons (Dr. Nicholas Katsanis, personal communication). No other Hsp40 subunits mapped to BBS loci. The mitochondrial heat shock protein homologs, *HSPD1* and *HSPE1*, are found on chromosome 2. Interestingly, *HSPD1* contains the cpn60/TCP1 domain; however, its specific chromosomal location is unknown. *HSPE1*, the 10 kDa subunit which associates with the *HSPD1* gene product, resides on 2q32, qter to the *BBS5* locus.

Little is known about the substrates which interact with the type II chaperonins. However, a few such proteins have been characterized including tubulins, actins, cyclin

E, α-crystallin and α-transducin. Therefore, all genes known to encode these proteins were investigated to determine chromosomal location. A number of these are located within the critical intervals of some BBS loci. *KIFC3*, a tubulin cytoskeleton-associated gene found in the retina, retinal pigment epithelium, kidney and lung is located on 16q21 (Hoang *et al.*, 1998). It was screened for mutations in *BBS2* families. However, no alterations were identified in the splice sites or coding regions (Dr. Nicholas Katsanis, personal communication). Another tubulin-associated gene which also maps within a BBS loci is *ARL2*. ARL2 (ADP-ribosylation factor-like 2) is a GTP-binding protein which aids in the folding of tubulin and maps to the *BBS1* interval. It is a small protein of 184 amino acids having ubiquitous expression (Clark *et al.*, 1993). It is not known to be folded by chaperonins, but it is small enough to fit inside the inner cavity of a TCP-1 protein complex and it is probably functionally important in vesicular transport, like its ARF (ADP-ribosylation factor) relatives (Amor *et al.*, 1994). None of the numerous actin-associated genes (other than *FLNB*) map within the critical regions of known BBS loci; nor did the cyclin E, α-crystallin or the α-transducin genes.

4.4.2 Other Positional Candidate Genes

Thus far in the published literature, six genes (excluding *MKKS/BBS6*, *BBS2* and *BBS4*) have been screened for mutations in BBS patients (Table 4.3). In five of these, no

Table 4.3 Candidate genes previously screened for mutations in BBS patients.

Gene	Locus	Reference
myosin IXA (MYO9A)	15q22-q23	Gorman *et al.*, 1999
Immunoglobulin superfamily containing Leucine-rich Repeat (ISLR)	15q22-q23	Nagasawa *et al.*, 1999
Nuclear Receptor subfamily 2, group E, member 3 (NR2E3)	15q22-q23	Haider *et al.*, 2000
EH-Domain containing 1 (EHD1)	11q13	Haider *et al.*, 1999
Recoverin	17p13.1	Parminder *et al.*, 1997
peripherin/RDS	6p21	Sohocki *et al.*, 2001

mutations were identified. Interestingly, recently, a mutation (R13W) in *peripherin/RDS* on 6p21 was identified in a BBS family (Sohocki *et al.*, 2001). However, it was not stated if the patient was heterozygous or homozygous for the mutation. No information describing clinical evaluations, family history or other genetic testing on the kindred was provided. It was difficult, therefore, to interpret whether the mutation was suggested to cause BBS or was co-segregating with a BBS gene. More information is needed in order to characterize *peripherin/RDS* as a possible BBS gene. Furthermore, no BBS locus has yet been identified on 6p21.

Identification and mutation screening of 24 transcripts within the 1 cM region of the *BBS1* interval was not successful in characterizing this elusive gene (Dr. Nicholas Katsanis, personal communication). With the continuing cataloging of new genes, thanks in large part to the Human Genome Project, other candidate genes have been identified, but remain unscreened. Some of these candidates, located within the critical regions of the BBS genes, are expressed in the appropriate tissues and/or have a function which could cause the manifestations encountered in this syndrome.

One such gene, located in the small but gene-rich *BBS1* critical interval, is *MAP4K2* (*GCK/RAB8IP*). By Northern blot analysis, a transcript was observed in all adult tissues tested, including kidney, heart, brain, pancreas, skeletal muscle, lung, liver and placenta tissues (Katz *et al.*, 1994). Cell fractionation and immunofluorescence analyses indicated MAP4K2 is present both in the cytosol and as a peripheral membrane protein, concentrated in the Golgi region and basolateral plasma membrane domains

(Tibbles and Woodgett, 1999). *MAP4K2* is composed of 32 exons and is qter to *PYGM* (Kedra *et al.*, 1997).

First identified in the germinal center of B lymphocytes, *MAP4K2* encodes a mitogen-activated protein kinase kinase kinase kinase. It was initially determined to bind to RAB8/MEL, a member of the RAS superfamily, which comprises small GTP/GDP-binding proteins. The latter may play a role in the transport of proteins from the endoplasmic reticulum to the Golgi and the plasma membrane. Two of MAP4K2 's substrates are casein and myelin basic protein (Katz *et al.*, 1994); neither of these map to a known BBS locus. Thus, RAB-regulated protein phosphorylation by MAP4K2 is possibly important for vesicle targeting or fusion. Moreover, MAP4K2 may serve to modulate secretion in response to stress stimuli (Katz *et al.*, 1994). Interestingly, Beales *et al.* (1999) noticed that 25% of their patients had asthma - three times the national UK prevalence - and these were exclusively BBS1 patients. It is known that allergic asthma is dependent on an IgE (immunoglobulin E) response controlled by T and B lymphocytes and activated by the interaction of antigen with mast cell-bound IgE molecules. *MAP4K2* is expressed in germinal center B cells where Ig genes hypermutate, acquiring somatic mutations in heavy and light Ig chains that may alter the specificity/affinity of B cell antigen receptors (Katz *et al.*, 1994).

MAP4K2, a serine-threonine protein kinase, is also known to modulate other mitogen activating protein kinases (MAPKs). It is associated with the SAPK (stress activated protein kinase), p38 and ERK (extracellular signal regulated kinase) pathways

(Tibbles and Woodgett, 1999). Mammalian MAPK pathways regulate an extensive range of cellular processes including gene transcription, cytoskeletal organization, cell growth, immune activation, development and apoptosis. MEKKs (MAPK and ERK kinase kinases) and MLKs (mixed lineage kinases) are regulated by MAP4K2. These, in turn, phosphorylate the next protein kinases in the above pathways, ultimately leading to the regulation of transcription factors.

There are also upstream regulators of MAP4K2. Upon receptor binding at the cell surface, MAP4K2 can be recruited by TRAF2 (tumour necrosis factor α receptor-associated protein 2). TRAF2 is capable of binding to numerous members of the TNFR (tumour necrosis factor receptor) superfamily and was hypothesized to be the point of bifurcation of the signals to the NFκB (nuclear factor kappa B) transcription factor and the activation of SAPK (Tibbles and Woodgett, 1999). Interestingly, the gene for TANK/I-TRAF (TRAF family member associated NFκB activator/TRAF-interacting protein), a molecule which has been shown to augment or block NFκB activation mediated by TRAF2 (Rothe *et al.*, 1996), is located on 2q31, within the *BBS5* critical interval. Thus, two possible positional candidates genes, *MAP4K2* and *TANK*, are linked in a common pathway.

These two genes could be members of a novel molecular pathway incorporating all BBS proteins (Fig 4.4). Binding of a theoretical cell membrane-bound receptor by its ligand could initiate the intracellular pathway. The receptor and agonist could be prolific members of a known family of receptor-ligands that act in the systems affected in BBS,

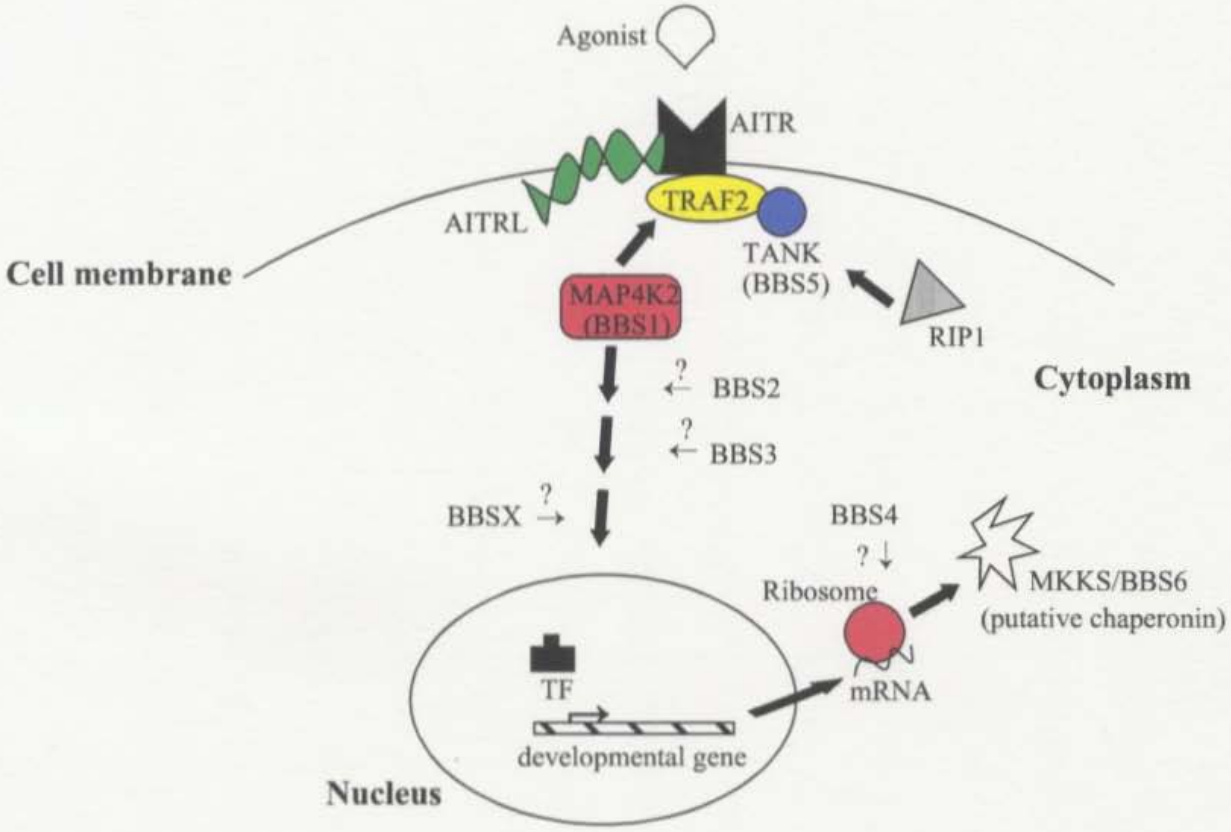


Fig 4.4 Possible molecular pathway involved in the causation of BBS, incorporating candidate genes known to be in the MAPK pathways at known and putative BBS loci.

such as TNFR1 and TNFα. This interaction could attract a receptor-associated factor such as TRAF2 which subsequently recruits MAP4K2 (BBS1?) and TANK (BBS5?). Perhaps additional associated factors are also recruited in a cell type and temporal manner. This combination of molecules would initiate a specific kinase dependent pathway which could ultimately lead to the activation of a transcription factor (TF). This TF may activate transcription of a gene(s) needed in response to environmental cues in development (e.g. the joining of the collecting tubules and the secreting tubules resulting in decreased proliferation of renal cells in the developing kidney). The putative chaperonin, *MKKS/BBS6*, may form a homo-multimeric protein which would properly fold the TF, the protein transcribed by the TF, or another pathway specific polypeptide. The other BBS protein products not mentioned - BBS2, BBS3, BBS4 and yet unidentified BBS gene(s) - could be incorporated into such a model as protein kinases, accessory proteins, other transcription factors or target genes, or chaperonin-related members.

A report was recently published of six BBS patients in a multiplex kindred who inherited a pericentric inversion (PEI) of chromosome 1 (p36.3q23) (Tayel *et al.*, 1999). The two mothers of the affected individuals also carried the PEI but were not affected. However, none of the five unaffected sibs in the two sibships tested carried the rearrangement and neither did the only father tested. The correlation between BBS and this PEI may be coincidental. However, 1p36 contains no less than five TNFRs and 1q23 contains two ligands of TNFRs. One of these ligands, a transmembrane protein called *TNFSF18/AITRL* (tumor necrosis factor (ligand) superfamily member 18/activation

inducible TNF family member), has its receptor, *TNFRSF18/AITR* (tumor necrosis factor receptor superfamily member 18/activation inducible TNFR family member), on 1p36.3. Thus far, AITR and its ligand seem to be important for interaction between activated T lymphocytes and endothelial cells (Gurney *et al.*, 1999; Kwon *et al.*, 1999). However, to date, only the two studies which initially identified these two novel proteins have been published. Although the breakpoints are not mapped in this PEI, it is interesting that a receptor and its ligand could be affected by the rearrangement. PEIs are mostly balanced, but familial translocations and inversions can predispose to the formation of uniparental disomy, whereby autosomal recessive mutations can be reduced to homozygosity (Cavenee *et al.*, 1983; Spence *et al.*, 1988; Pentao *et al.*, 1992). Perhaps if uniparental disomy did occur and either one of these receptors or ligands, or possibly both, were affected by the PEI, then such a phenomenon may cause BBS in this family.

On 18p11.3, within a putative BBS locus (tested in **Chapter 3.5**), there is also a member of a MAPK pathway - *RALABP1/RIP1* (RalA binding protein 1/Ral-interacting protein 1). This serine/threonine kinase interacts with Fas and TNFR1, stimulating apoptosis via the SAPK and NFκB pathways *in vitro*. *rip1* deficient mice appear normal at birth but fail to thrive, displaying extensive apoptosis in both the lymphoid and adipose tissue (Kelliher *et al.*, 1998). RIP1 is known to interact with TRAF1, 2 and 3 (Arch and Thompson, 1998). Because of the already known complexities and intricacies of the MAPK pathways, it is feasible that alterations in proteins confined to specific cells at specific times during development, and possibly during adult life, in a novel but related

pathway, could lead to the BBS phenotype.

Other good candidate genes, presently unrelated to the MAPK pathways, are also located within the various BBS critical regions. The *DUTT1* gene is a member of the Ig superfamily and a new member of the cell adhesion molecule family NCAM. It is located on 3p12, within the *BBS3* critical region. It contains multiple fibronectin III domains and putative transmembrane and intracellular domains (Sundaresan *et al.*, 1998). Adult human tissue expression is evident in all tissues tested, but weaker in kidney. Also, when mouse embryo tissues were tested, there were identical expression patterns as in adults, with the maximal expression on day 11, but none on or before day 7 (Sundaresan *et al.*, 1998). This pattern was similar to the pattern found with the *mkks* transcripts in mice (Dr. Nicholas Katsanis, personal communication). There is also some evidence that *DUTT1* is involved in dimer formation, providing other opportunities for protein interaction (Sundaresan *et al.*, 1998). Such a gene, expressed in many tissues during development, localized to the *BBS3* interval and putatively part of an unknown pathway, makes an appealing *BBS3* candidate.

Another interesting candidate, positioned within the *BBS5* region on 2q31, is an α-integrin gene, *ITGAV* (Fernandez-Ruiz and Sanchez-Madrid, 1994). It undergoes post-translational cleavage to yield disulfide-linked heavy and light chains that combine with multiple integrin β-chains to form various integrins (Sims *et al.*, 2000). Among the known associating β-chains (β-chains 1, 3, 5, 6, and 8) each can interact with extracellular matrix ligands. The most studied ligands are associated with the $\alpha_v\beta_3$

heterodimer and include fibrinogen, thrombospondin, von Willebrand factor and vitronectin; none of these are known to map to BBS loci. This α-integrin is known to function in angiogenesis and vasculogenesis (Sims *et al.*, 2000). Notably, two other α-integrin genes map to 2q31 - *ITGA6* and *ITGA4* - possibly comprising a cluster of such genes (Fernandez-Ruiz and Sanchez-Madrid, 1993).

Initially, the *BBS5* locus was assigned in close proximity to the *HOX*D-gene cluster on chromosome 2q31. This led to the suggestion that these nine homeobox genes of the *Drosophila antennapedia* class, and other closely located genes *(EVX2* and *DLX1/DLX*2) that are involved in patterning of the embryo, were candidate genes for *BBS5*. Recent findings that duplication of the *HOXD13* gene causes synpolydactyly (Akarsu *et al.*, 1996) earmarked it as a promising gene candidate, given that syndactyly and/or polydactyly are congenital manifestations of BBS. However, refined mapping of *BBS5* within the 13 cM interval *D2S156–D2S1238* placed it several centimorgans up-stream from the *HOXD13* gene that is positioned at the proximal end of the *HOX*D-gene cluster (Spurr *et al.*, 1996). Refined mapping of the recombinant ancestral chromosome excludes all genes within the *HOX*D-gene cluster as being candidate genes for *BBS5*.

Though the identification of *MKKS/BBS6* has directed researchers to specific candidates that are possibly related to chaperonins themselves or their function, no clear candidates genes have been assessed. More information about the function of *MKKS/BBS6* and with what it interacts with is required before other candidate genes can be put forward with certainty.

During the writing of this thesis, *BBS2* and *BBS4* were identified on 16q21 (Nishimura *et al.*, 2001) and 15q23 (Mykytyn *et al.*, 2001), respectively. The open reading frame of the *BBS2* gene (AF342736) is composed of 2163 bp comprising 17 exons. Two frameshift, one substitution and two nonsense mutations were identified in five unrelated BBS families. One frameshift and the two nonsense mutations were in exon eight. When the BBS2 protein sequence was compared to other chaperone or chaperone-like proteins, no significant similarity was found to any other genes with known function. It was concluded that *BBS2* is a novel gene whose function could not be determined by comparison of primary sequence with any other known gene. Also, no obvious possible interactions with *MKKS/BBS6* were identified. Due to the identification of *BBS2* it will now be possible to screen families B6, B7, B11 and B14 for mutations.

The identification of *BBS4* came not long after that of *BBS2* (Mykytyn *et al.*, 2001). *BBS4* (AF359281) consists of 16 exons spanning 52 kb with an open reading frame of 519 codons. Mutations were found in five consanguineous families and consisted of a two exon deletion (exons 3 and 4), a substitution mutation (R295P), and two splice site alterations. Interestingly, the 6kb deletion was found in both an Italian family and an unrelated Israeli Arab family. Furthermore, the deletion breakpoints were within Alu repeat elements in introns 2 and 4. Also, in an additional non-consanguineous family, only a single mutation was identified (585-586insTG) and no mutations were found in a consanguineous family in which there was evidence for homozygosity at the *BBS4* locus. It was also shown that *BBS4* is ubiquitously expressed, with the highest

level of expression found in the kidney.

The BBS4 predicted protein sequence (AAK58868) is most similar to O-linked N-acetylglucosamine (O-GlcNAc) transferase (OGT; Mykytyn *et al.*, 2001). OGT in *Arabidopsis*, called SPINDLY, has been shown to be a signal transduction protein involved in a variety of developmental processes (Jacobsen *et al.*, 1996). OGT is a nucleocytoplasmic glycosyltransferase which adds a single GlcNAc to hydroxyl groups of serine and threonine residues. Proteins that are modiifed by OGT include transcription factors, kinases, cytoskeletal proteins, and nuclear pore proteins (Roos and Hanover, 2000). This posttranslational modification may prevent protein interactions required for transcription activation and protein degradation (Han and Kudlow, 1997; Su *et al.*, 1999). Particularly, OGT may function in glucose metabolism since it has been shown to act upon proteins involved in this pathway, including glycogen synthase kinase 3 (GSK3), casein kinase II (CKII), and insulin receptor substrate 1 and 2 (IRS1 and 2)(Lubas *et al.*, 1997; Patti *et al.*, 1999). Thus, OGT has been implicated in insulin resistance and may play a role in diabetes (Lubas *et al.*, 1997; Hanover *et al.*, 1999).

Like the OGT protein, BBS4 contains a structural motif called the tetratricopeptide repeat (TPR). The TPR motif is a degenerate, 34 amino acid repeat that is found in proteins involved in a wide range of cellular functions with the majority of them participating in cell cycle control, transcription, protein transport, protein folding and regulatory phosphate turnover (Blatch and Lassle, 1999). This motif has been shown to be important in protein-protein interactions between a TPR protein and one or more

non-TPR proteins. One of the major types of complexes that involve TPR proteins is the molecular chaperone complex. The co-chaperone STIP1 (stress-induced-phosphoprotein 1) is an Hsp70/Hsp90 organizing protein, perhaps forming a 'bridge' between the two chaperones (Smith *et al.*, 1993). Although BBS4 contains only one TPR motif, and multiple TPRs are needed for protein-protein interactions (Scheufler *et al.*, 2000), it is possible that BBS4 oligomerizes to bring multiple TPRs together. Therefore, BBS4 could possibly operate in a chaperone complex. However, TPR domains interact with carboxy EEVD motifs (Scheufler *et al.*, 2000) which are not present in the BBS2 and BBS6 proteins.

4.5 Possible Cause of BBS

Clues to the cause of BBS may be ascertained by considering human embryology, especially during the time of organogenesis in the second and third month of development. At five weeks of fetal development, the hand plate forms, the optic cup and lens vesicle begin to invaginate, the cerebral hemispheres are well marked, the metanephros begins to develop and the cloacal folds and genital tubercle have appeared (Larsen, 1997). Subsequently, in the sixth week, the foot plate develops and the hand rays form. The major and minor calyces form and the kidney begins to ascend. Genital ridges appear, the neural layer of the retina begins to develop and pigment appears in the

outer layer of the optic cup (Larsen, 1997; Sadler, 2000).

By the end of the eighth week, the mesenchymal core of the peripheral part of the platelike enlargement at the extremity of the limb bud has become increasingly condensed to outline the digits, and the thinner intervening areas break down from the circumference toward the centre. This apoptotic process, occurring in the necrotic zones between the rays, may be carried out incompletely, causing the syndactyly and brachydactyly present in BBS patients. Eventually, the blind ends of the secreting tubules in the developing kidney must establish communication with the blind ends of the collecting tubules derived from the metanephrogenic cap of the ureteral diverticulum. Failure to do so leads to the formation of calyceal cysts, a characteristic renal finding in BBS patients (Parfrey *et al.*, 1997).

Although the definitive layers of the retina do not develop until late fetal life, there is some differentiation observable in the stratification of this tissue. The inner and outer neuroblastic layers are evident, as are the pigment retina, nerve fiber layer, and the internal and external limiting membrane (Larsen, 1997). The disarray of this stratification and the malfunctioning of the photoreceptors, which are evident in the retinal dystrophy in BBS, could begin around this time in embryonic eye formation.

The male and female genitalia are virtually indistinguishable in their development up to this juncture, but after the twelfth week they begin to differentiate more recognizably. The shaft of the penis grows and the scrotum forms, and in the female, the vaginal plate elongates and canalizes, opening up into the uterus (Larsen, 1997; Sadler,

2000). Lack of growth in the genitalia during this period, or previous to this, could lead to the hypogonadism observed in male BBS patients. In females, the structural malformations causing hydrometrocolpos would most likely occur at this time, as the uterus and vagina are developing.

The time between when the fingers form and the blind ends of the tubules and the collecting ducts join in the kidney is a relatively long duration. How, when, and if the systems affected in BBS intersect is difficult to determine. However, between the eighth and tenth weeks after conception, the hands and feet, the layers of the retina, the genitalia, and the kidneys have significantly developed and the cerebral cortex is differentiating. It is possible that a fundamental developmental pathway that should be operational at this time, during the maturation of several organs, may be defective in BBS. This prediction by Parfrey *et al.* (1997) is supported by preliminary results on the expression of *MKKS/BBS6* during development. The *MKKS/BBS6* gene was ubiquitously expressed in humans after week seven of gestation. In the mouse, *mkks* transcripts were detected by stage 8.5 in the heart, in limb buds and forebrain by stage 9.0, and in every tissue by stage 11.5 (Dr. Nicholas Katsanis, personal communication).

An alternative hypothesis is that the BBS genes are regulated at different times during development. For example, they could be turned on in the hand during week six to seven when the fingers are differentiating, and in the kidney at week ten when the forming nephron joins the collecting tubule - a tissue and time specific system. Such a process seems more complex than the possibility that the affected systems have a

temporal intersection during their development. However, even if such an overlap does not exist, a mishap at some time previous to the formation of some affected systems, but during the development of others, could result in a chain reaction that would eventually cause the malformations observed in these later forming tissues. When and where the defect in the putative developmental pathway resulting in BBS occurs will remain speculative until studies on the function of BBS genes are undertaken.

4.5.1 Further Research into the Cause of BBS

The observation that mutations at any one of a minimum of six independent BBS loci can result in nearly identical phenotypes suggests that the products of these genes are involved in a common biochemical or developmental pathway or that they are related as part of a multi-subunit complex. The apparently unrelated pathologies characteristic of BBS made it difficult to predict potential candidate genes until the discovery that mutations in *MKKS* can cause BBS. This should accelerate the identification of other BBS genes through the identification of proteins that cooperate with *MKKS/BBS6*, *BBS2* and/or *BBS4* to form a multi-subunit chaperone, or that require these genes for correct folding.

One commonly used technique to identify proteins that interact with a protein of interest is the yeast two-hybrid system. In this library-based method, in order for

transcription of a reporter gene to occur, there must be attachment of both a DNA-binding protein and an activation protein to an upstream promoter region. The binding protein is attached to the gene of interest and the activator domain attaches to a number of test proteins. Only a protein that binds to the test protein will result in the transcription of the reporter. Another commonly implemented method is phage display technology. Again this is a library-based method in which members of a cDNA library are cloned into a phage gene which encodes a phage coat protein. The recombinant gene will be displayed on the virus' outer covering. The protein of interest, with a reporter molecule attached, can be used to screen the recombinant phage library and any proteins interacting with the probe-reporter molecule can be purified. A less sophisticated, but robust, approach to identifying proteins interacting with *MKKS/BBS6*, *BBS4* or *BBS2* is the classical co-immunoprecipitation method. Cell lysates are generated, antibody is added, the antigen is precipitated and washed, and bound proteins are eluted and analyzed.

An important technology that could be utilized in the study of BBS gene expression and function is the creation of genetically modified animals. By gene targeting using homologous recombination, it is possible to alter or inactivate a gene and monitor the effect of the mutation on the development of the animal. The *Cre-lox*P site-specific recombination system is a powerful tool in this type of gene targeting. The function of the Cre recombinase is to mediate recombination between two *lox*P sequences that are in the same orientation, leading to excision of the intervening sequence between the two *lox*P sites. Using gene targeting, *lox*P sequences can be positioned into a desired

gene or chromosomal location. When the *Cre* product is provided, an artificially generated site-directed recombination event will occur. Because of the important nature of the BBS gene products during development, the creation of an *mkks* knock-out mouse may result in an organism which is unviable. Therefore, cell type- and tissue-specific knock-out mice may be required. Mice with a Cre recombinase gene linked to a tissue specific promoter are mated with mice containing the sequence of interest flanked by *lox*P sites. Offspring which contain both the *lox*P flanked sequence and the *Cre* gene will express the *Cre* gene in the desired tissue type, resulting in the tissue specific inactivation of the target locus. By using a tetracycline responsive promoter to express Cre recombinase, a temporal as well as a tissue specific expression pattern can be created. In this way, it would be theoretically possible to determine the effects of mutations in *mkks* in the kidney, extremities, brain and any other desired tissue at different periods of development.

Additional clinical information from BBS families in Newfoundland may also accelerate the accumulation of knowledge regarding the etiology of BBS. Several investigators have compared the phenotypes among and between families with different forms of BBS. Carmi *et al.* (1995) tried to identify specific clinical features that were indicative of a particular BBS locus using the original three Arab-Bedouin kindreds linked to *BBS2*, *BBS3*, and *BBS4*. They concluded that mutations in *BBS3* was associated with polydactyly of all four limbs, whereas in BBS4 patients, polydactyly was usually limited to the hands. They also indicated that the BBS4 form was associated with early-

onset morbid obesity while BBS2 appeared to present the "leanest" form of BBS. The identification of *BBS2* revealed that this association does not hold outside the Bedouin population [non-Bedouin BBS patients with obvious obesity were homozygous for *BBS2* mutations (Nishimura *et al.*, 2001)]. Also, the "BBS3 phenotype" described by Carmi *et al.* (1995) was not observed in any of the five affected members of the Newfoundland BBS3 family (Young *et al.*, 1998). Additionally, in a survey of 44 Scandinavian individuals with BBS, Riise *et al.* (1997) were unable to find any distinctive clinical features that could separate individuals linked to *BBS4* from those unassigned to a particular locus. Finally, only subtle phenotypic differences were noticed in a study of 18 BBS families linked to *BBS1*, *BBS2* and *BBS4* (Beales *et al.*, 1997). The most striking difference was that affected offspring were taller than their parents in the BBS1 category, whereas affected subjects in the BBS2 and BBS4 groups were significantly shorter than their parents. This led Beales *et al.* (1997) to speculate that the different genes responsible for BBS may influence growth characteristics such as height.

Because environmental conditions and the genetic background of the Newfoundland population are probably more similar than in other such studies, a comparison of the clinical and phenotypic features of affected individuals with BBS on the island may determine the extent of variation within a locus and between loci. Also, a study of the possible phenotype of heterozygotes may be ascertained more readily in Newfoundland for the above reasons and because of the availability of large families with multiple affected and unaffected siblings.

Identification of additional BBS genes should also aid in determining the molecular basis of BBS. More candidate genes must be screened. Determining the location of at least one additional BBS gene (*BBS7*) should also expedite this process. Like the B13 family, the B6 kindred, which was excluded from all known BBS loci, could be used in a genome-wide scan. Although it will not provide statistically significant evidence for linkage, it could be used in conjunction with other unlinked families identified by collaborating groups. If a putative locus for *BBS7* is identified, it could be tested in these families for confirmation and for possible refinement of the critical region.

4.6 Concluding Remarks

Using Newfoundland families, it was possible to characterize the first gene to cause Bardet-Biedl syndrome (*MKKS/BBS6*). Initially, a genetic survey of 17 BBS families with DNA markers linked to four known BBS loci *(BBS1-4)* was undertaken. As a consequence of this survey, evidence for a fifth BBS locus (*BBS5*) was established. Subsequently, this locus was mapped to 2q31 in a large consanguineous Newfoundland kindred. The *BBS1* locus was also narrowed to 1 cM and the *BBS3* locus was confirmed and further delimited to a 6 cM interval. Six BBS families were excluded from all five known BBS loci. One of these six families was used for a genome-wide scan,

implementing homozygosity mapping of pooled DNA samples. Through this method, a sixth BBS locus (*BBS6*) was identified on 20p12 and subsequently confirmed by five other BBS families. A candidate gene, *MKKS*, was identified within the 2 cM *BBS6* critical region and was screened for mutations in affected individuals from six families. All six families were shown to segregate *BBS6* alterations causing the disease in affected individuals. Surprisingly, three different mutations were identified. One kindred was excluded from *BBS1-6*, indicating a seventh BBS gene (*BBS7*), further demonstrating the genetic heterogeneity of BBS in Newfoundland. It was possible to categorize 14 of the 17 Newfoundland BBS families to a single known BBS gene or locus.

One practical application of this work is that it gives individuals from BBS6 families the option of carrier status testing. This will allow more accurate genetic counselling.

The information gathered in this thesis on the genetic basis of Bardet-Biedl syndrome, using Newfoundland kindreds, should assist in the elucidation of other BBS genes and their products. In addition, such information will aid in the unravelling of the mechanisms which govern the regulation and distribution of body fat, as well as retinal, limb, and kidney development.

Addendum

Eleven days prior to the oral defense of this thesis, an article by Katsanis *et al.* (2001) was published pertaining to an alternative model of inheritance in Bardet-Biedl syndrome (*Science* **293:** 2256-59). Specifically, the authors demonstrate that the BBS phenotype is due to triallelic inheritance in some families. Thus, they conclude BBS may not strictly have an autosomal recessive mode of inheritance, but one that spans the gap between classical Mendelian disorders and complex traits.

In this paper, eight families, mapping to the *BBS2* locus, were initially identified with having only a single mutant allele within the open reading frame and exon-intron boundaries of *BBS2*. Microsattelite analysis was also performed to determine if any of these pedigrees indicated linkage to *BBS2*, however only one of six informative families was consistent with linkage to this gene. Also, when additional microsattelite markers were tested on the five families unlinked to *BBS2*, three of these families were excluded from all BBS loci except *BBS1*, one family was consistent with mapping to *BBS3* only, and one to *BBS6* only. This suggested that there could be mutations in two different BBS genes in affected persons from these families. Therefore, they screened 19 BBS families which had one or two *BBS2* mutations, for mutations in *BBS6* (at this time only *BBS2* and *BBS6* had been identified). Interestingly, four pedigrees contained three mutant BBS alleles - two families with two *BBS2* mutations and one *BBS6* mutation, and two families with one *BBS2* mutation and two *BBS6* mutations. One Newfoundland family (B14), characterized in this thesis, which was shown to be HBD at the *BBS2* locus and also having a A242S alteration in *BBS6*, was one of these four triallellic kindreds. In addition, the argument for triallelic inheritance was augmented by the fact that in

one family, an affected individual and an unaffected sib had two nonsense mutations in *BBS2*, but only the affected patient had a mutation in *BBS6*.

Thus, Katsanis *et al.* (2001) provided some evidence for a trialleic mode of inheritance in this disease. However, it remains uncertain if such a mechanism is required to cause BBS in all circumstances, or if it is exhibited only with a certain combination(s) of BBS loci and/or mutations.

In Newfoundland, triallelic inheritance may not be an important BBS-causing mechanism. If considered a true autosomal recessive disorder, then 25% of offspring of parents who carry a BBS mutation, at a single BBS gene, should be affected with the disorder. In the 22 BBS families in Newfoundland, there are 43 affected individuals and 137 unaffected sibs, giving a segregation frequency of 31% (95% CI of 23.6 - 39.2). This value is similar to the expected frequency. If three individual mutations were necessary to cause a phenotype, than this frequency for an autosomal recessive condition should be lower than 25%, and closer to 12.5%. Interestingly, the B14 kindred was homozygous for a *BBS2* mutation (Y24X) and heterozygous for a *BBS6* mutation (A242S) which was also found in the unaffected sibling. It is uncertain whether this *BBS6* missense mutation is truly a mutation or a rare polymorphism (it was found on one Newfoundland control chromosome). As well, it was originally identified in both affected and unaffected sibs in an MKS family in the homozygous form, as part of a complex allele with H84Y (Stone *et al.*, 2000).

In order to accurately assess the occurrence of triallelic inheritance in Newfoundland, mutation screening of all families for all BBS genes must be performed. Such an endeavor should begin by screening the three characterized BBS genes (*BBS2*, *BBS4* and *BBS6*).

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*** These authors contributed equally to this work.**

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Electronic Database Information

URLs found in this work are as follows:

Association of Newfoundland and Labrador Archives (ANLA), http://www.anla.nf.ca/

Project 21, http://www.huronweb.com/genweb/p21/main.html

The Genome Database (GDB), http://gdbwww.gdb.org

GeneMap '99, http://www.ncbi.nlm.nih.gov/genemap/

Marshfield Center for Medical Genetics, http://research.marshfieldclinic.org/genetics/

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http://www-genome.wi.mit.edu/

Cooperative Human Linkage Consortium (CHLC), http://lpg.nci.nih.gov/CHLC/

BLAST algorithm, http://www.ncbi.nlm.nih.gov/BLAST/blast_databases.html

Electronic PCR (ePCR), http://www.ncbi.nlm.nih.gov/STS/

Primer v3 program, http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi

NCBI, Map Viewer, http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/maps.cgi?org=hum&chr=11

Conserved Domain Database (CDD), http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml

Cluster of Orthologous Groups (COGs) database, http://www.ncbi.nlm.nih.gov/COG/

GenBank accession numbers found in this work are as follows:

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thsA of *Pyrodictium occultum*, AJ006549

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MKKS mouse homolog cDNA, AF254074

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filamin exons 15, 16 and 17 cDNA, AF191606

BBS2 cDNA, AF342736

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Appendix A

Pedigrees and Haplotype Data on 17 Newfoundland BBS Families

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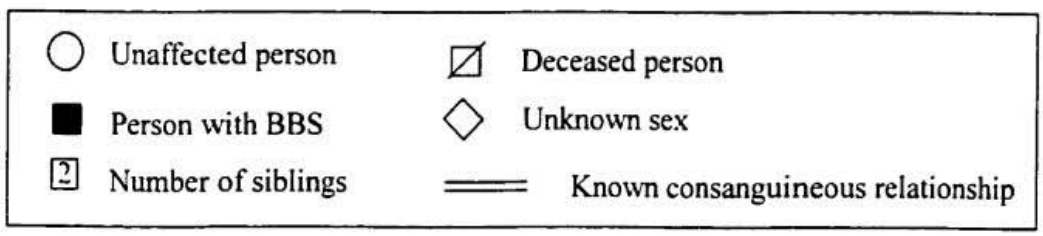

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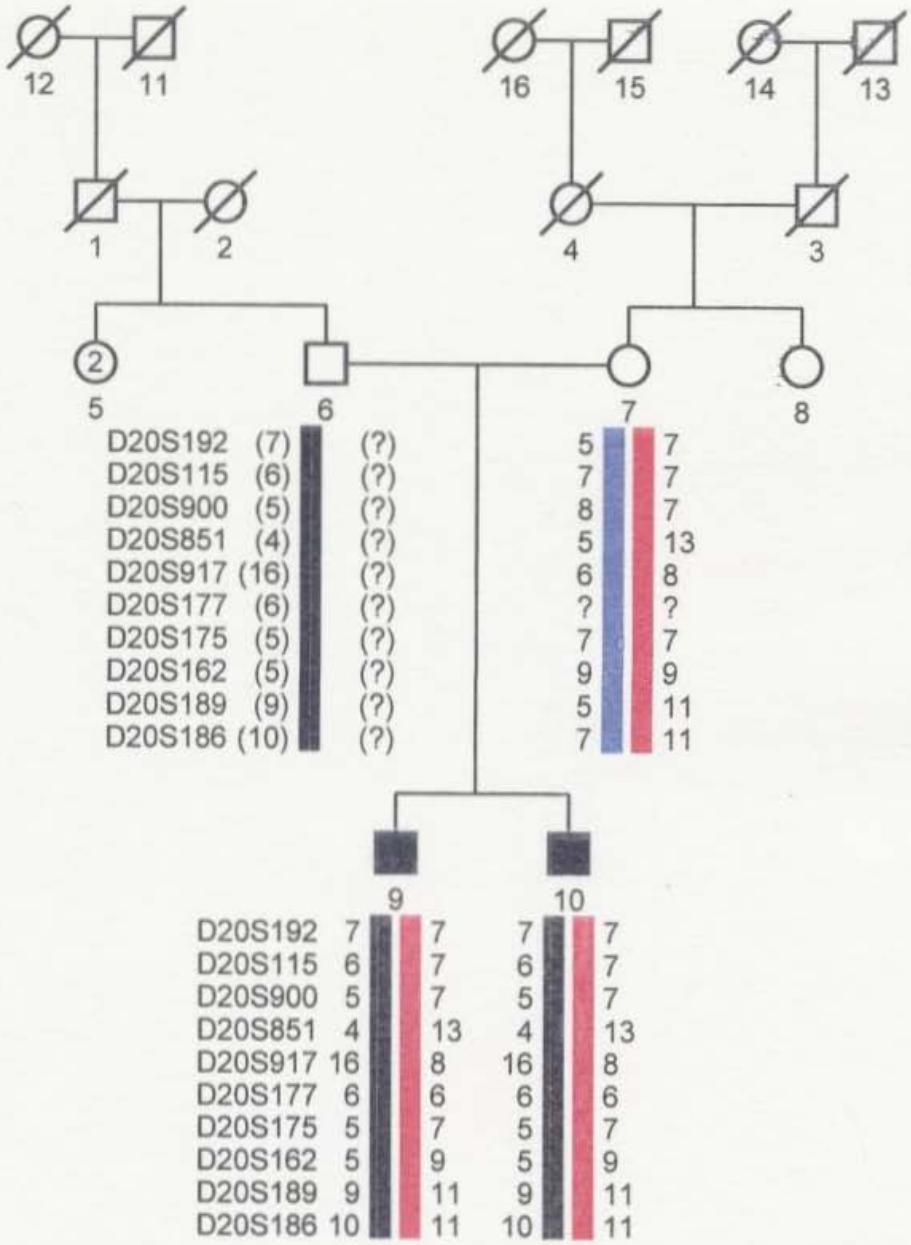

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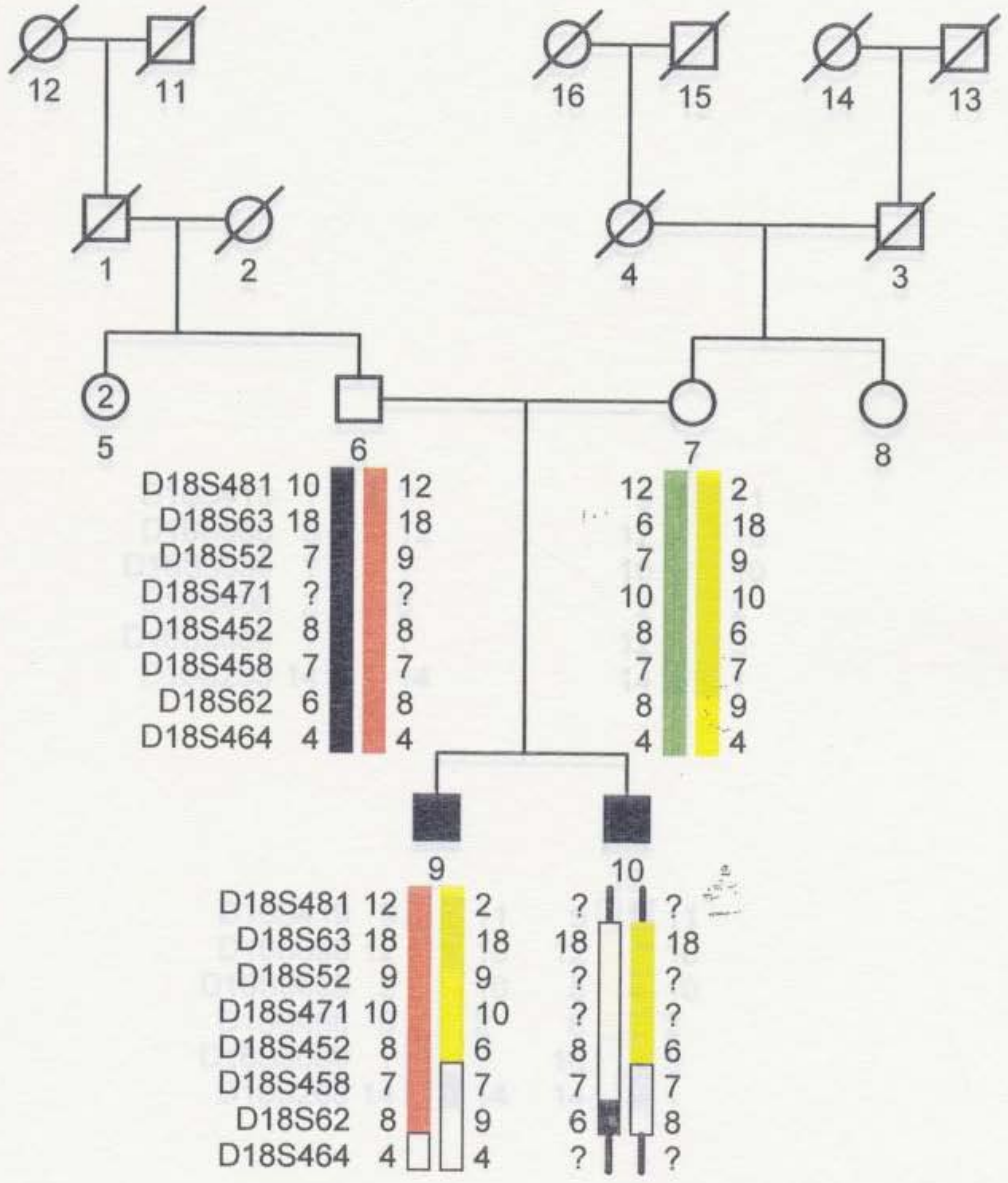

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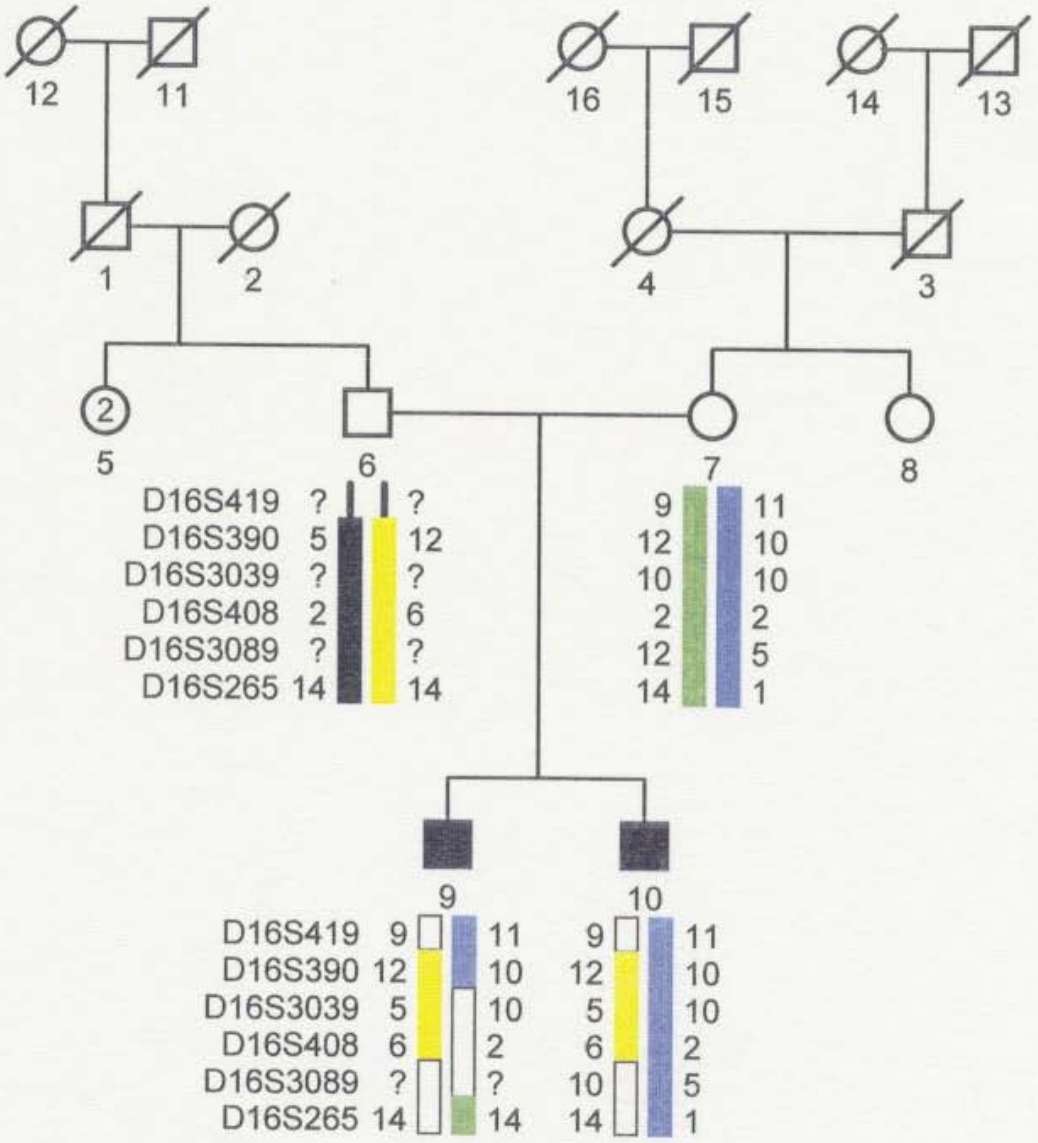

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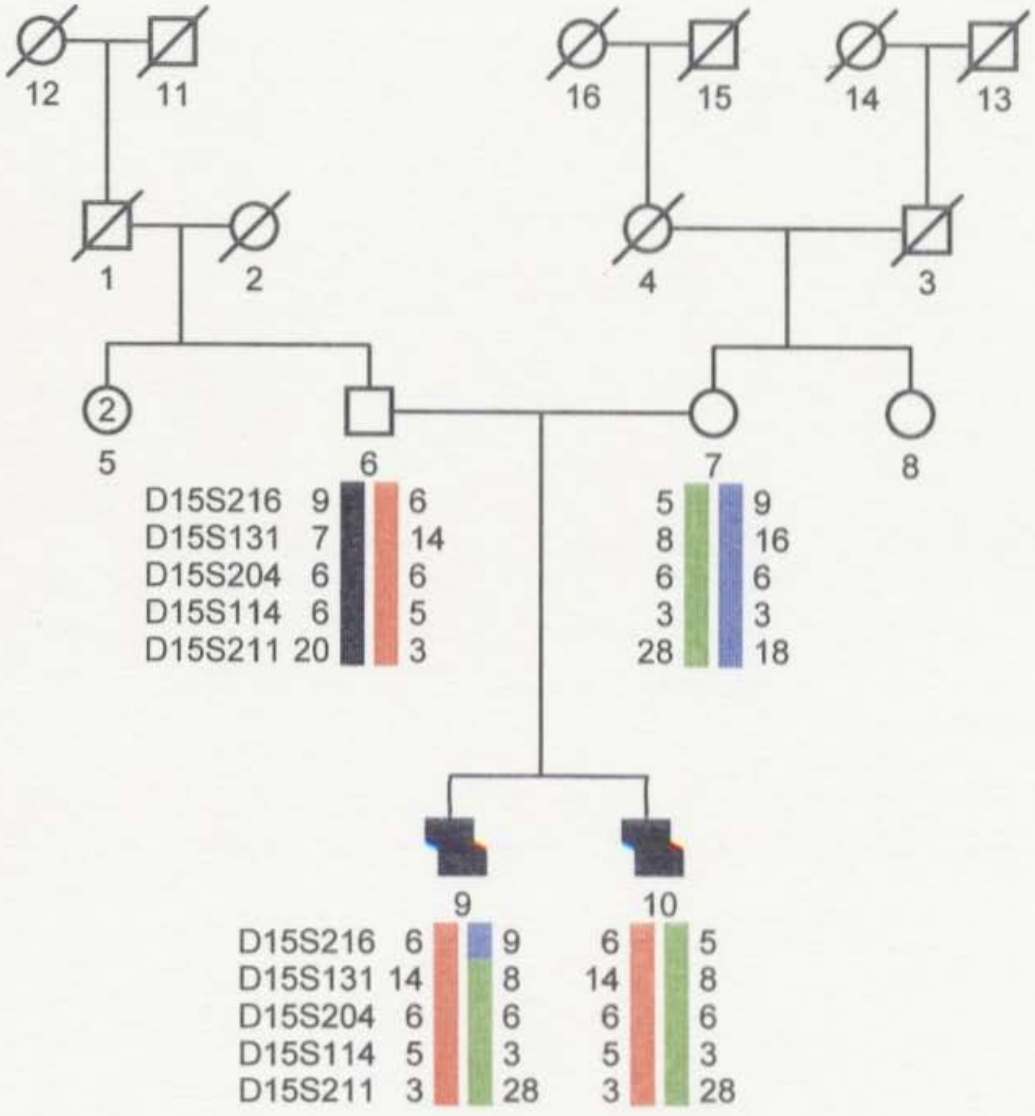

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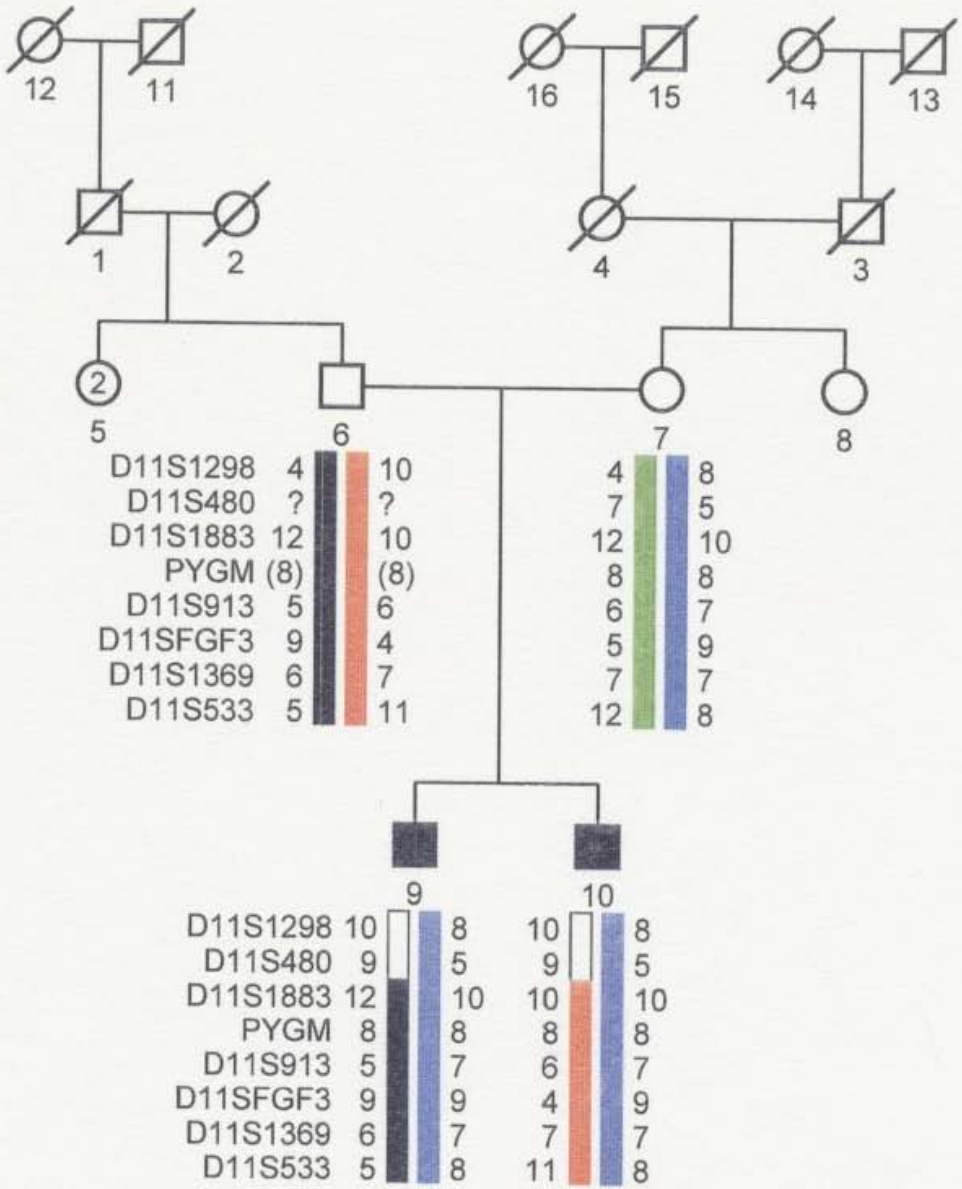

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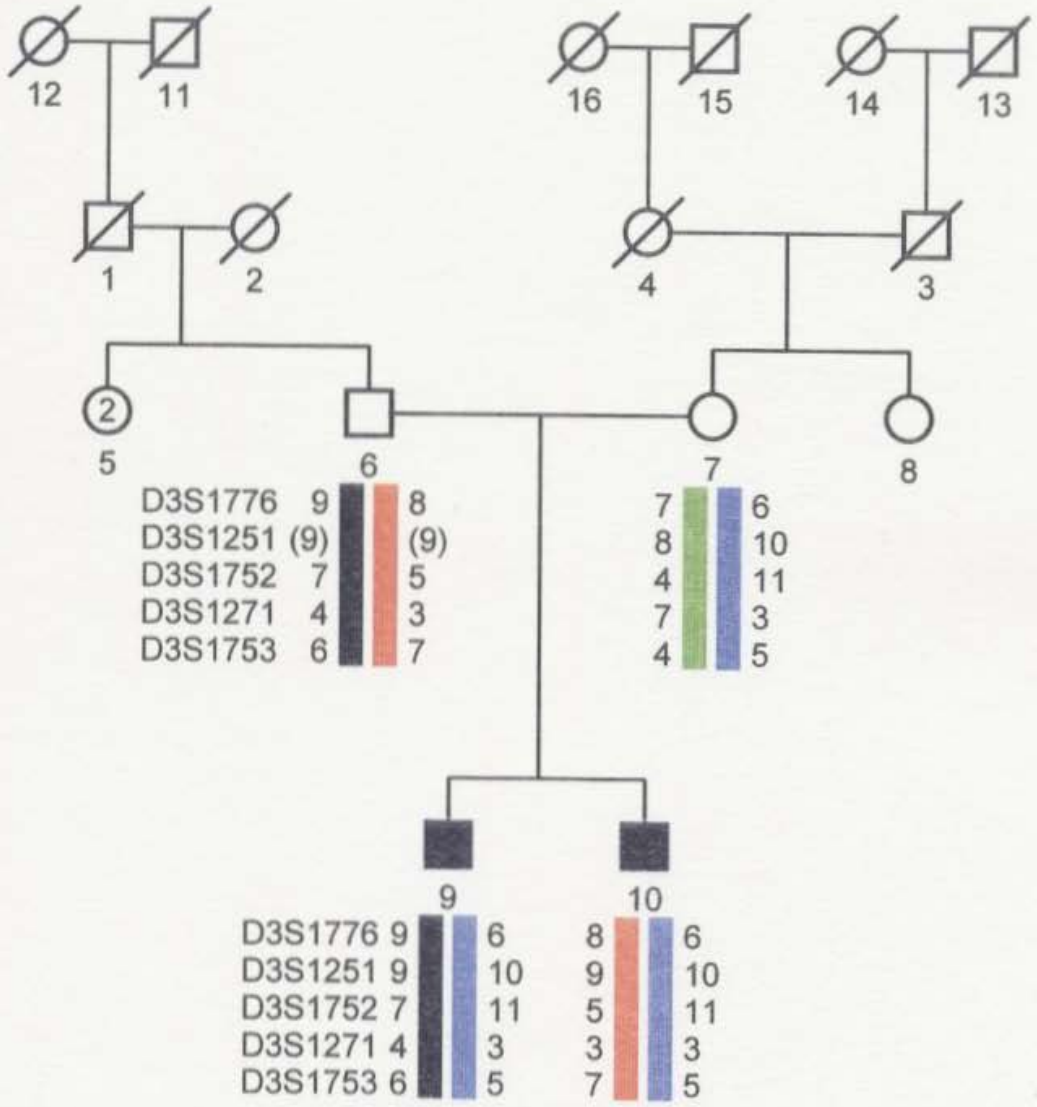

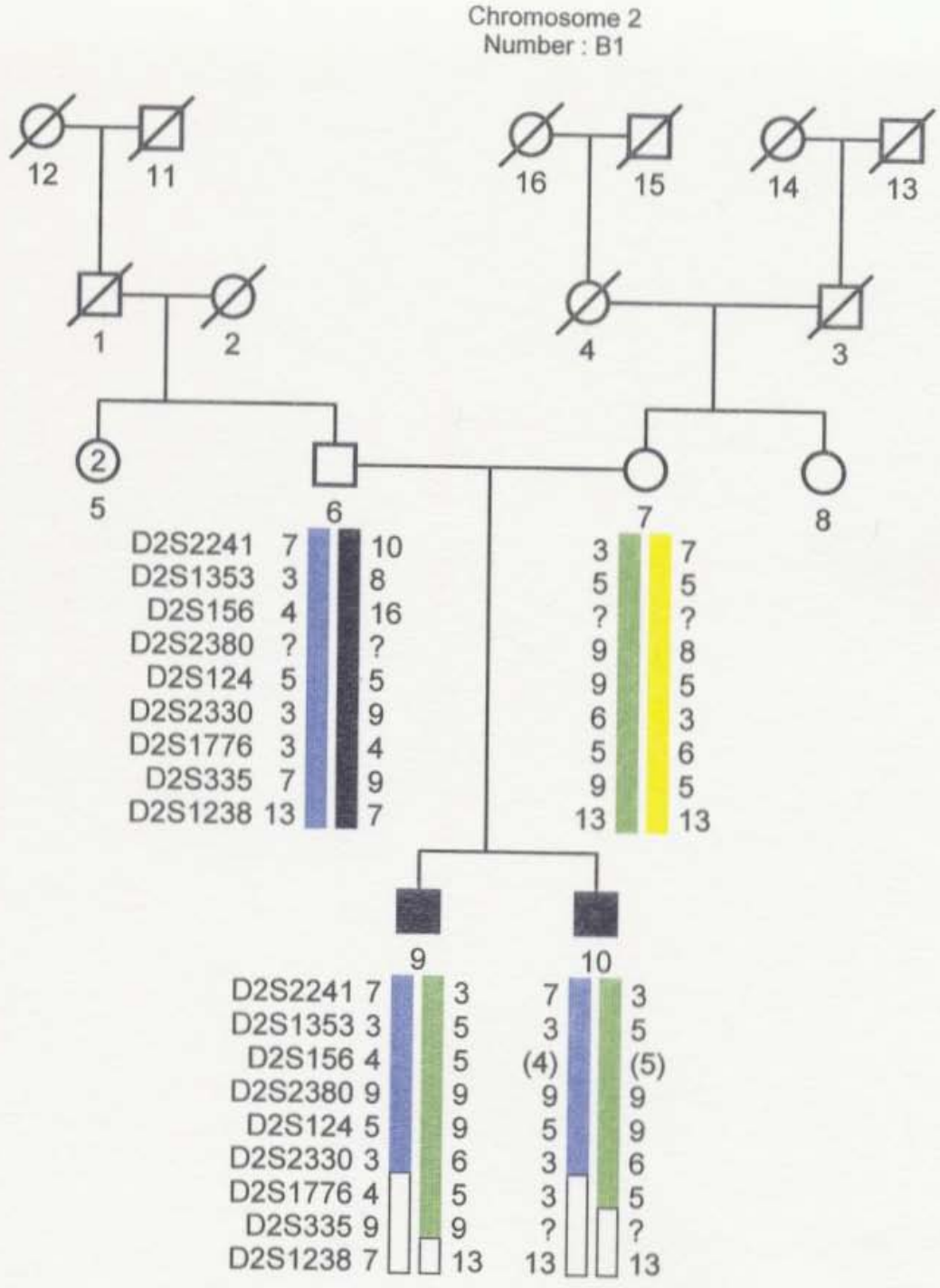
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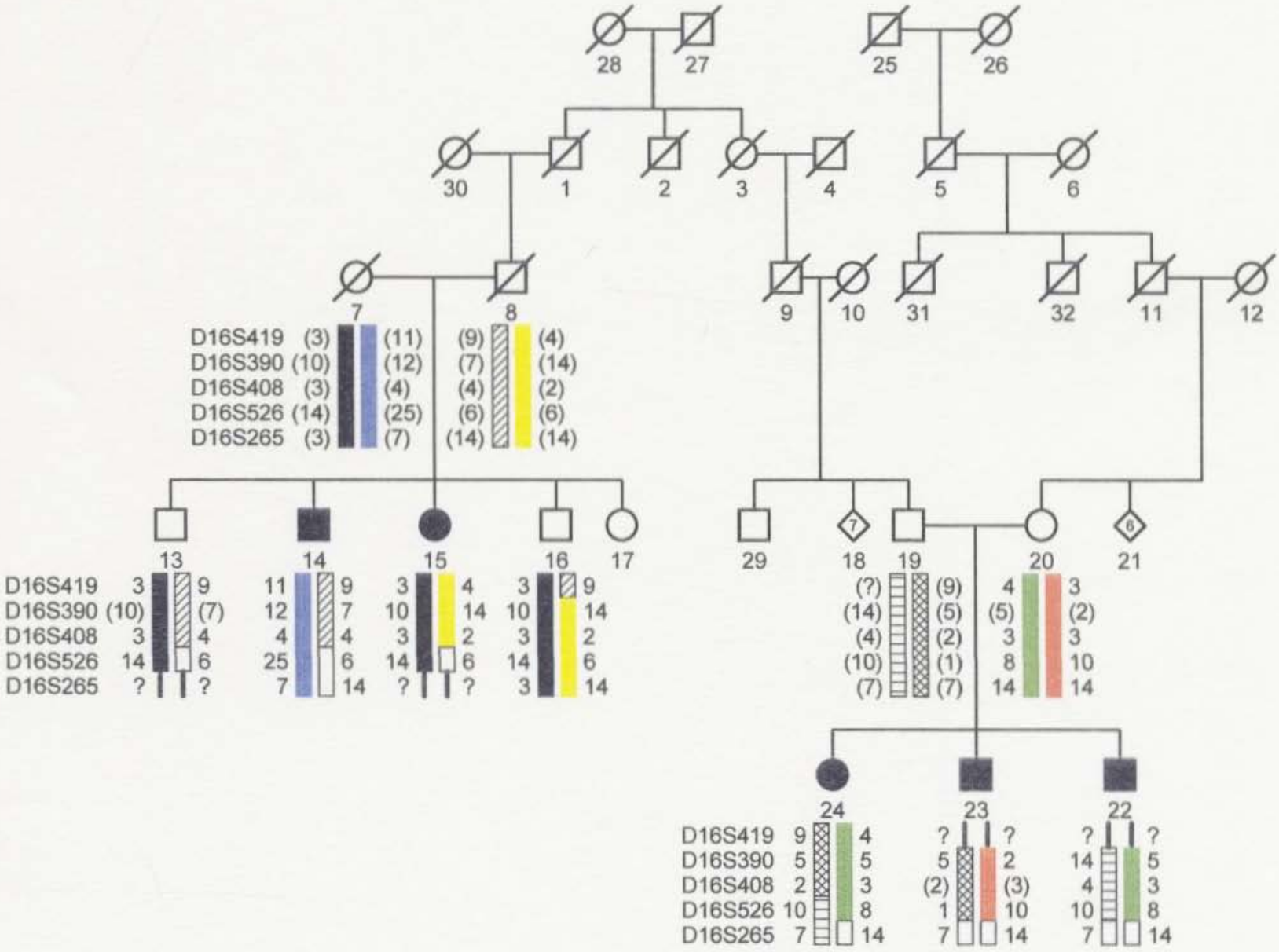

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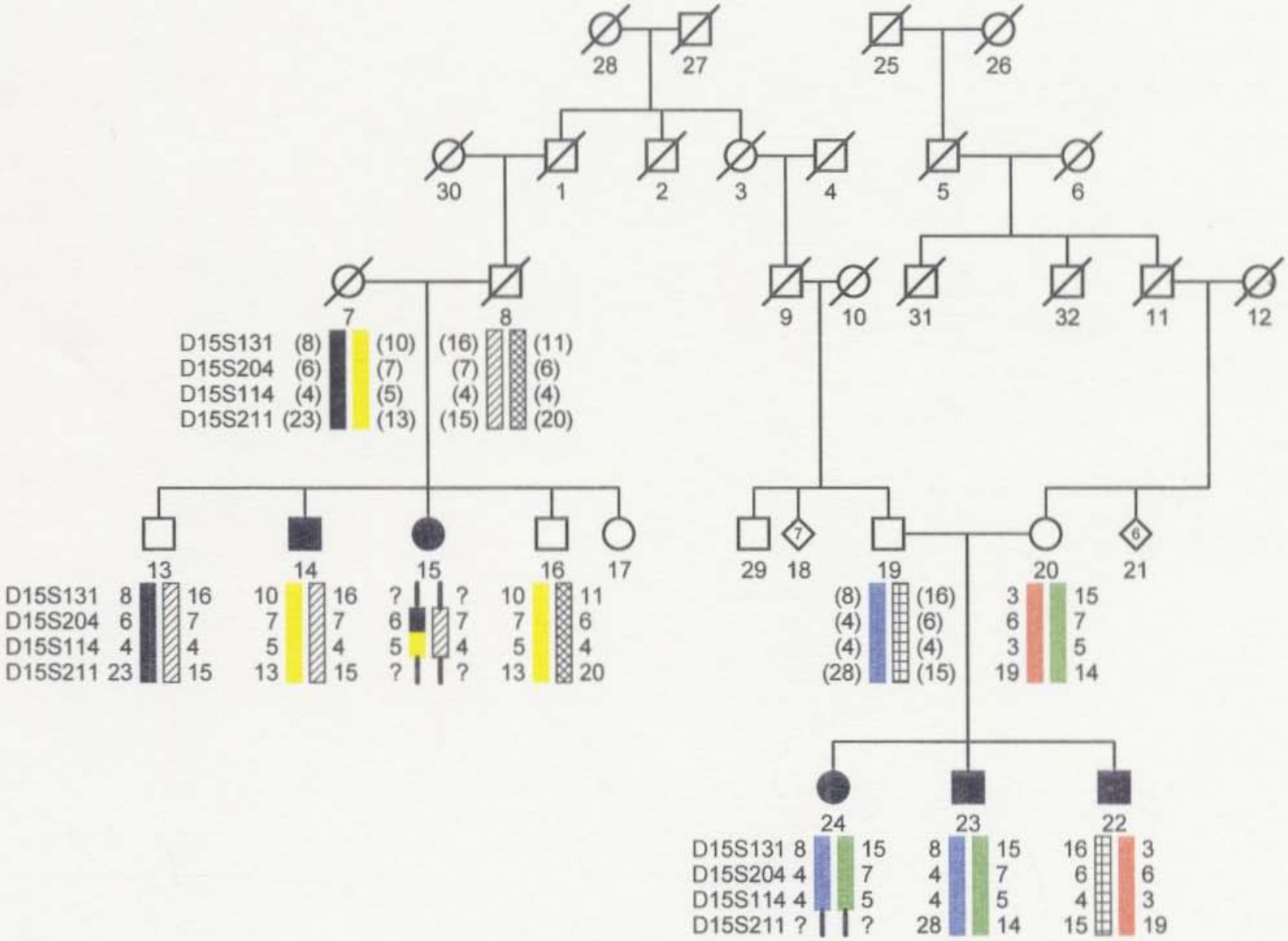

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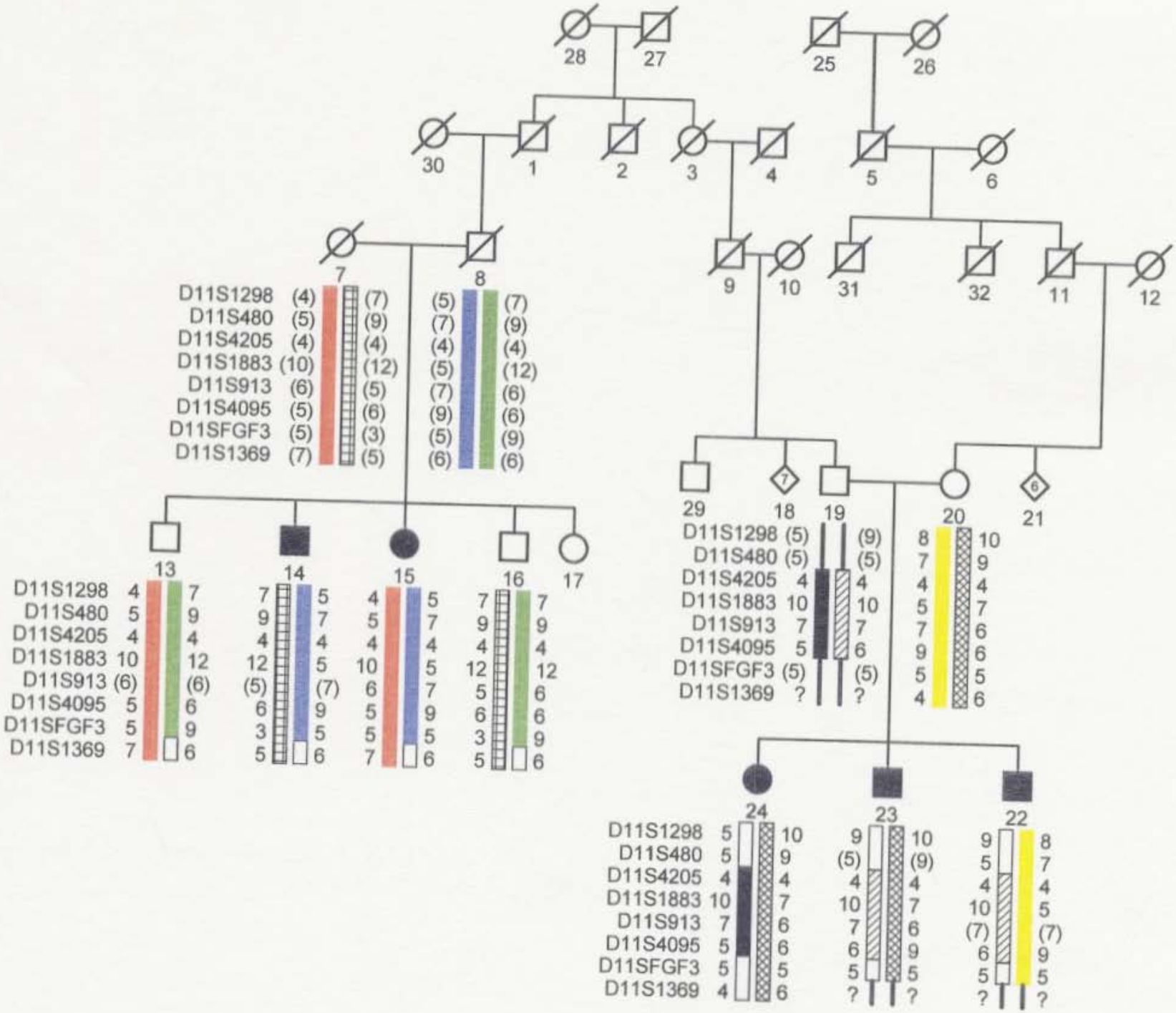

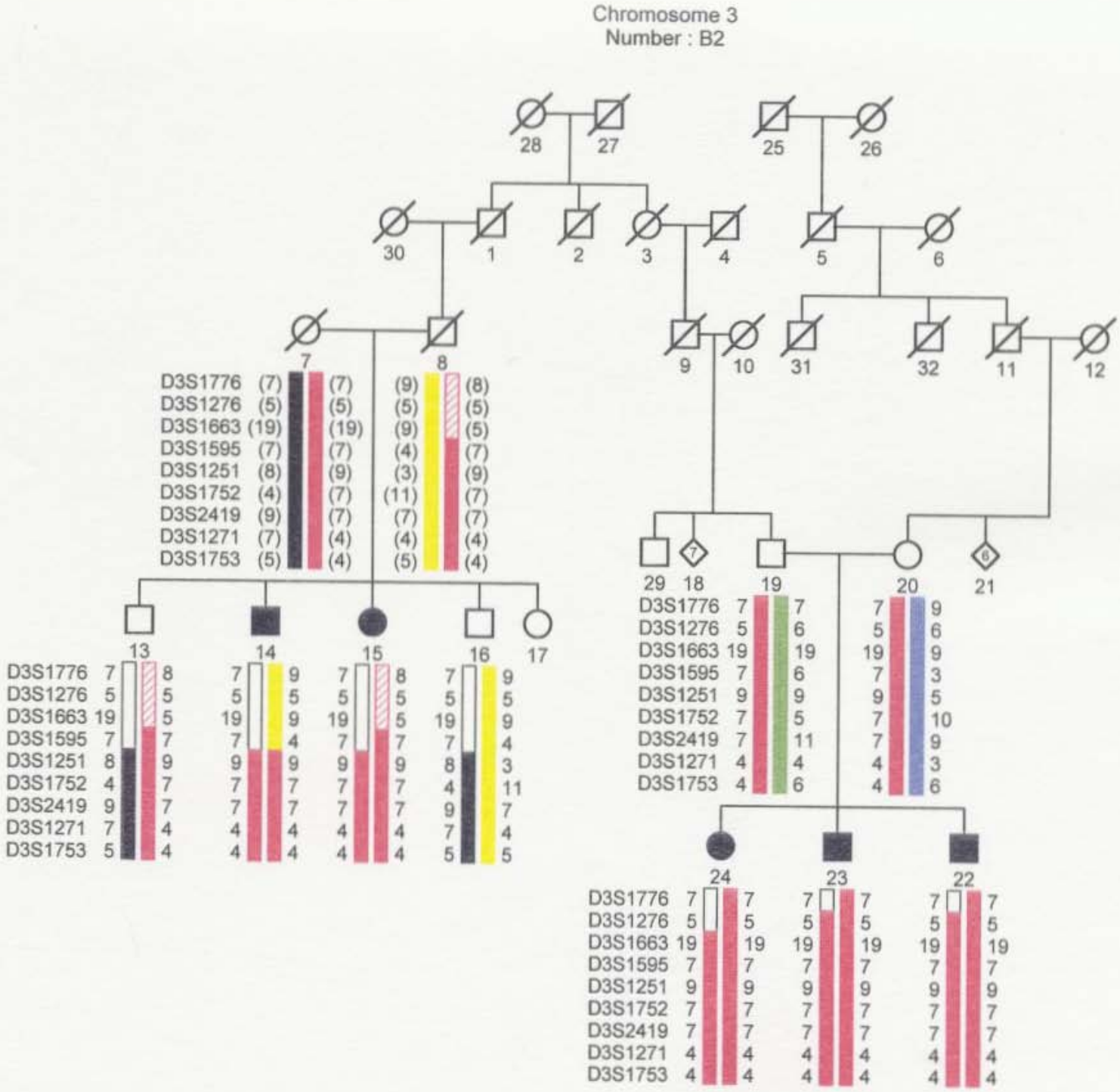
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D3S1251 (8) (9) (3) (9)
D3S1752 (4) (7) (11) (7)
D3S2419 (9) (7) (7) (7)
D3S1271 (7) (4) (4) (4)
D3S1753 (5) (4) (5) (4)
29
18
19
20
21
D3S1776 7 7 7 9
D3S1276 5 6 5 6
D3S1663 19 19 19 9
D3S1595 7 6 7 3
D3S1251 9 9 9 5
D3S1752 7 5 7 10
D3S2419 7 11 7 9
D3S1271 4 4 4 3
D3S1753 4 6 4 6
13
14
15
16
17
D3S1776 7 8 7 9 7 8 7 9
D3S1276 5 5 5 5 5 5 5 5
D3S1663 19 5 19 9 19 5 19 9
D3S1595 7 7 7 4 7 7 7 4
D3S1251 8 9 9 9 9 9 8 3
D3S1752 4 7 7 7 7 7 4 11
D3S2419 9 7 7 7 7 7 9 7
D3S1271 7 4 4 4 4 4 7 4
D3S1753 5 4 4 4 4 4 5 5
24
23
22
D3S1776 7 7 7 7 7 7
D3S1276 5 5 5 5 5 5
D3S1663 19 19 19 19 19 19
D3S1595 7 7 7 7 7 7
D3S1251 9 9 9 9 9 9
D3S1752 7 7 7 7 7 7
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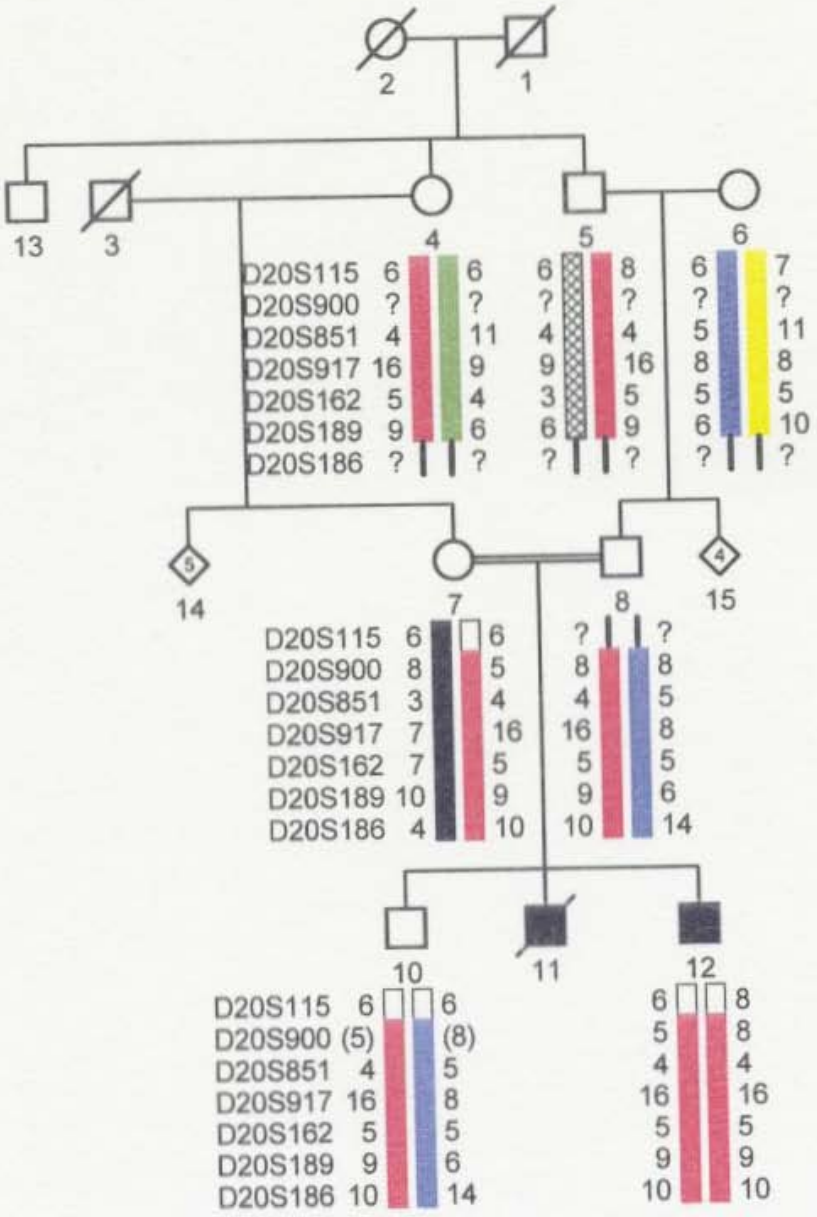
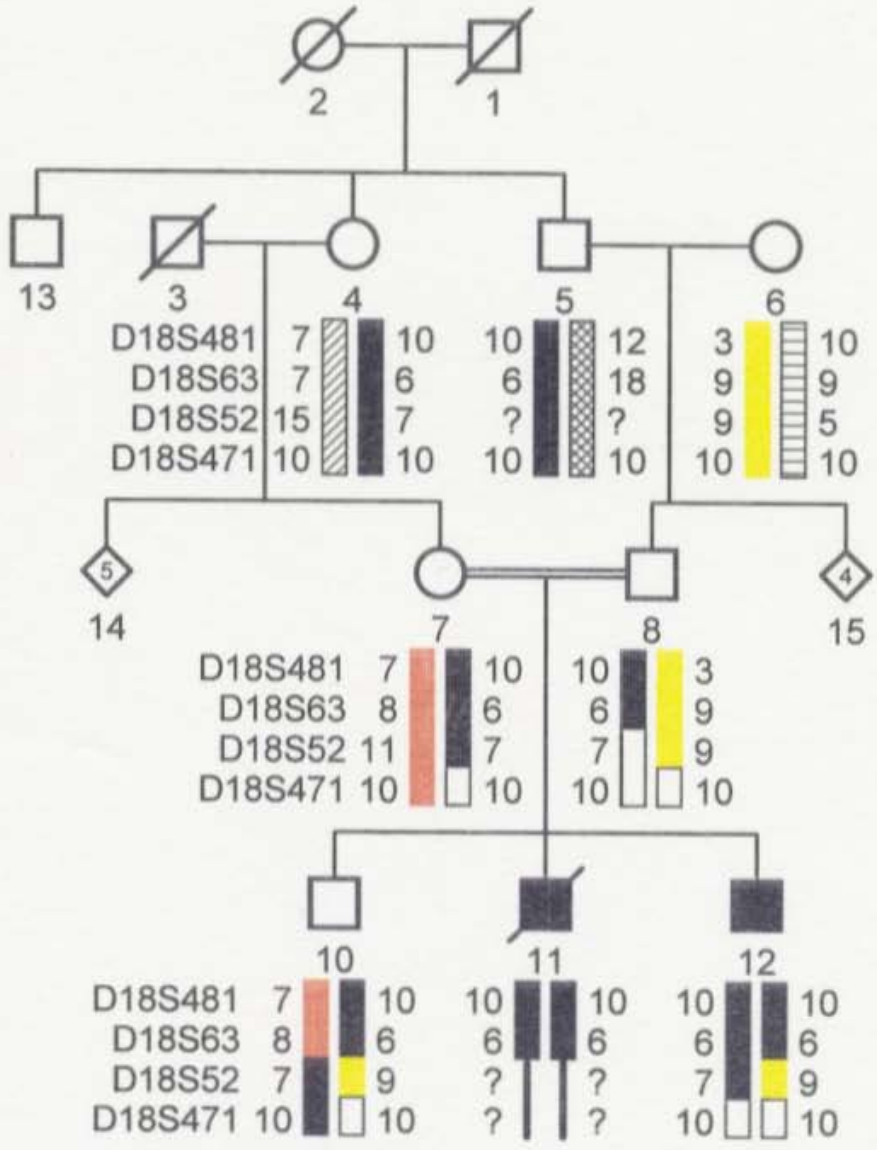
D3S1753 4 4 4 4 4 4

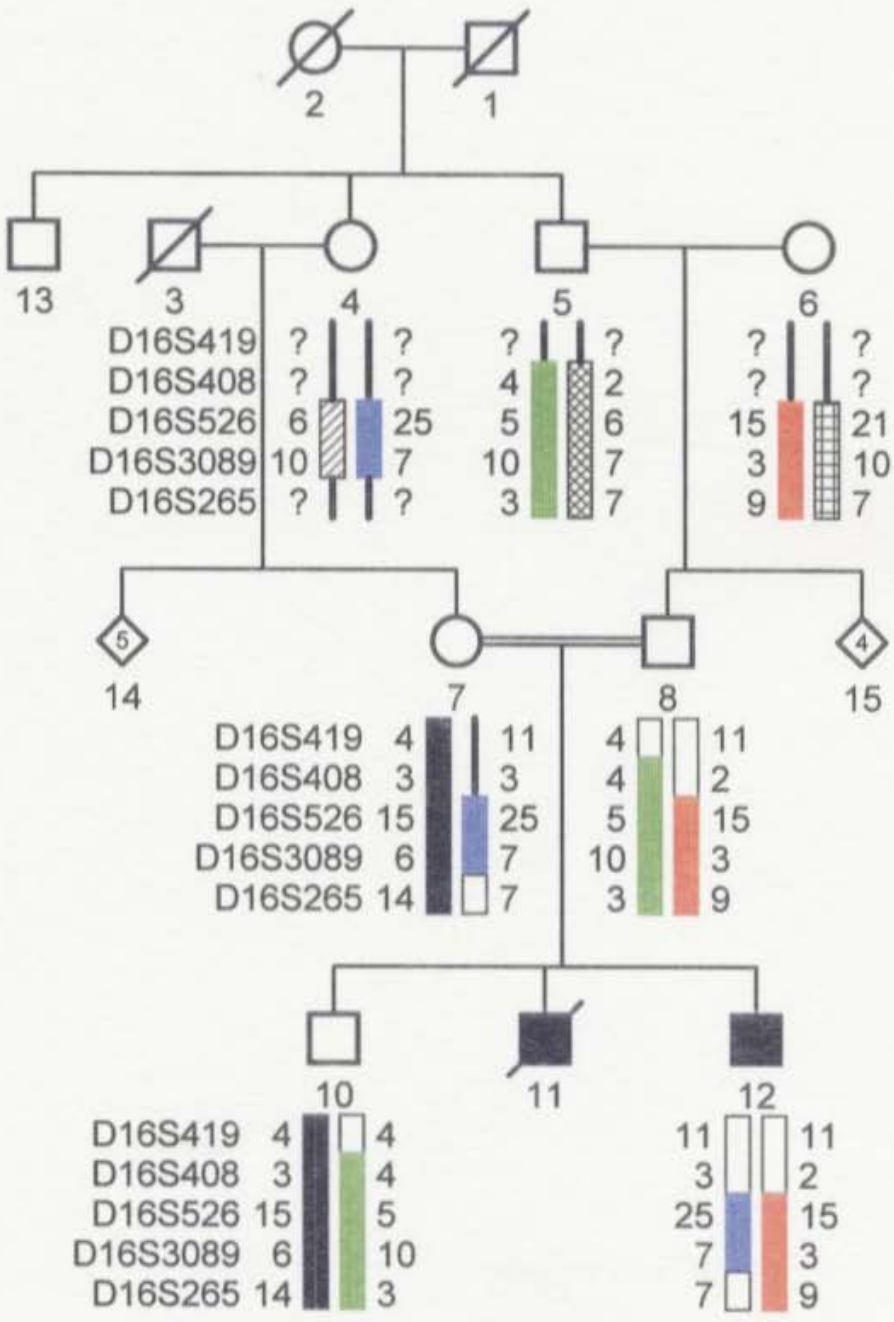
Chromosome 20
Number : B3



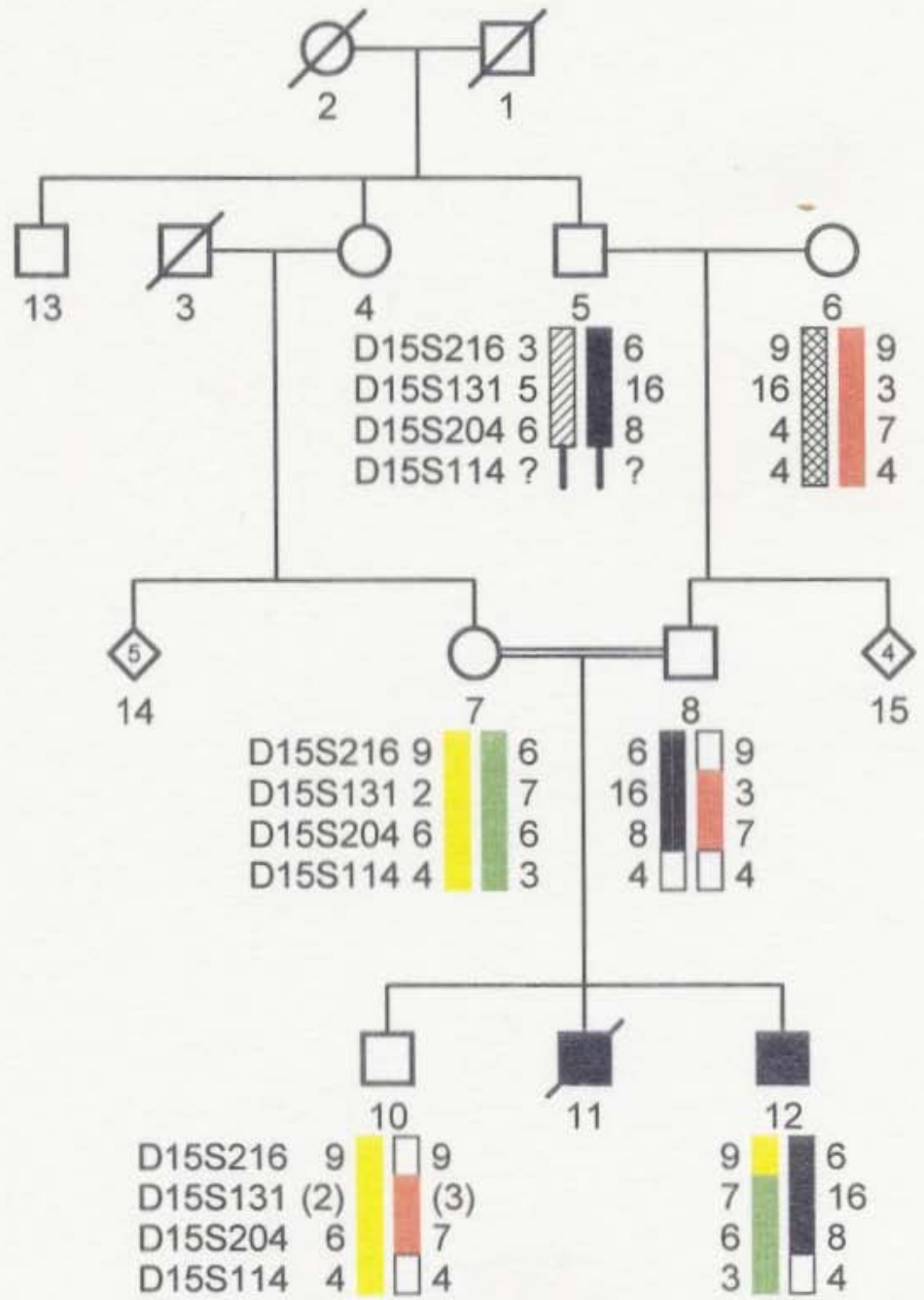
Chromosome 18
Number : B3



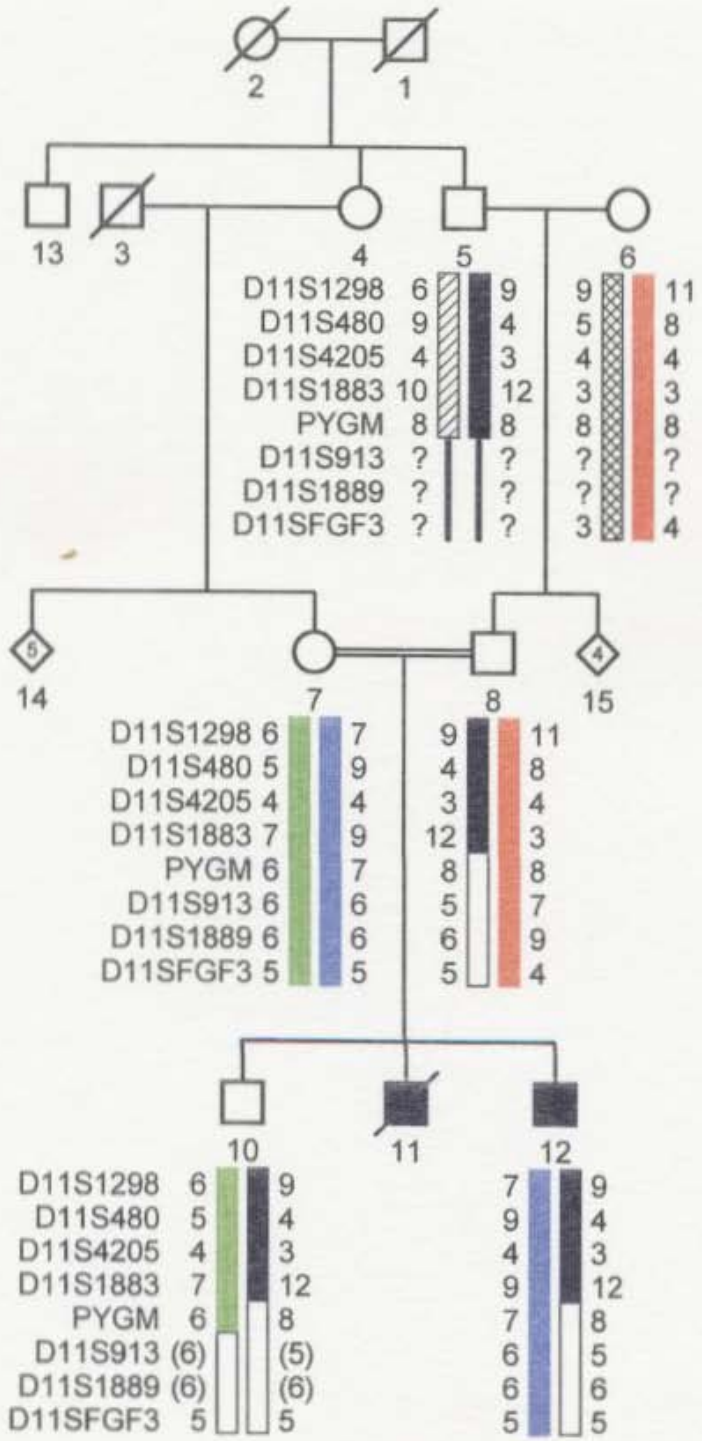
Chromosome 16
Number : B3



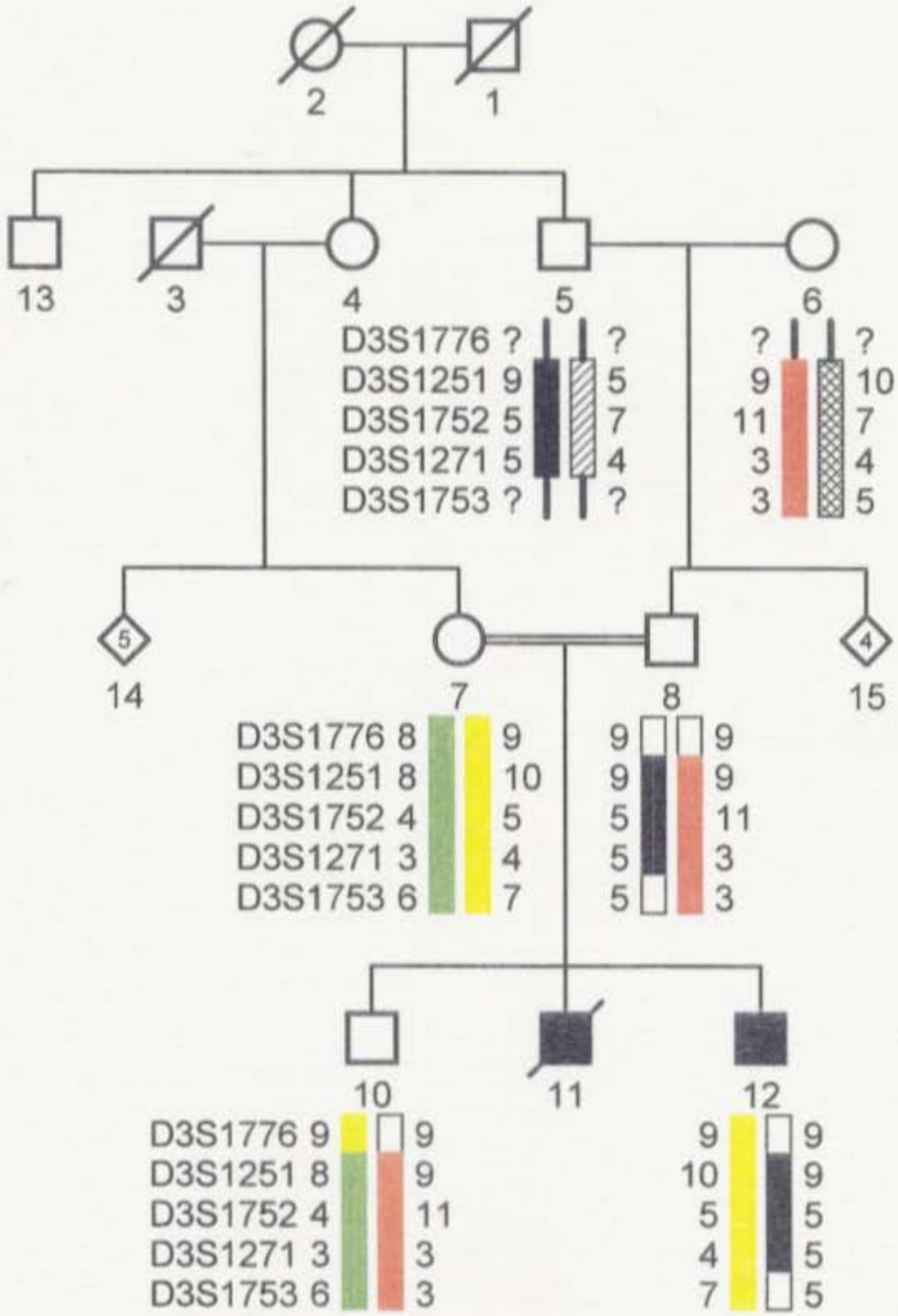
Chromosome 15
Number : B3



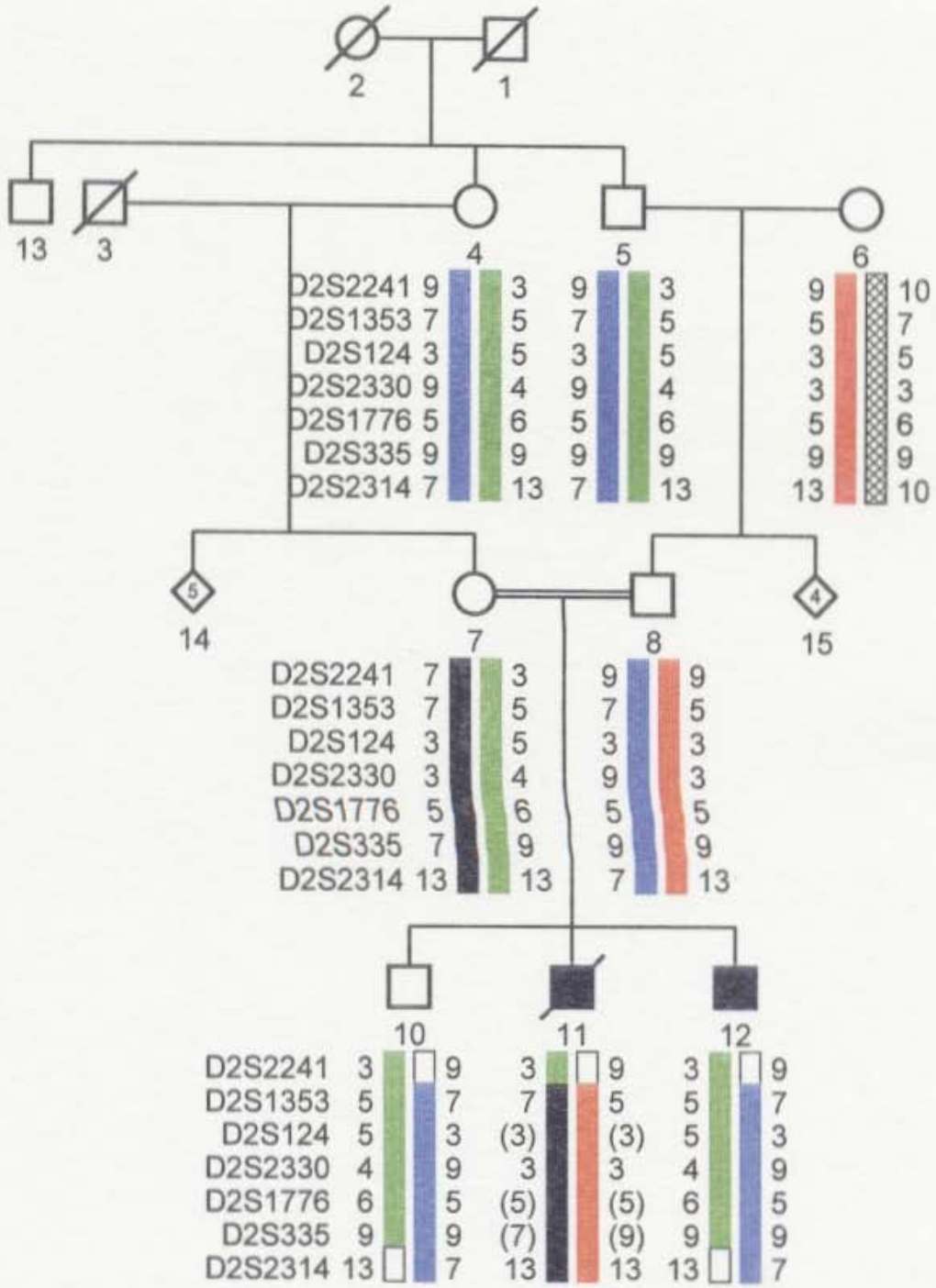
Chromosome 11
Number : B3



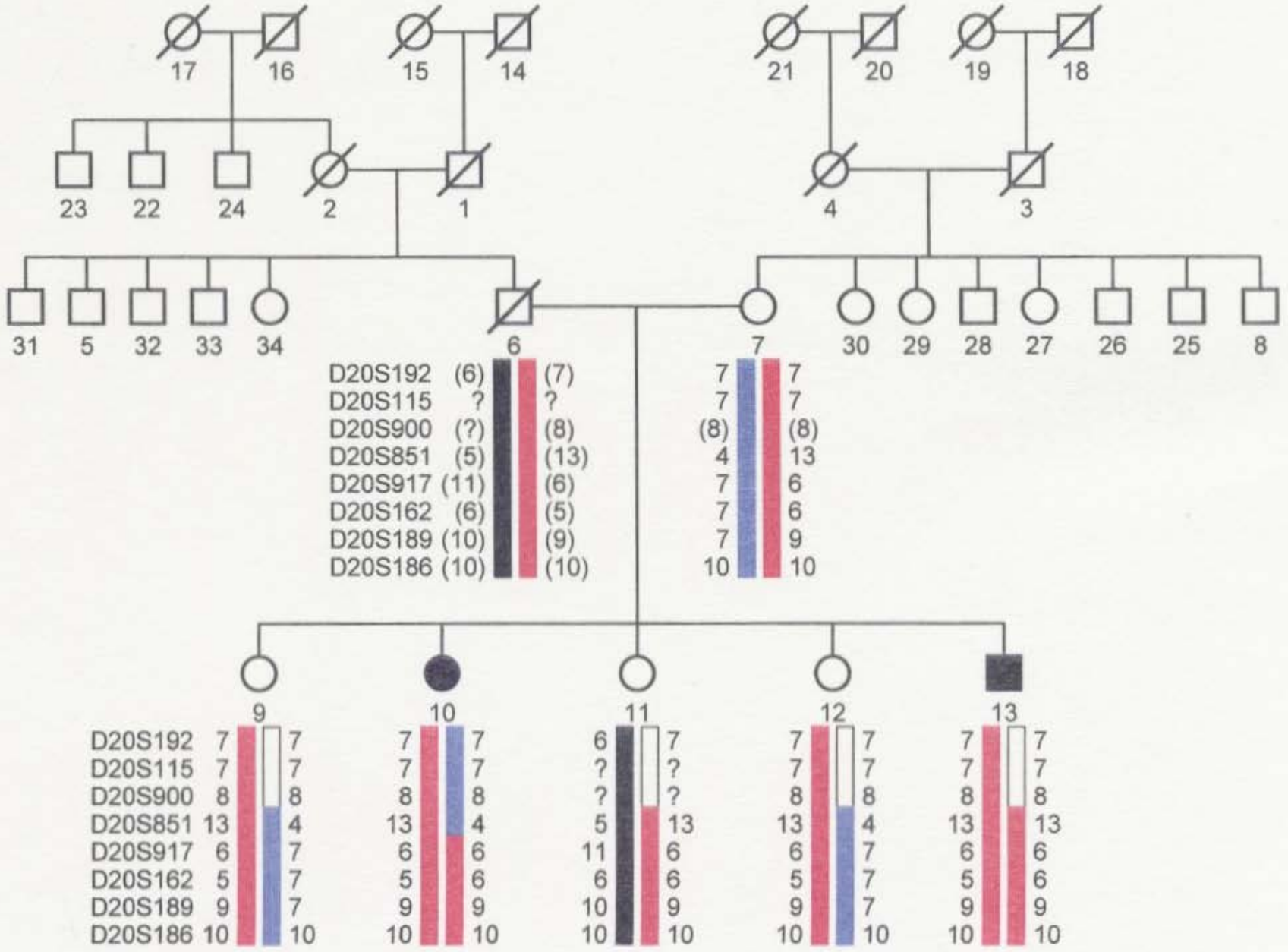
Chromosome 3
Number : B3



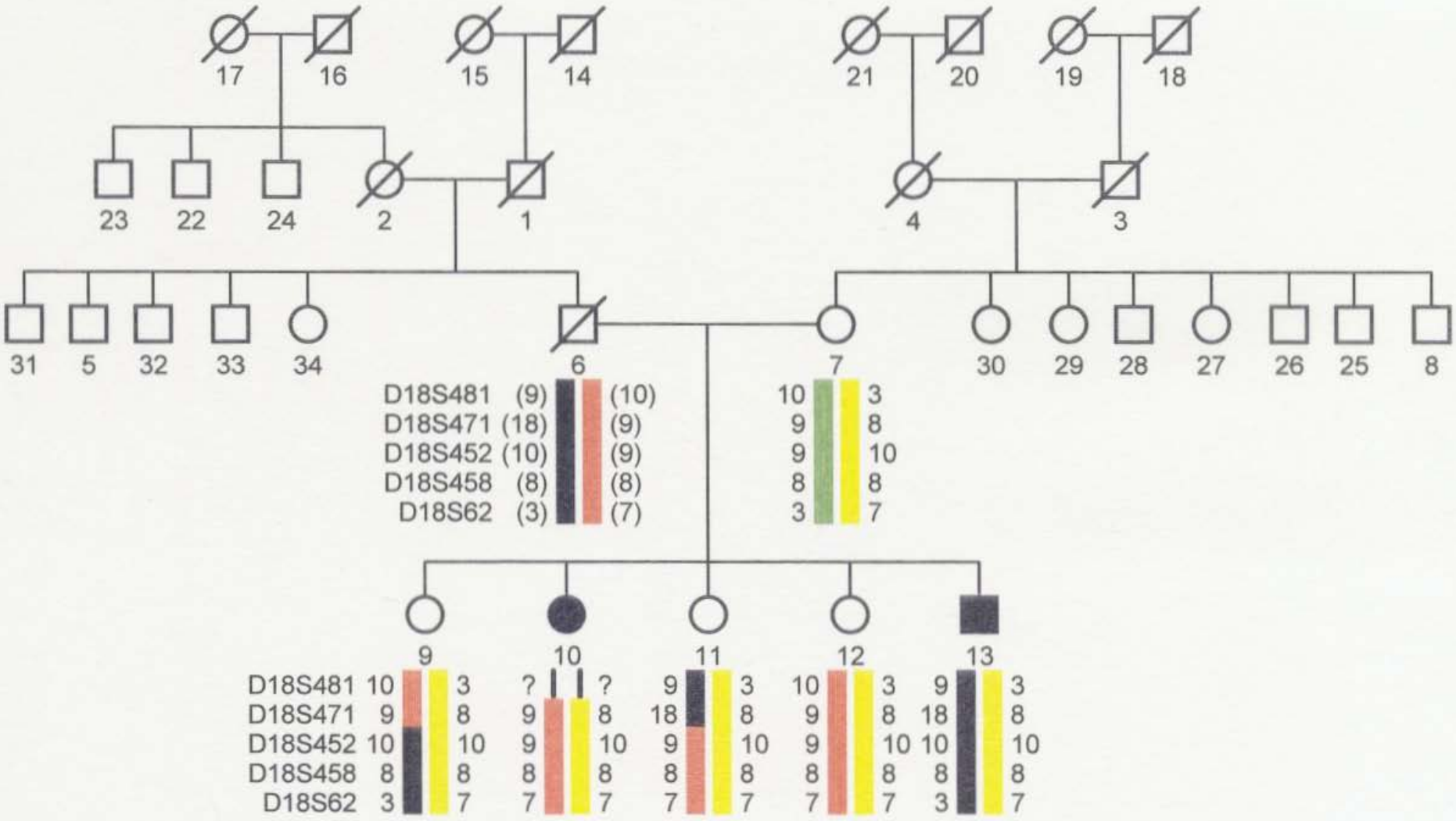
Chromosome 2
Number : B3



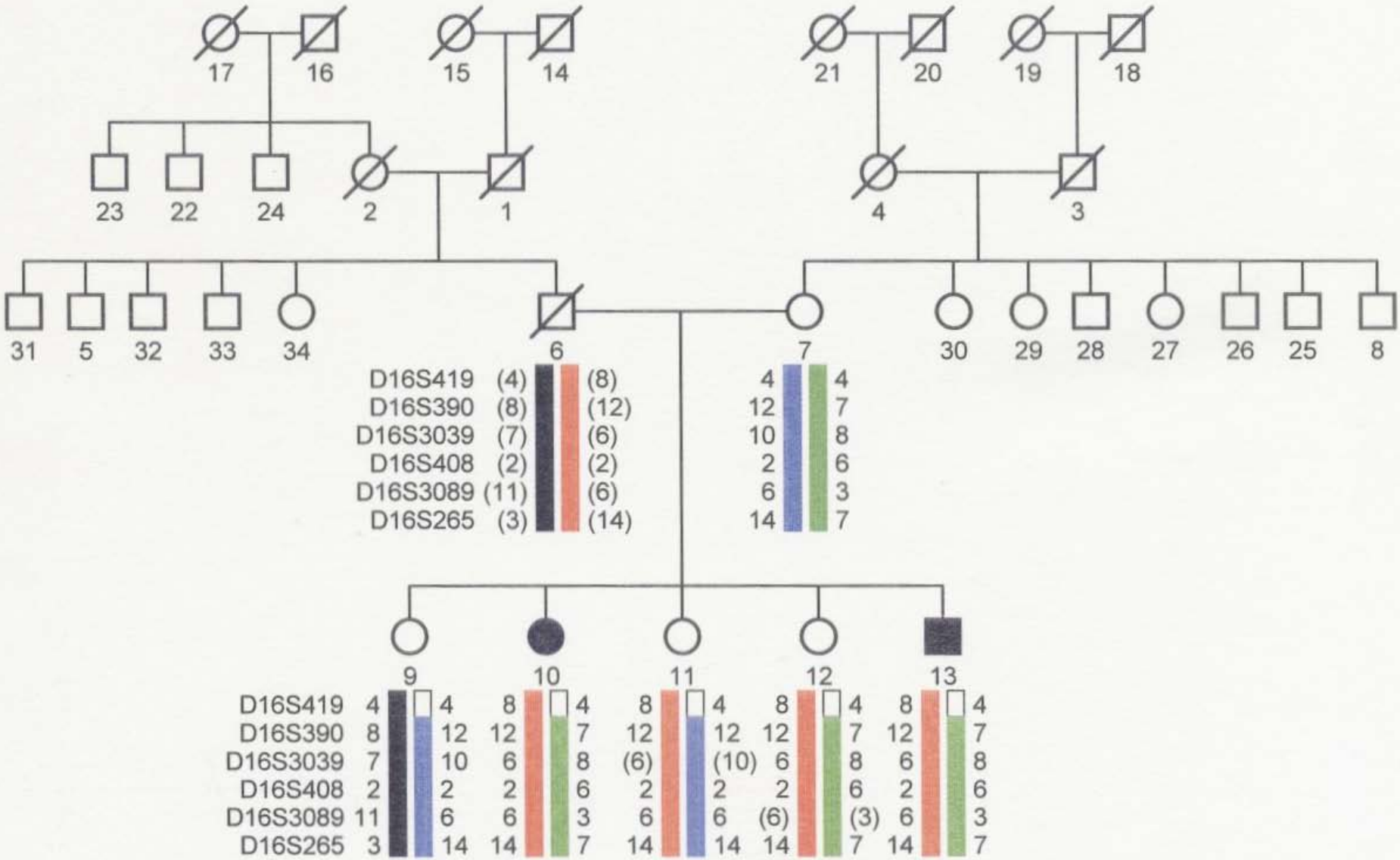
Chromosome 20
Number : B4



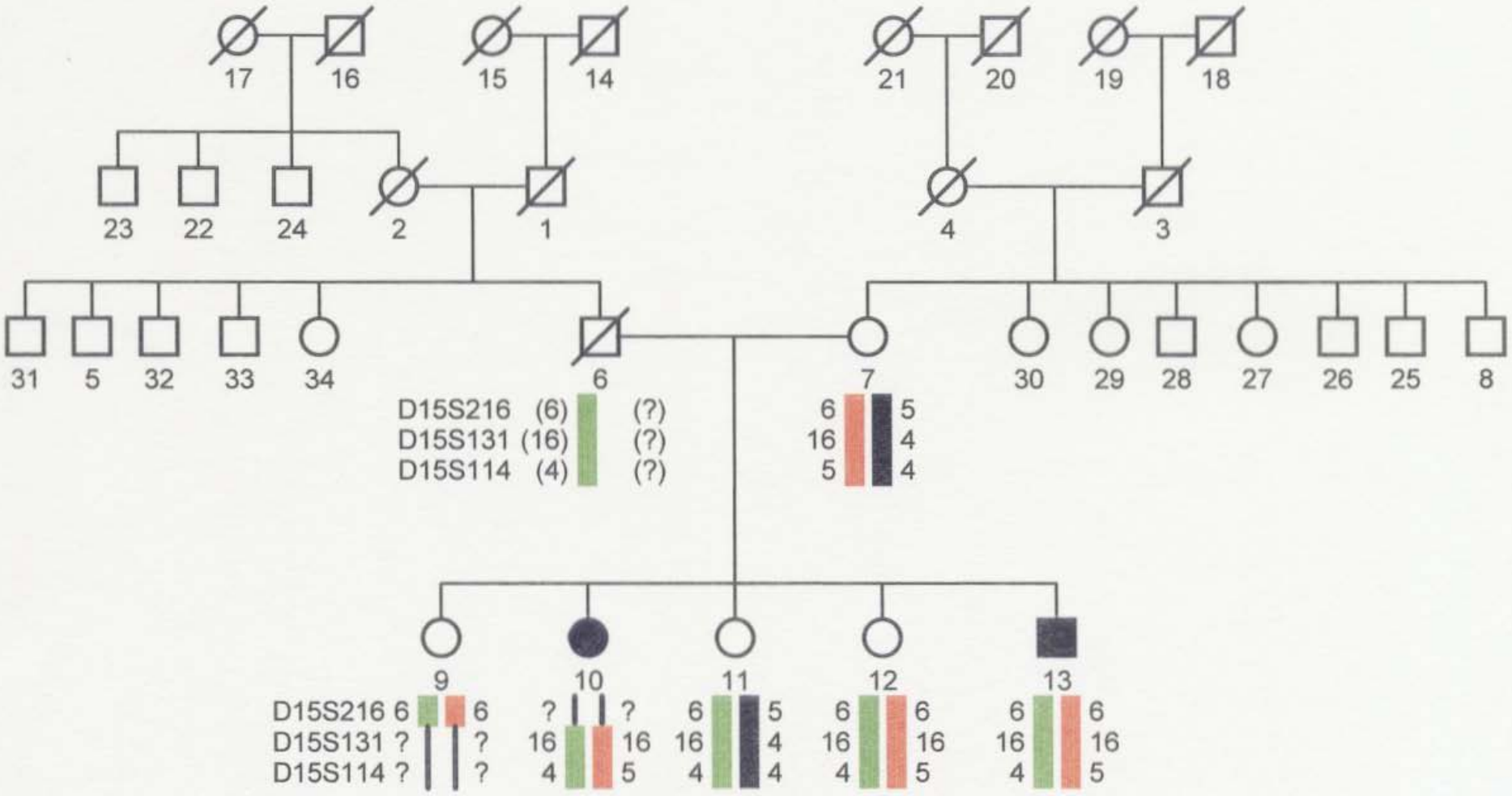
Chromosome 18
Number : B4



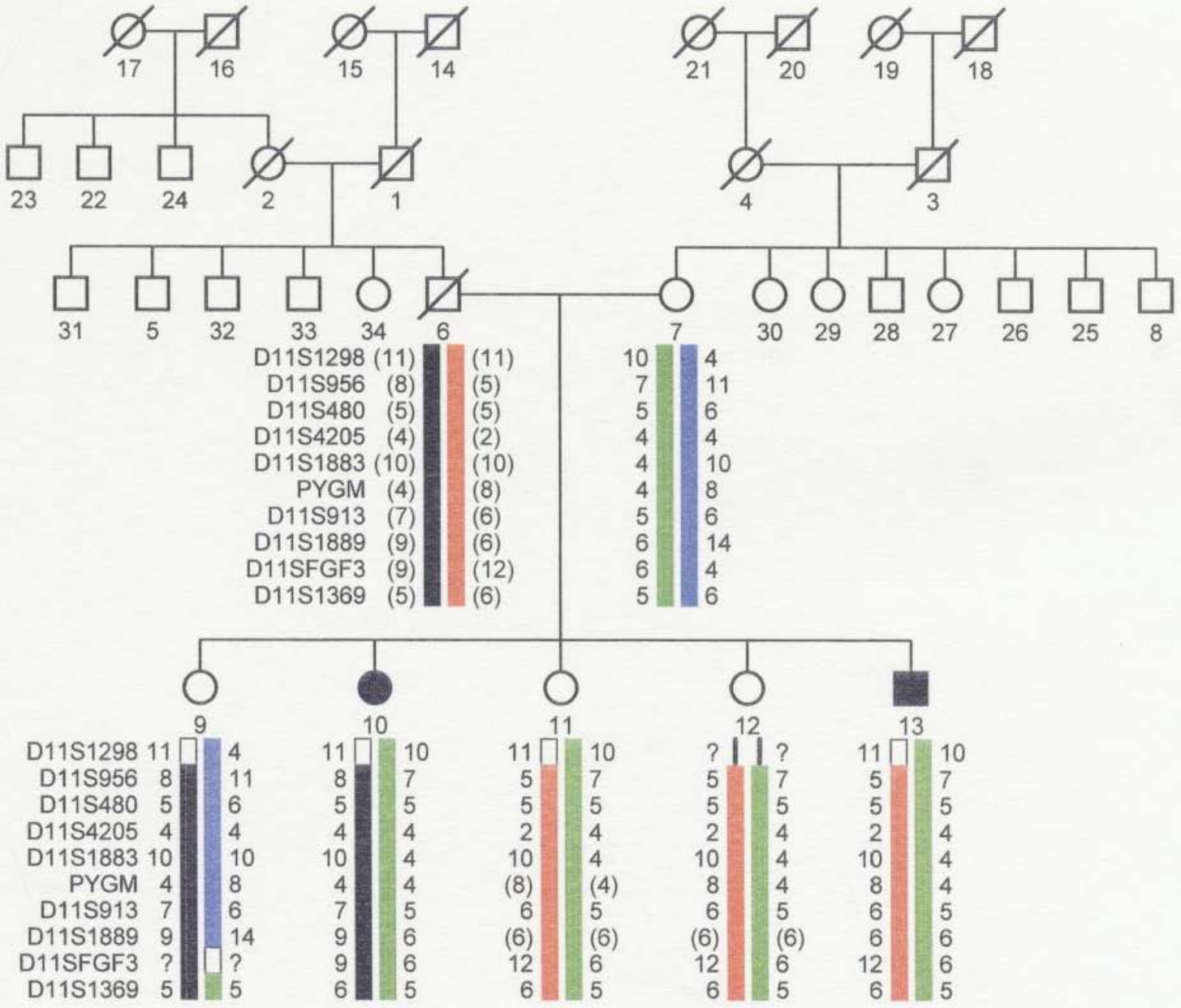
Chromosome 16
Number : B4



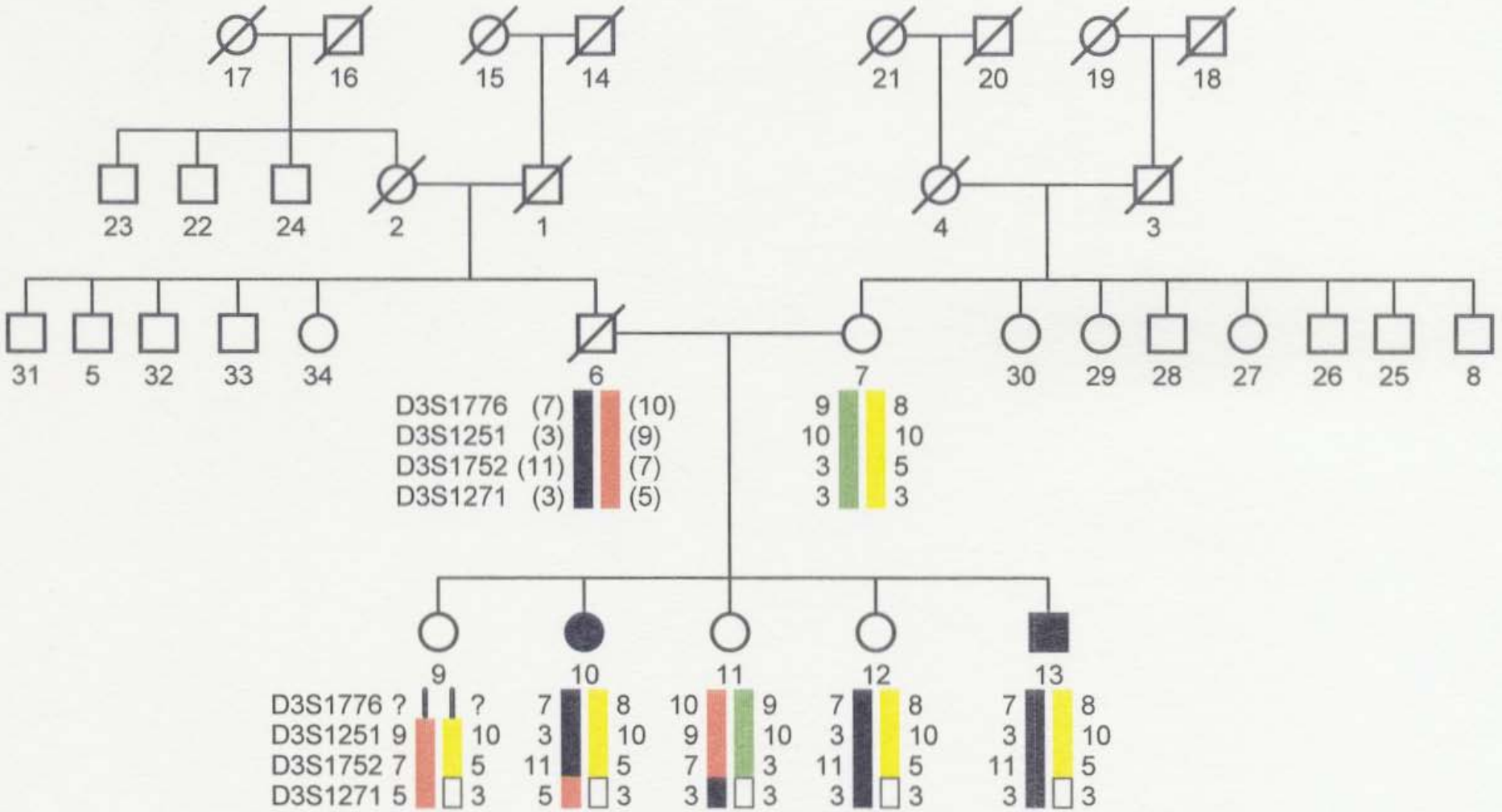
Chromosome 15
Number : B4



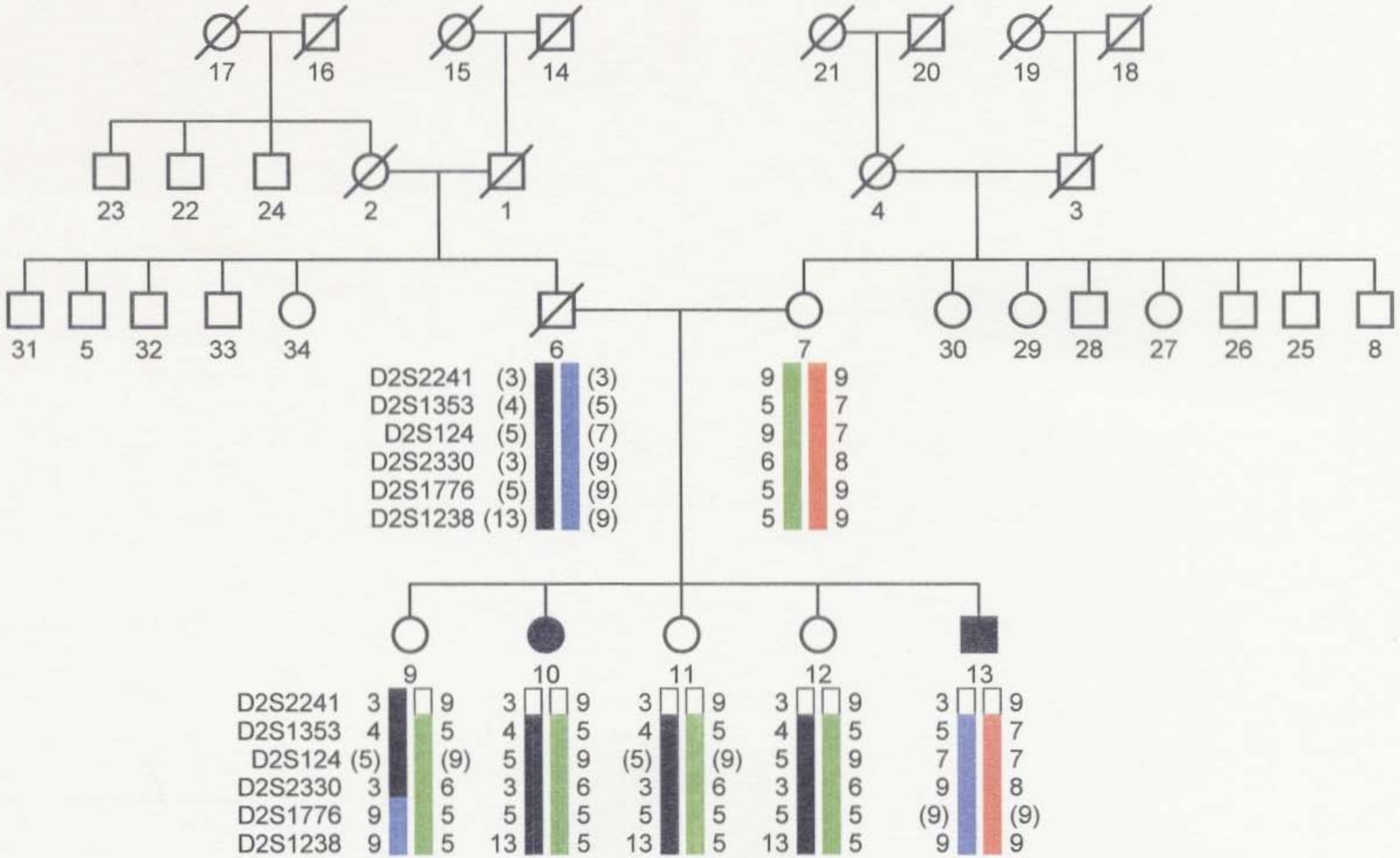
Chromosome 11
Number : B4



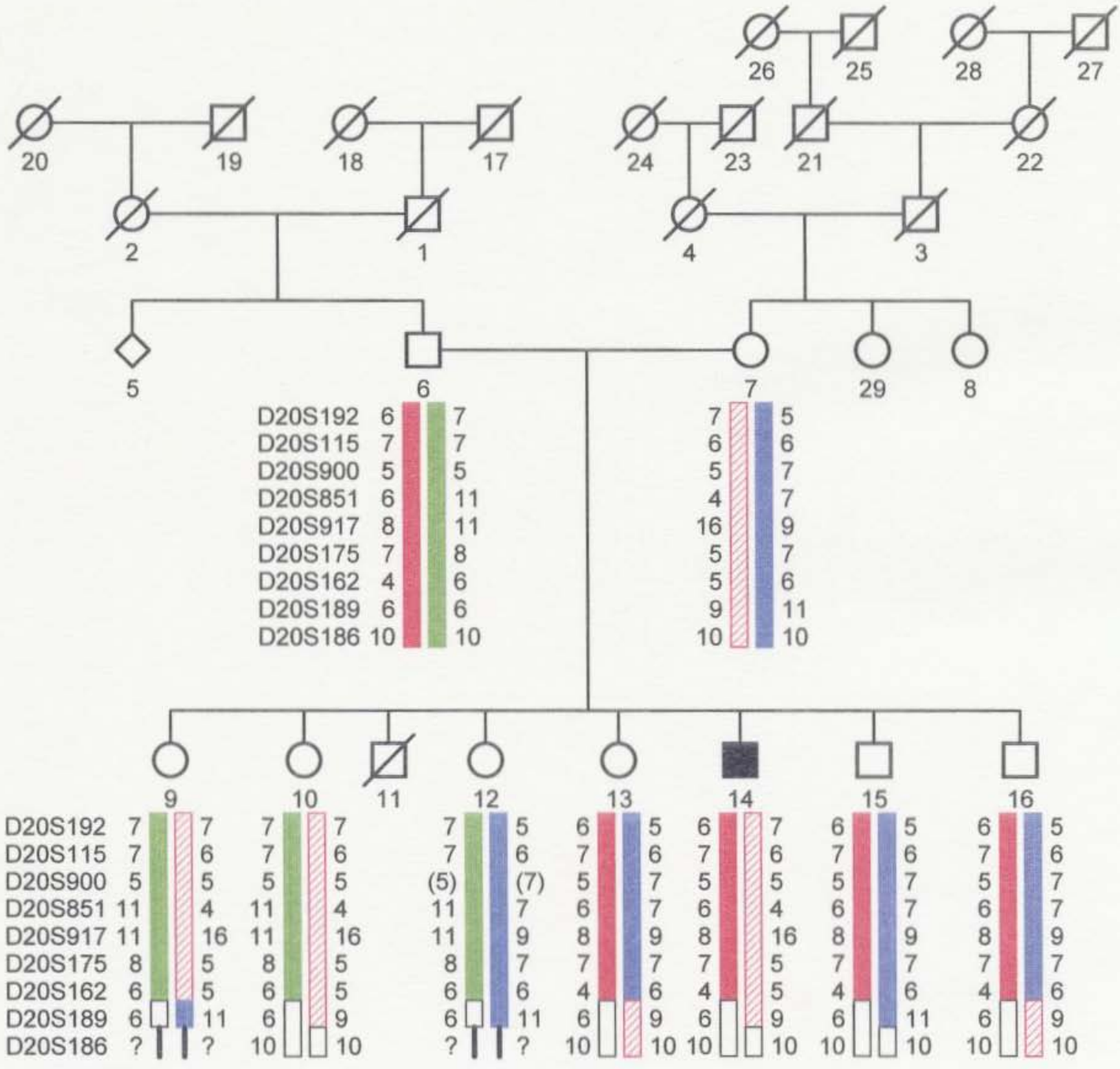
Chromosome 3
Number : B4



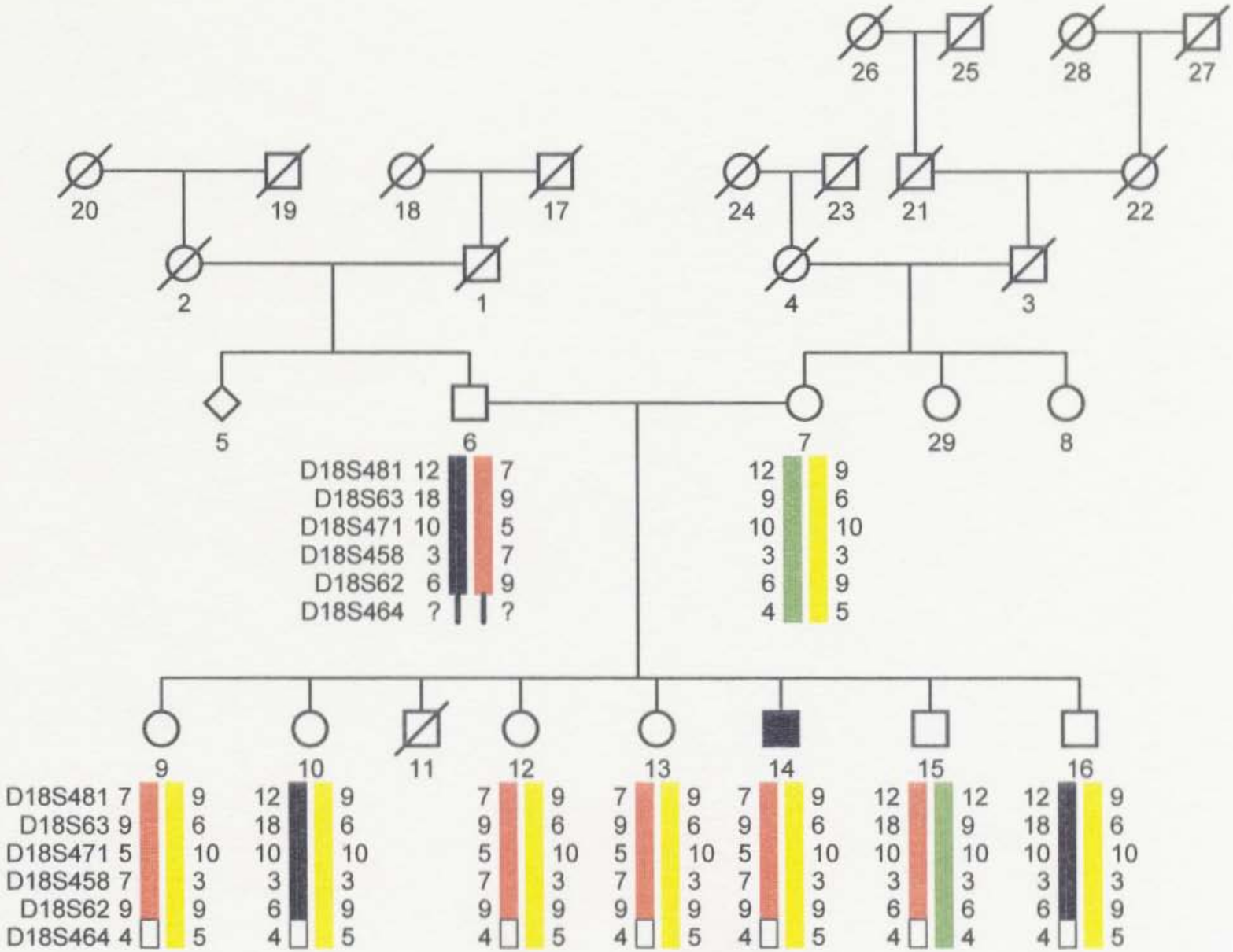
Chromosome 2
Number : B4



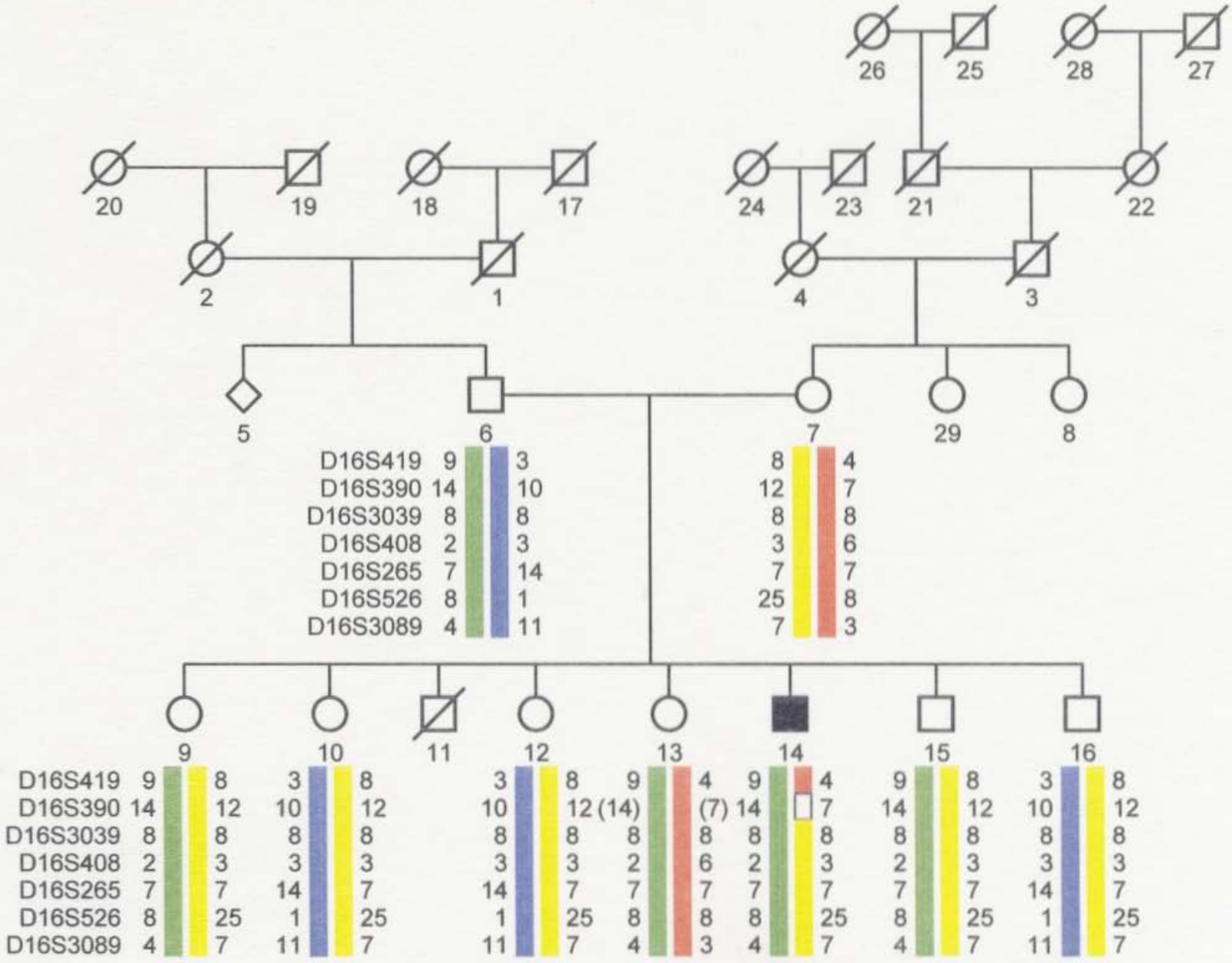
Chromosome 20
Number : B5



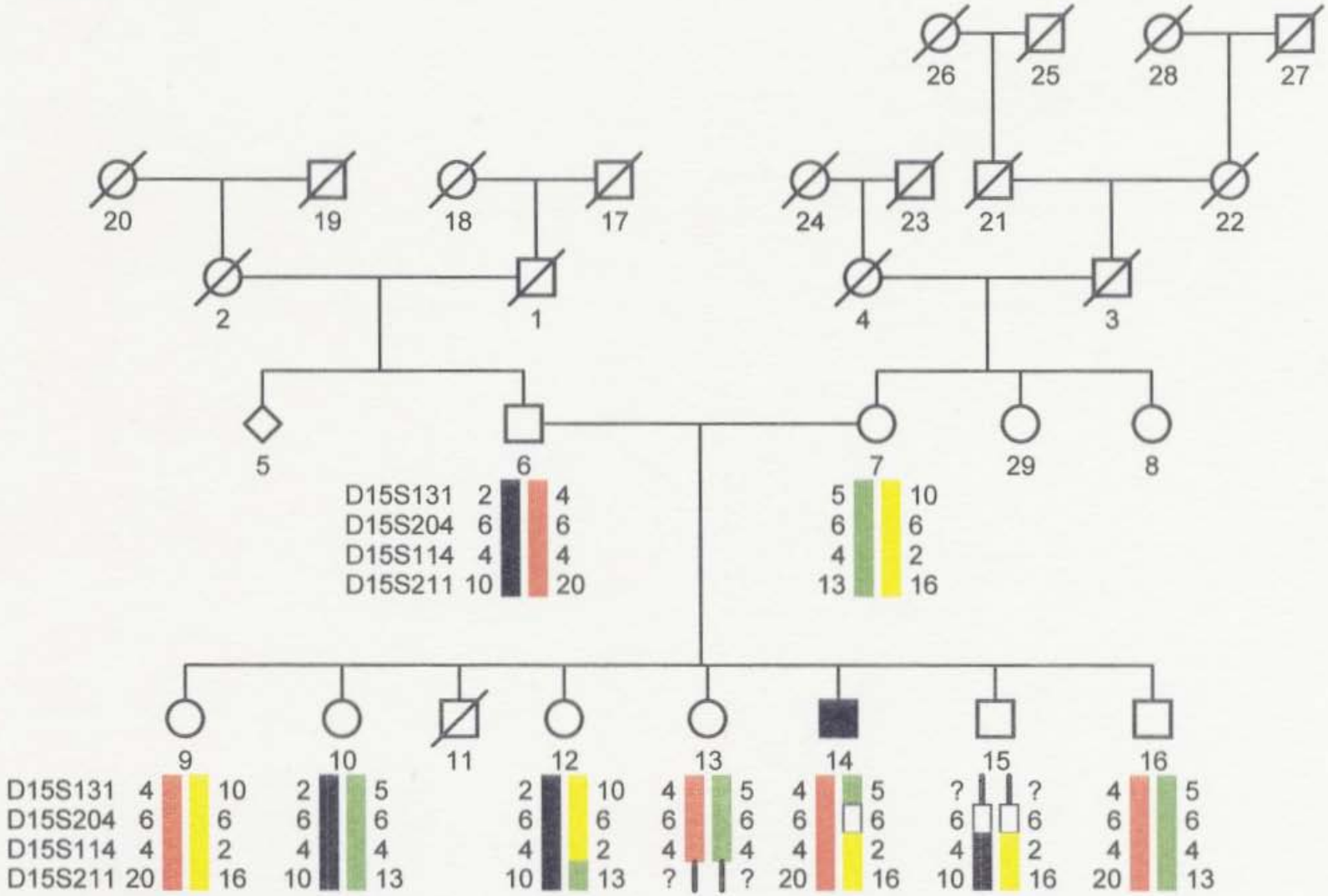
Chromosome 18
Number : B5



Chromosome 16
Number : B5



Chromosome 15
Number : B5



Chromosome 11
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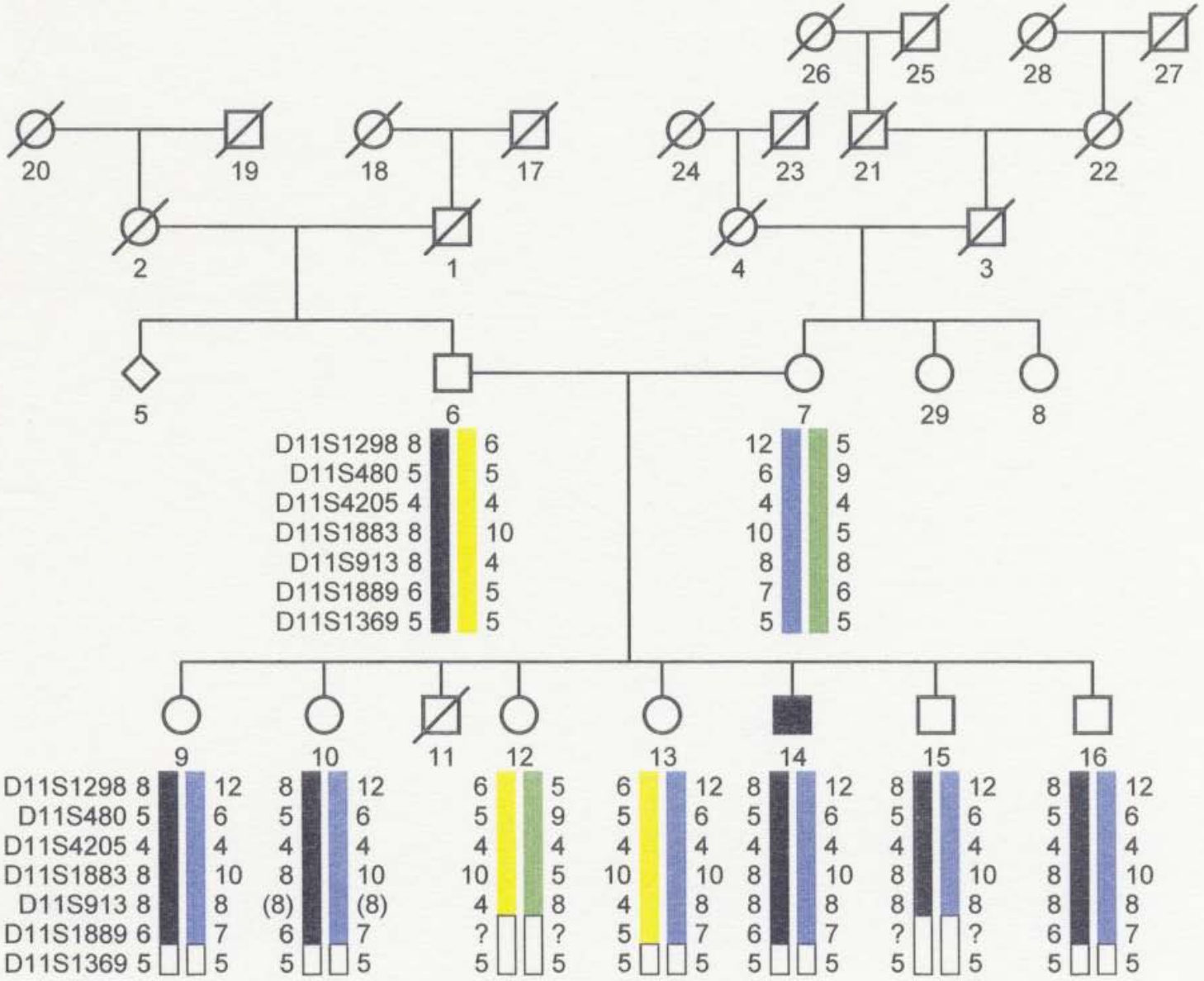
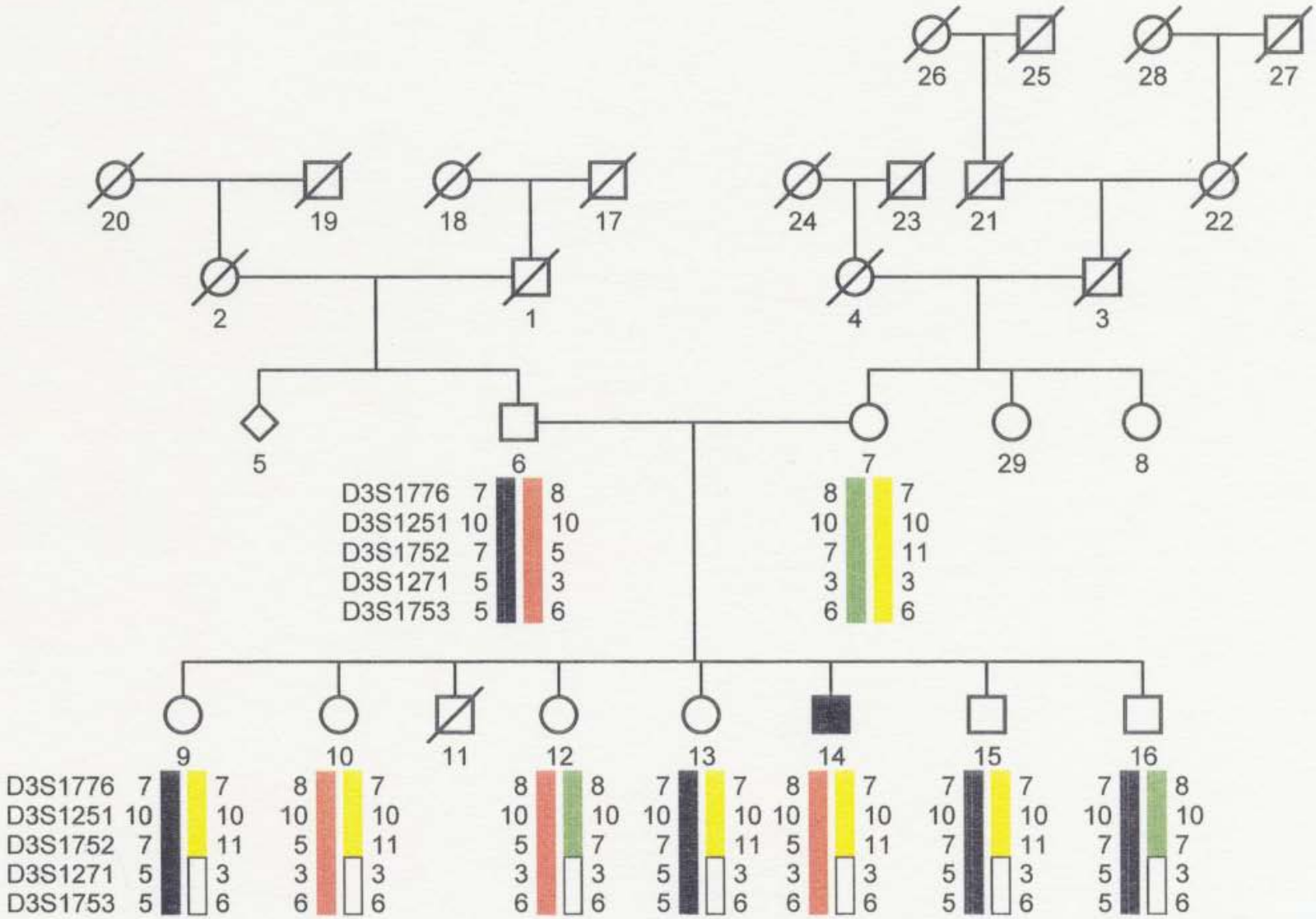
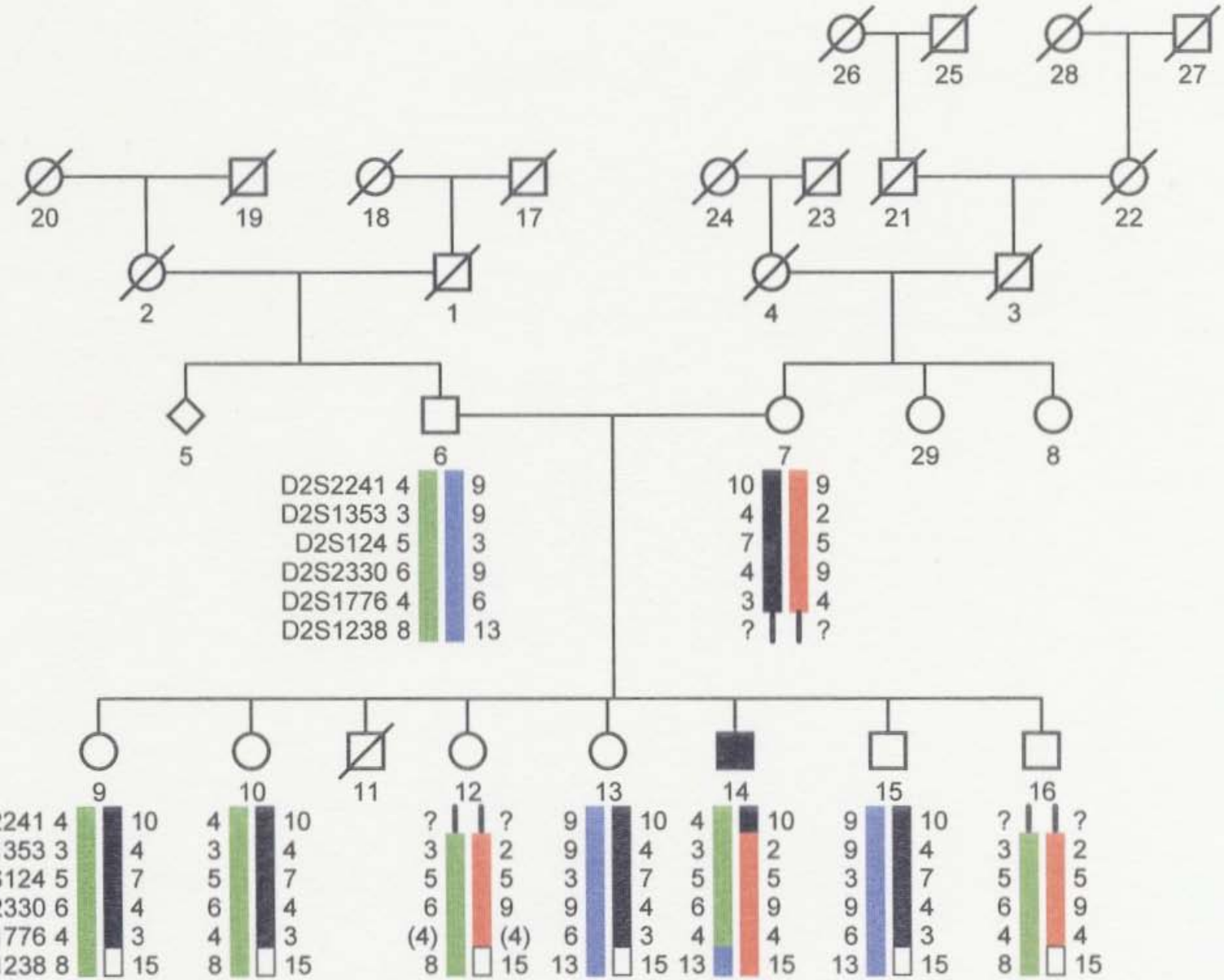


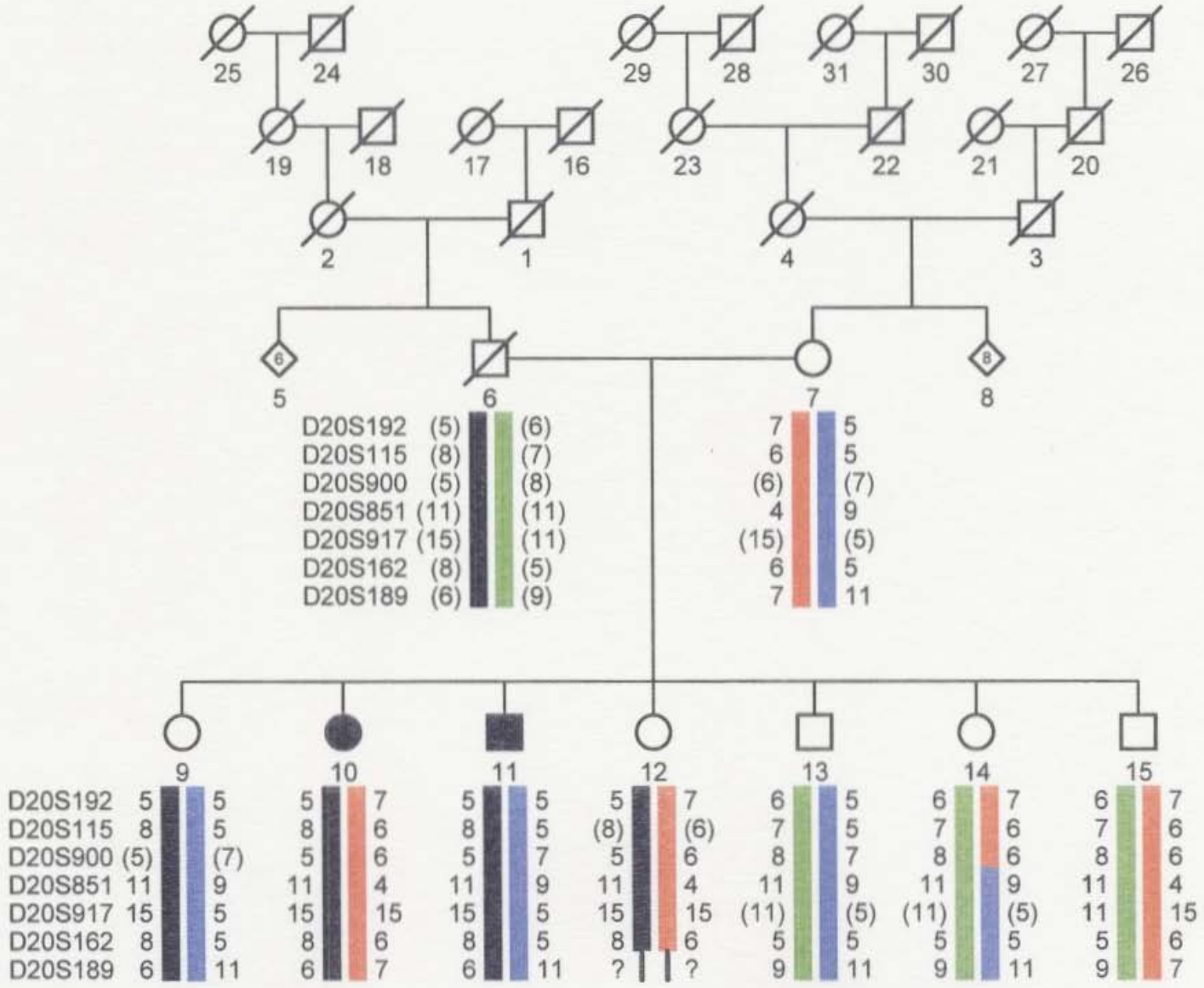
Chromosome 3
Number : B5



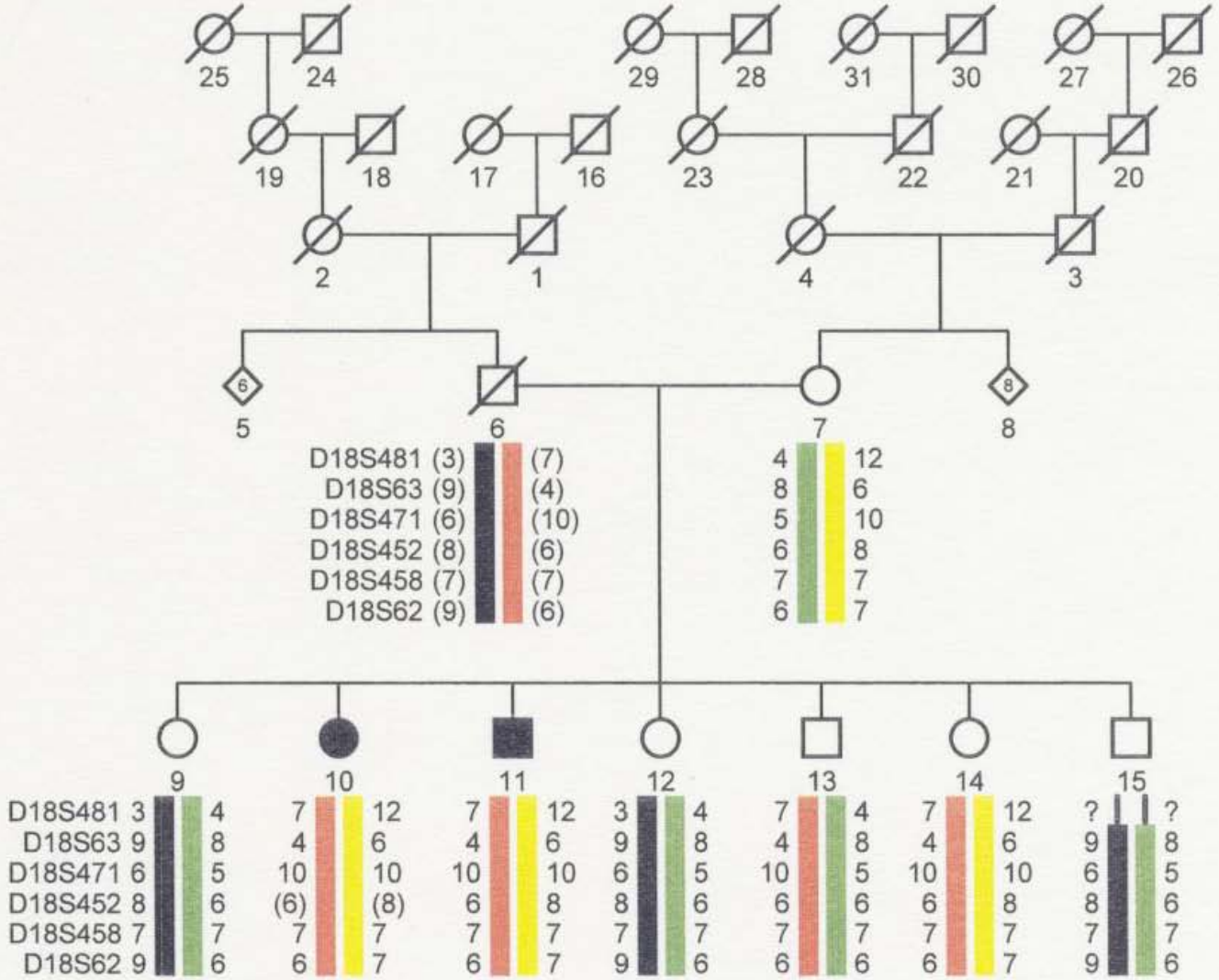
Chromosome 2
Number : B5



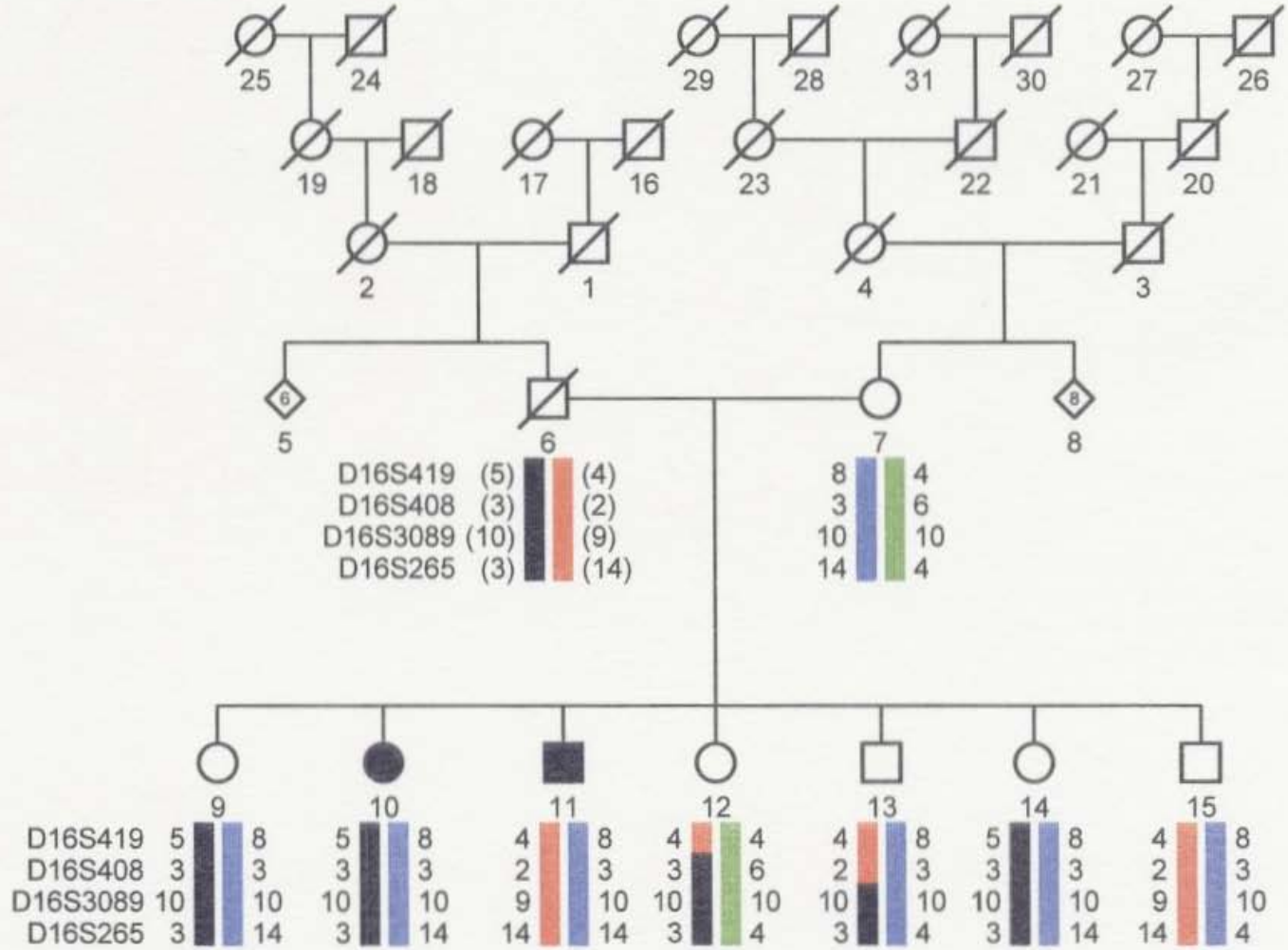
Chromosome 20
Number : B6



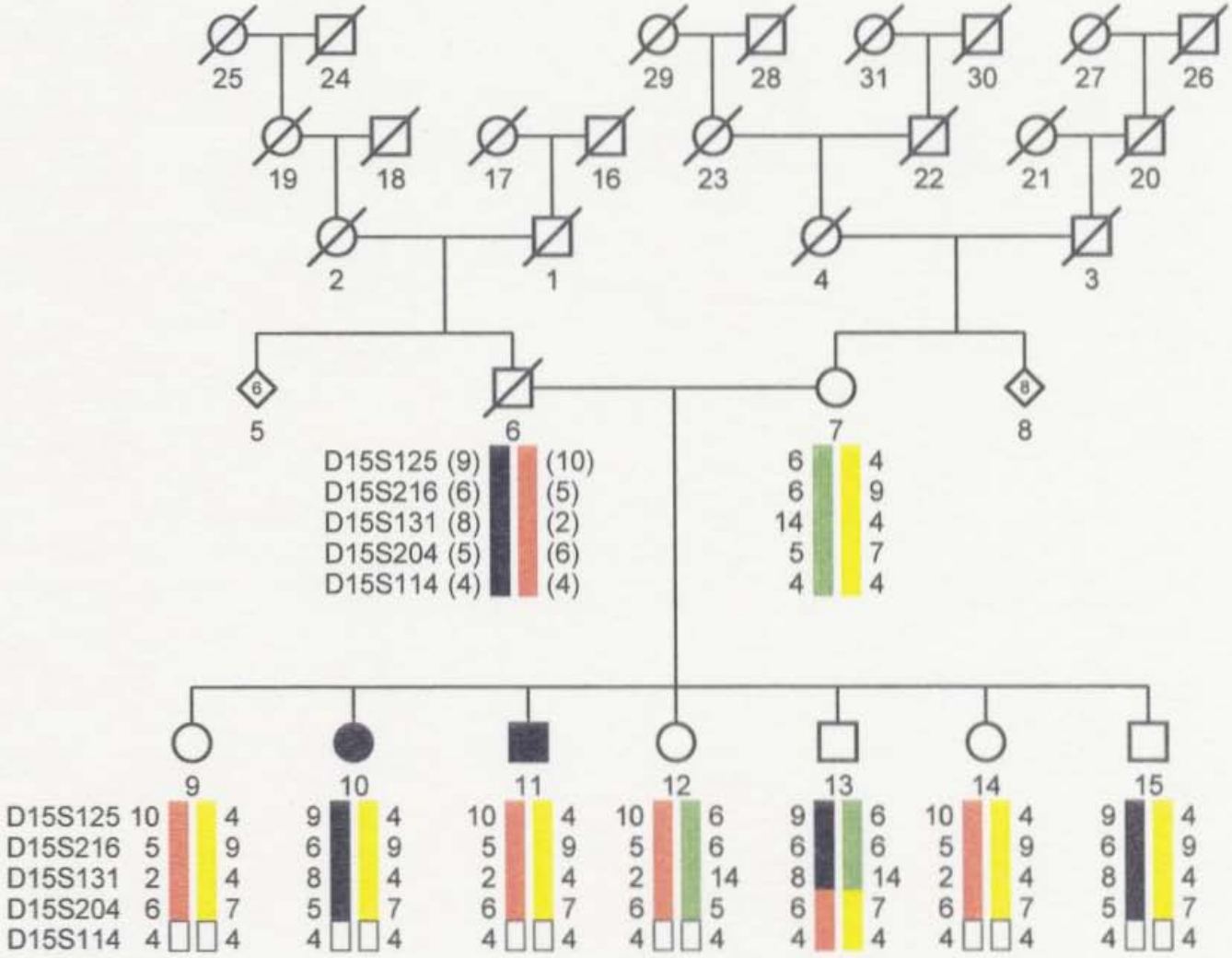
Chromosome 18
Number : B6



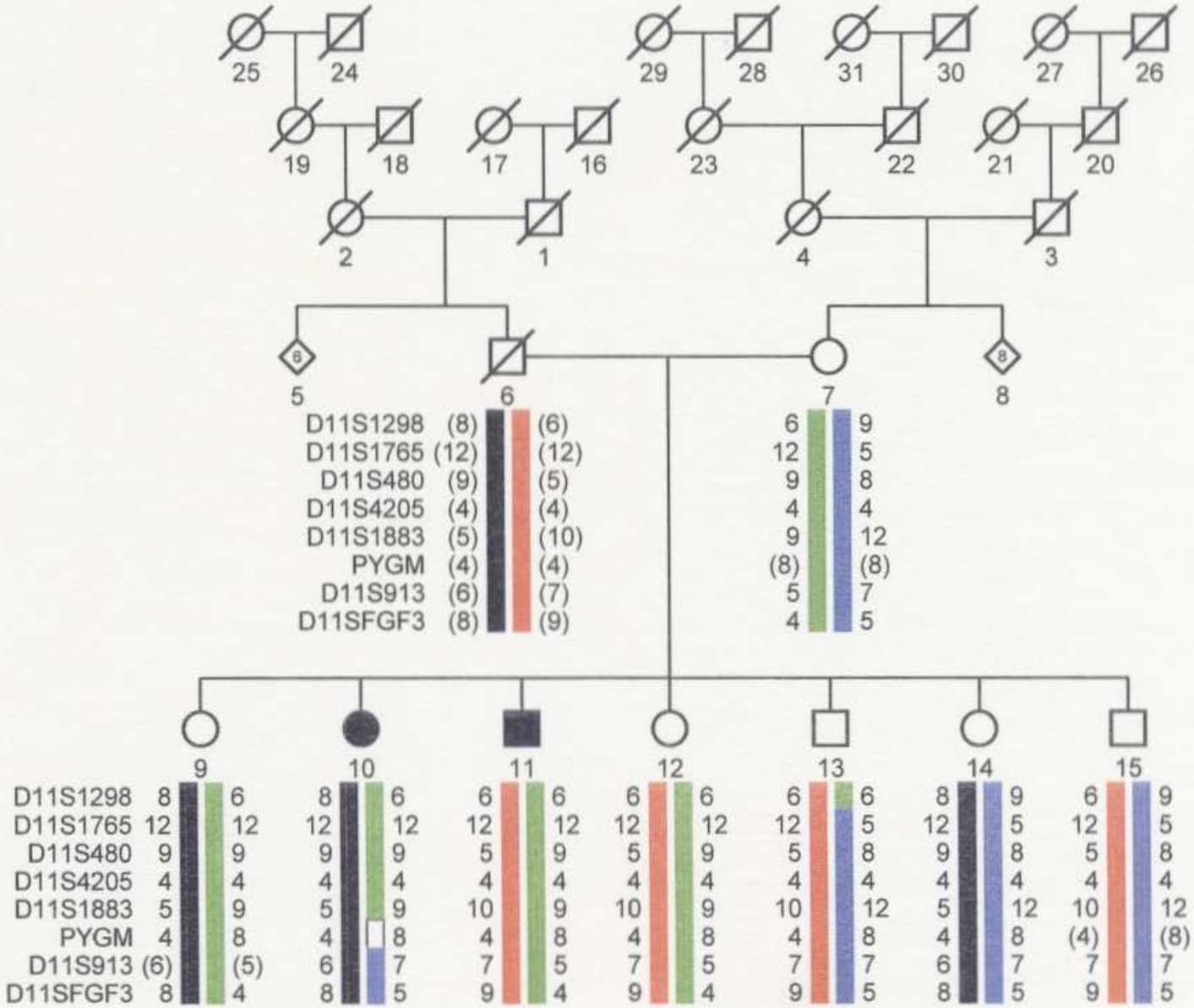
Chromosome 16
Number : B6



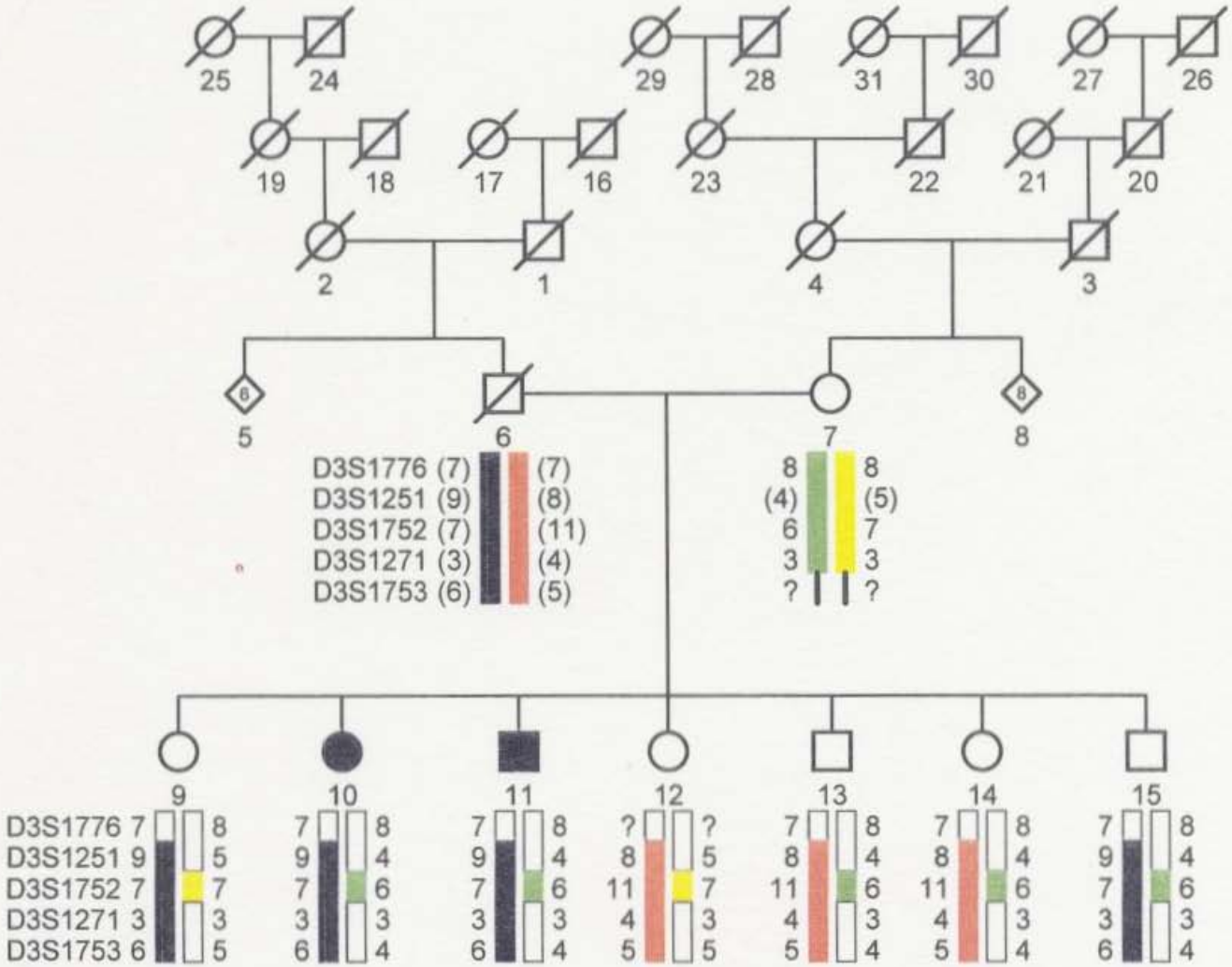
Chromosome 15
Number : B6



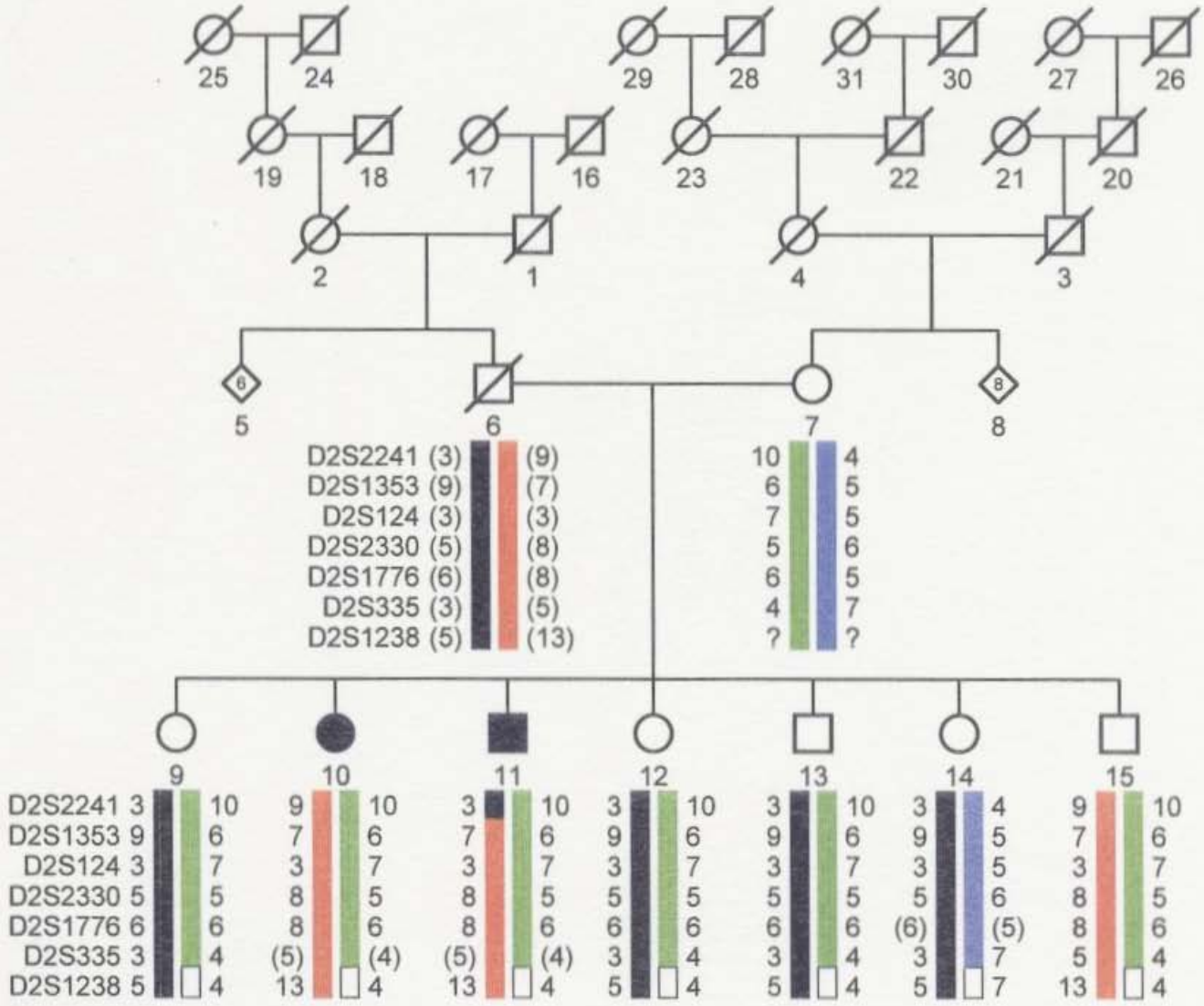
Chromosome 11
Number : B6



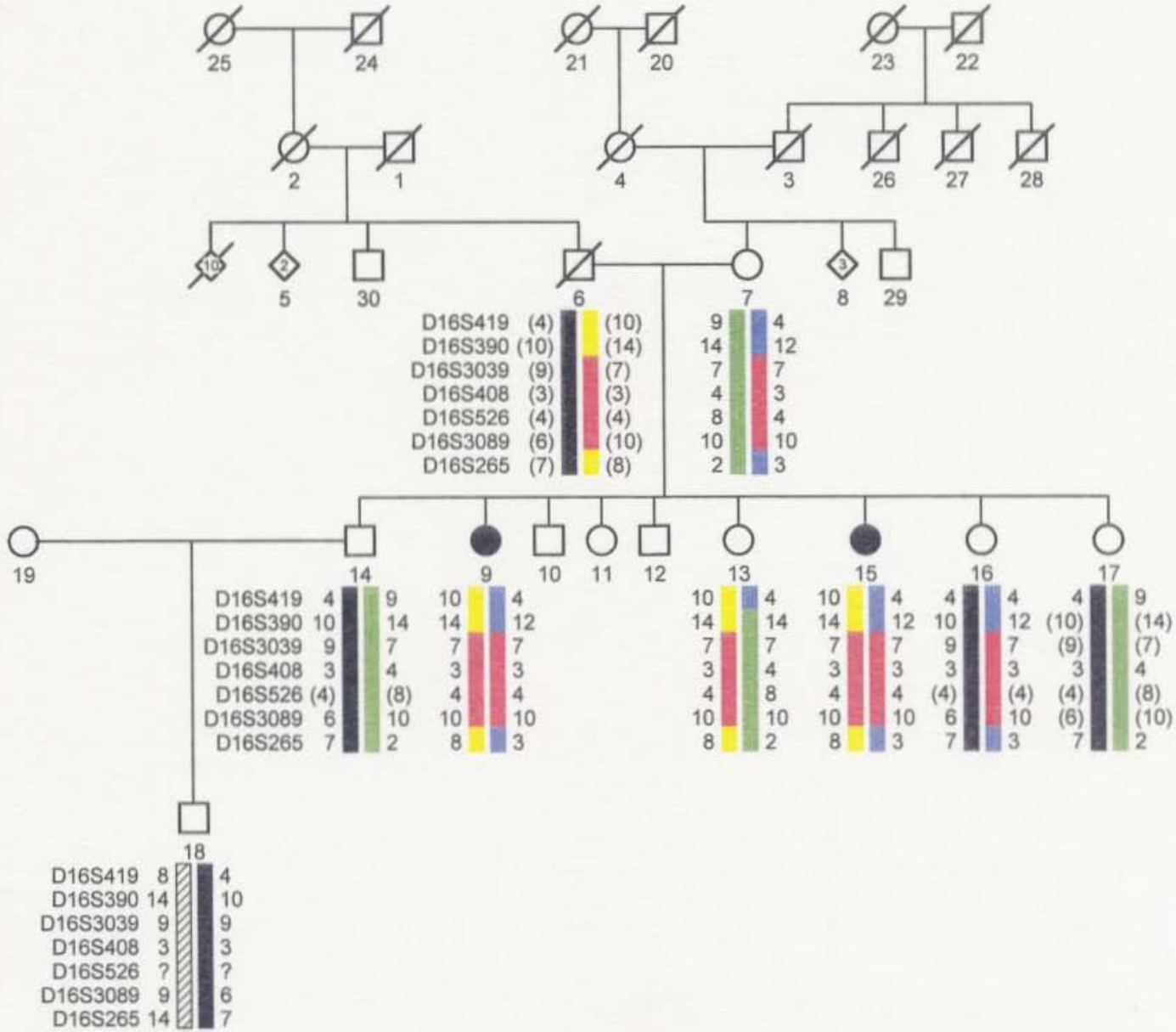
Chromosome 3
Number : B6



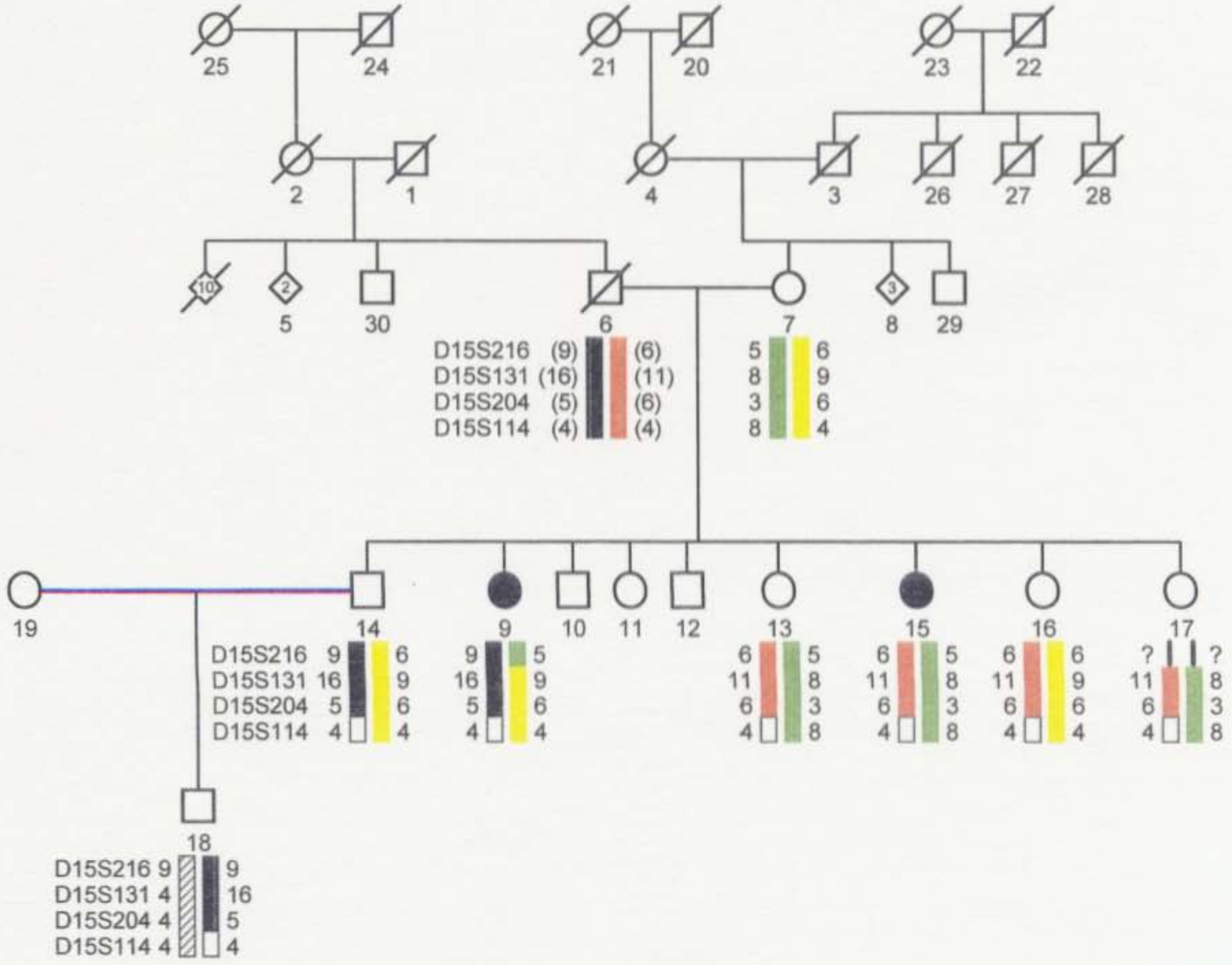
Chromosome 2
Number : B6



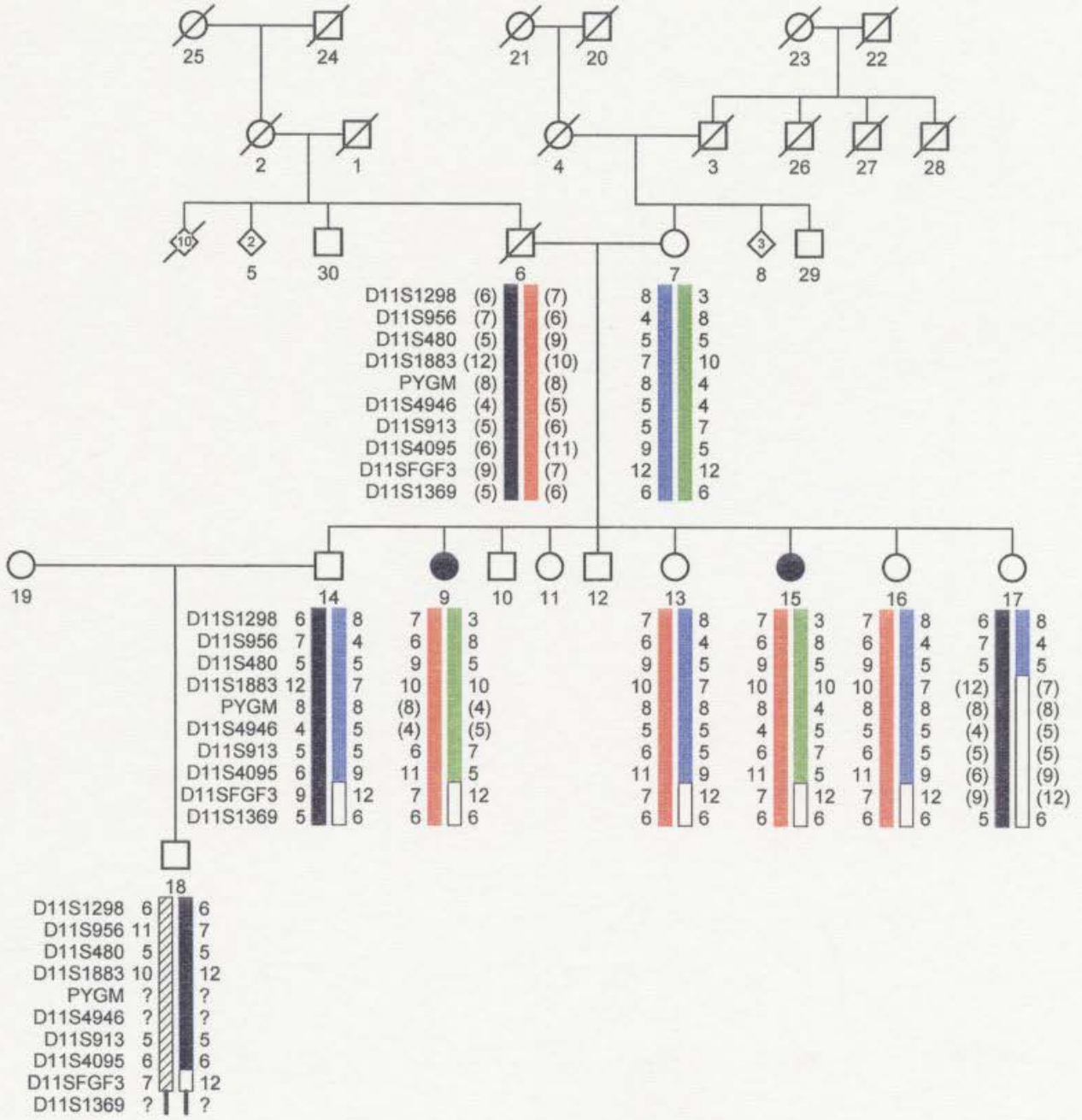
Chromosome 16
Number : B7



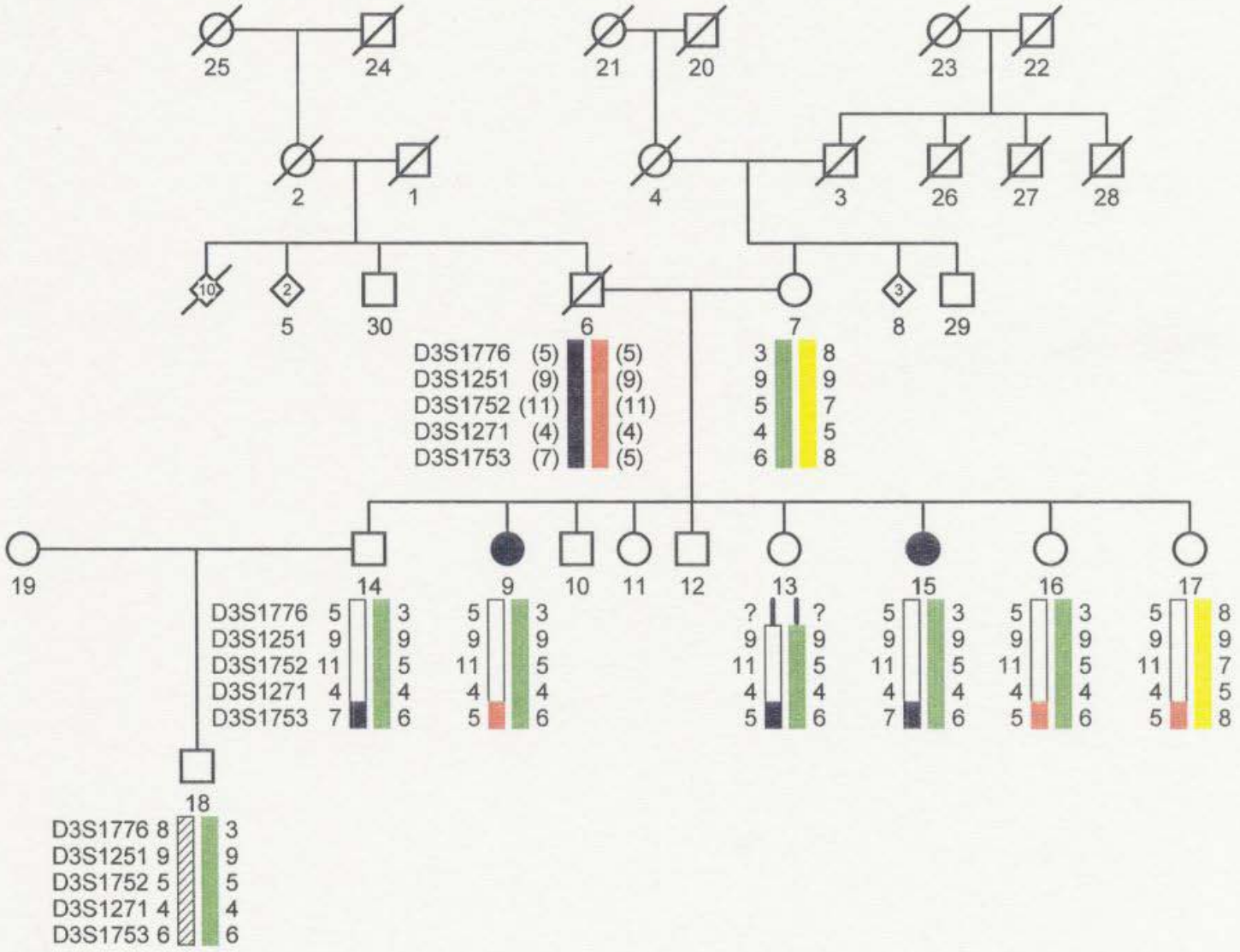
Chromosome 15
Number : B7



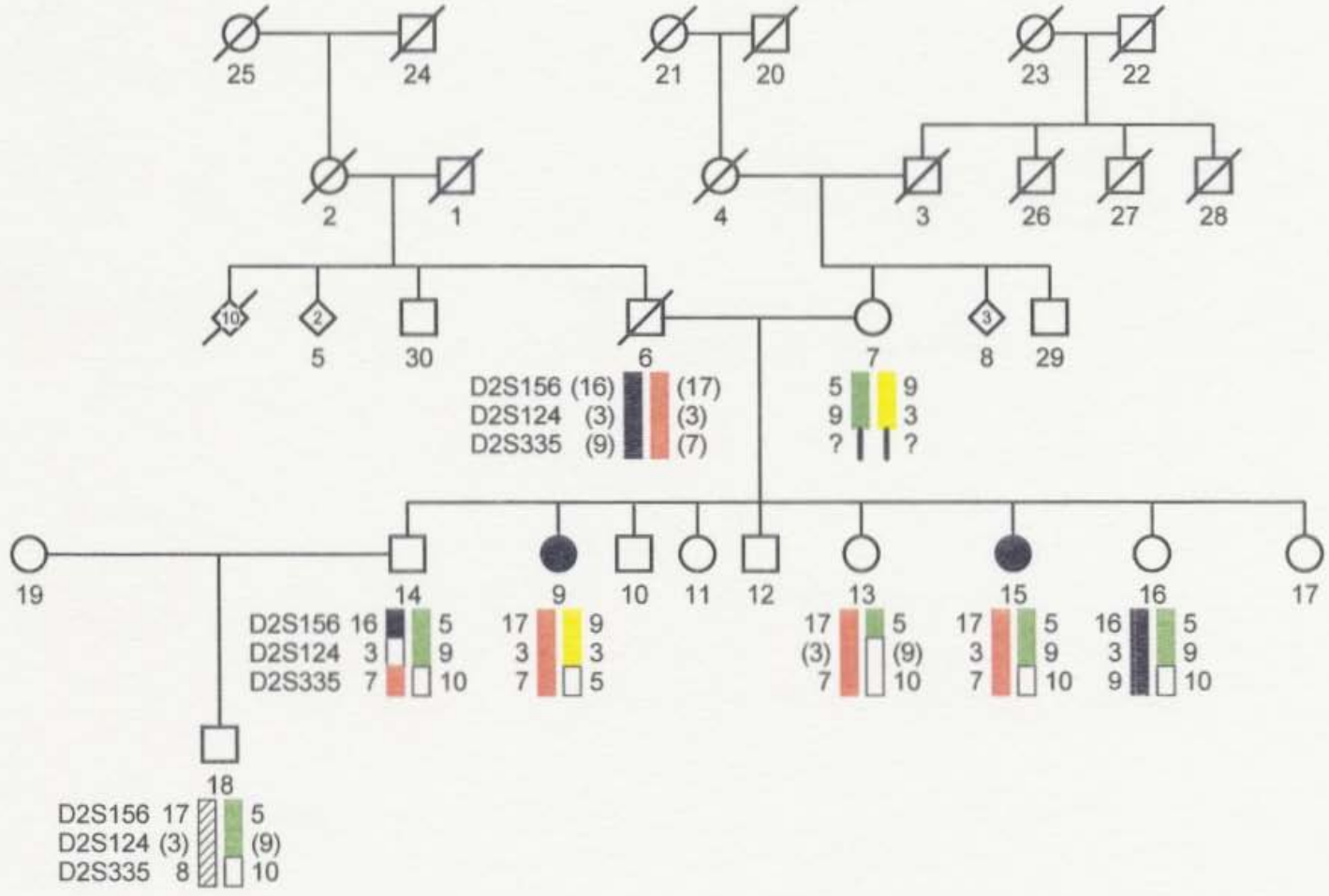
Chromosome 11
Number : B7



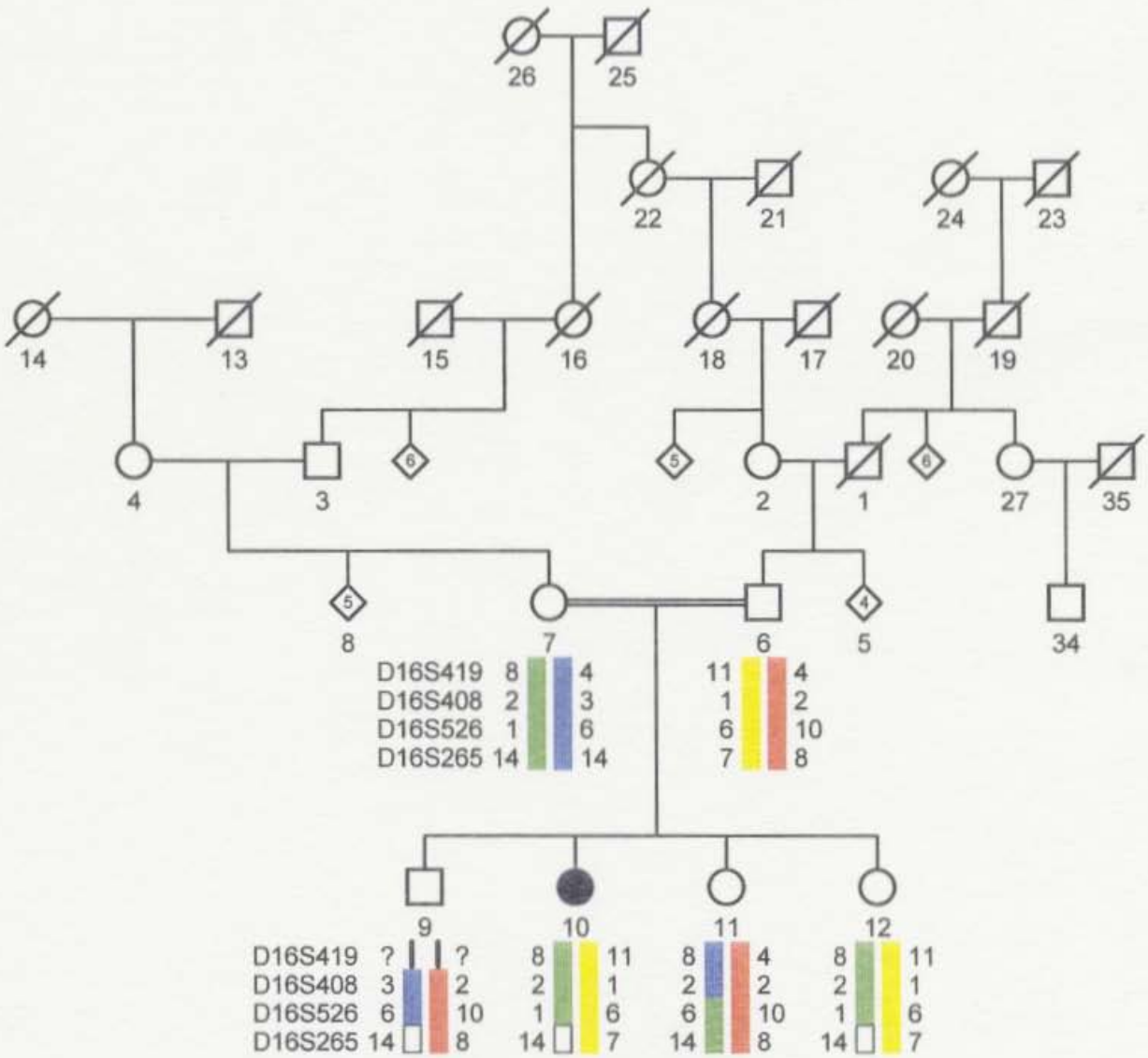
Chromosome 3
Number : B7



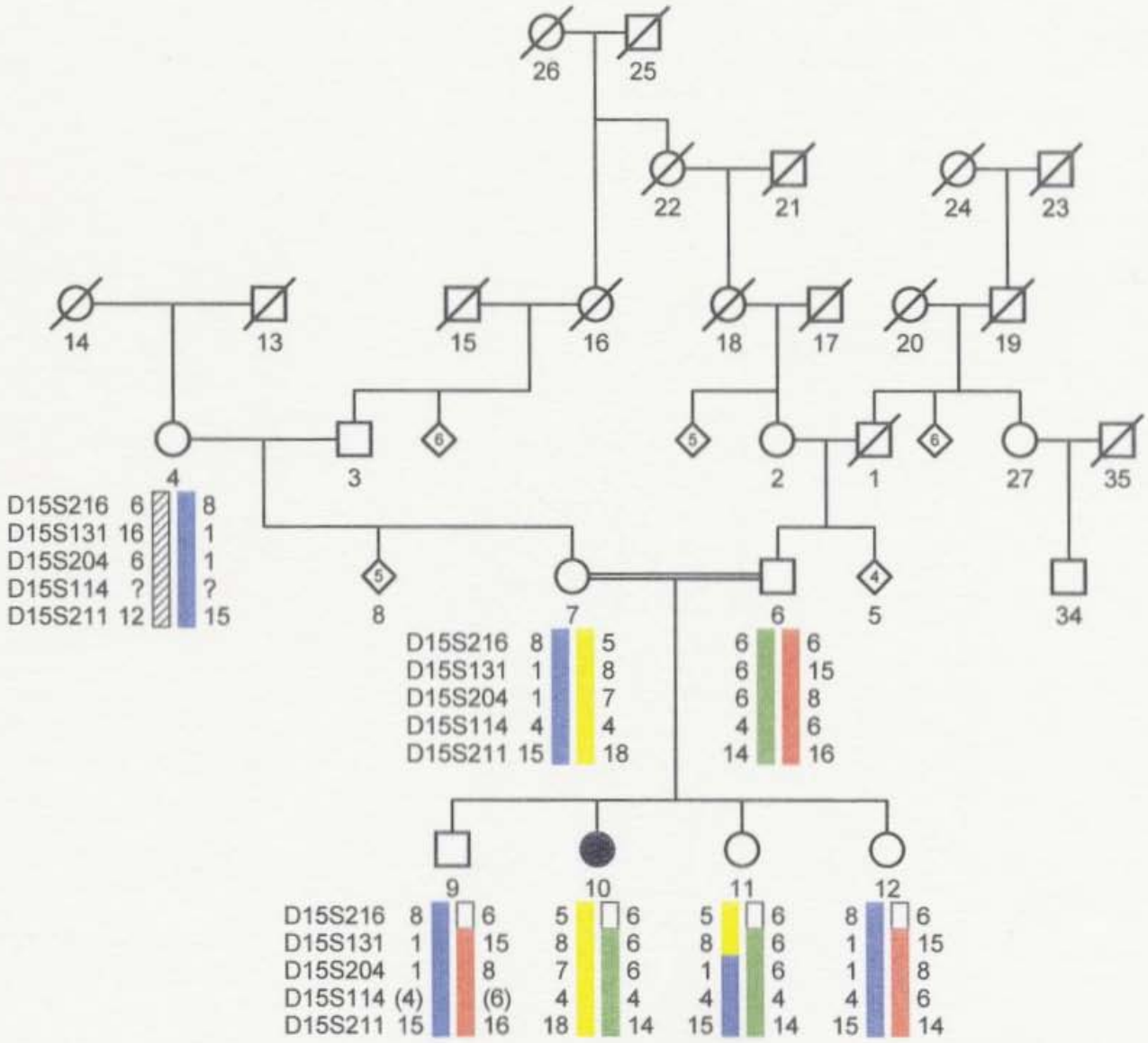
Chromosome 2
Number : B7



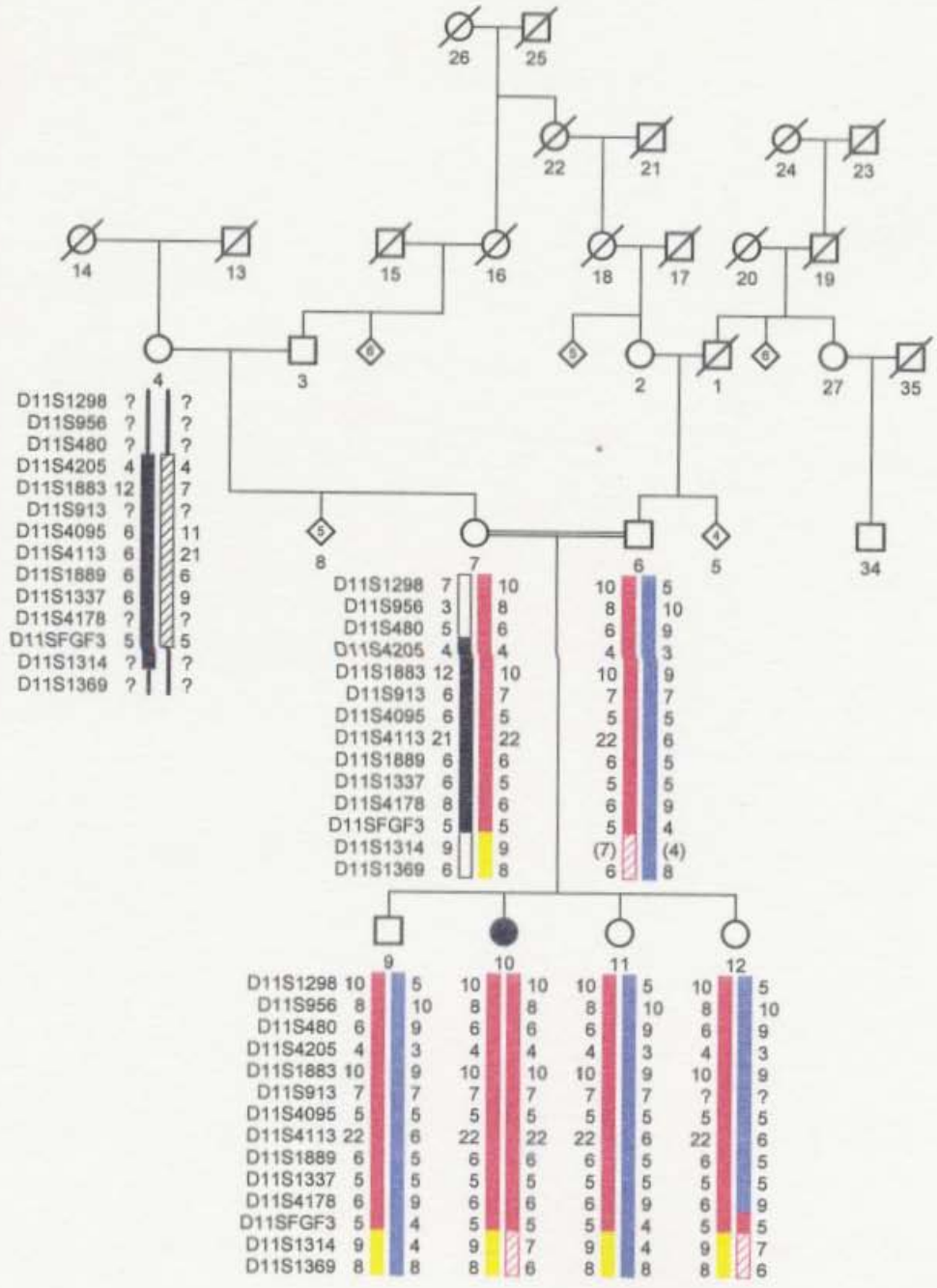
Chromosome 16
Number : B8



Chromosome 15
Number : B8



Chromosome 11
Number : B8



Chromosome 3
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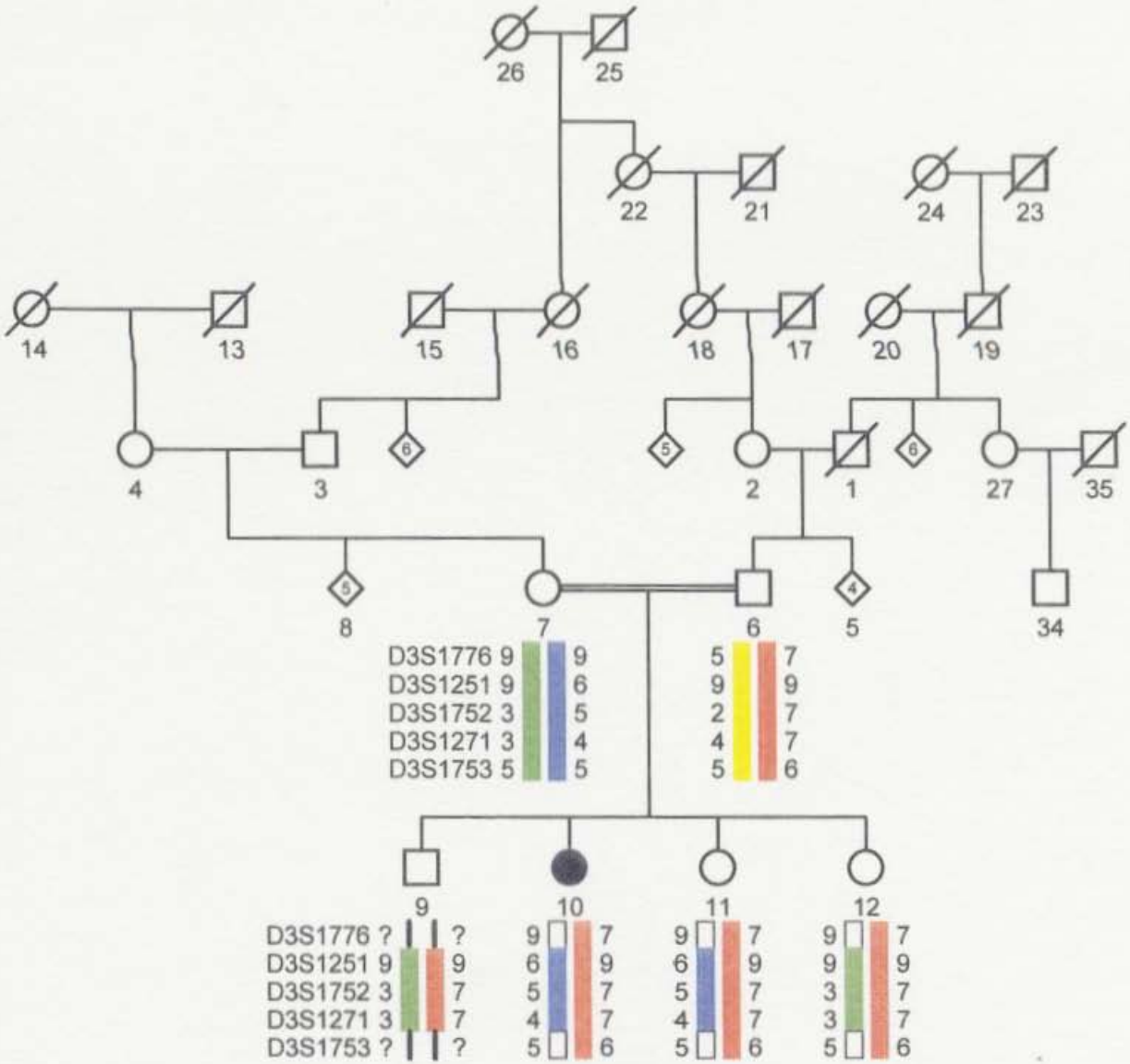

Chromosome 16
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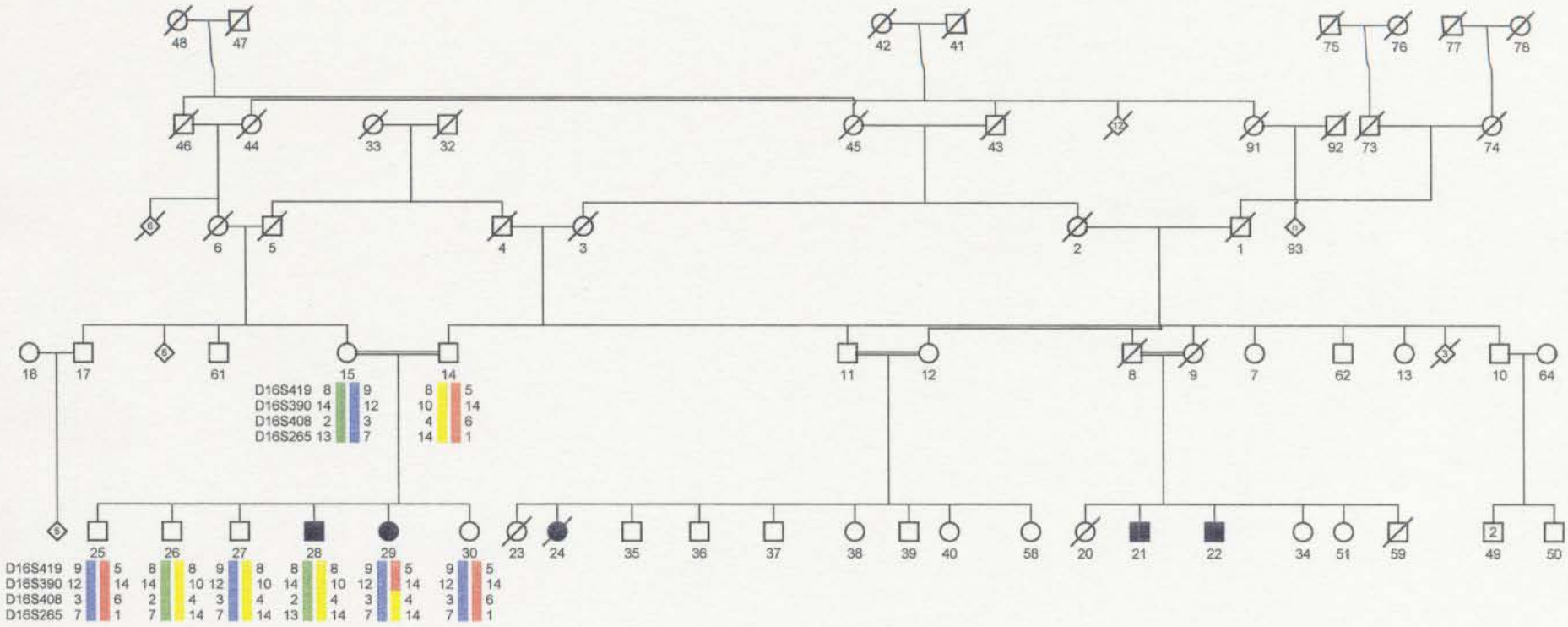
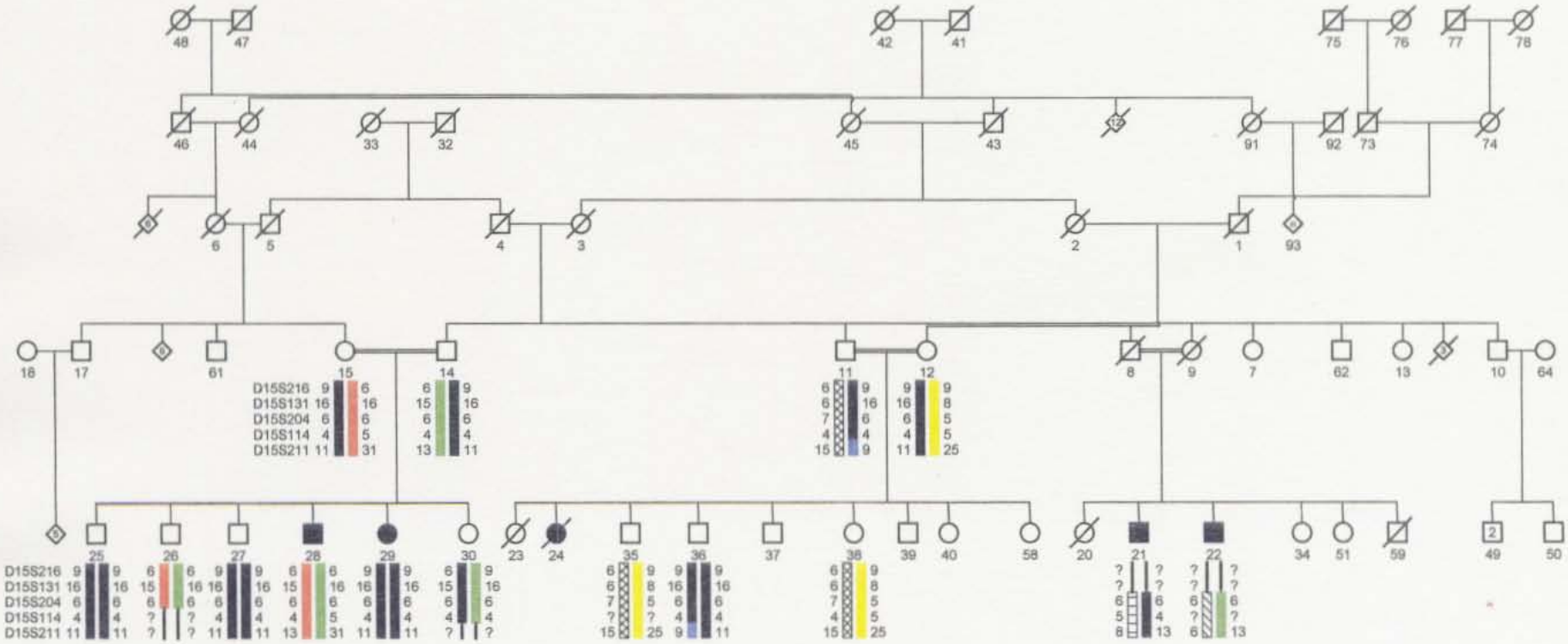
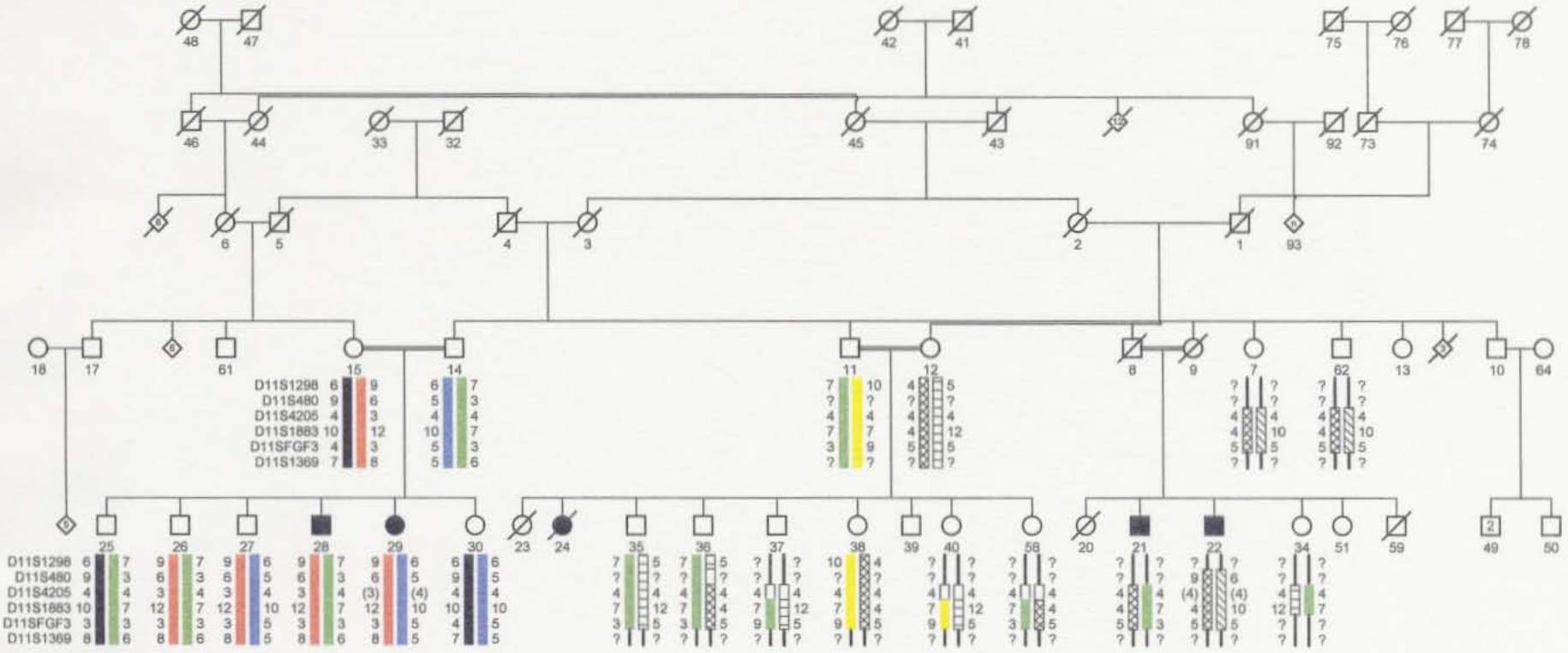


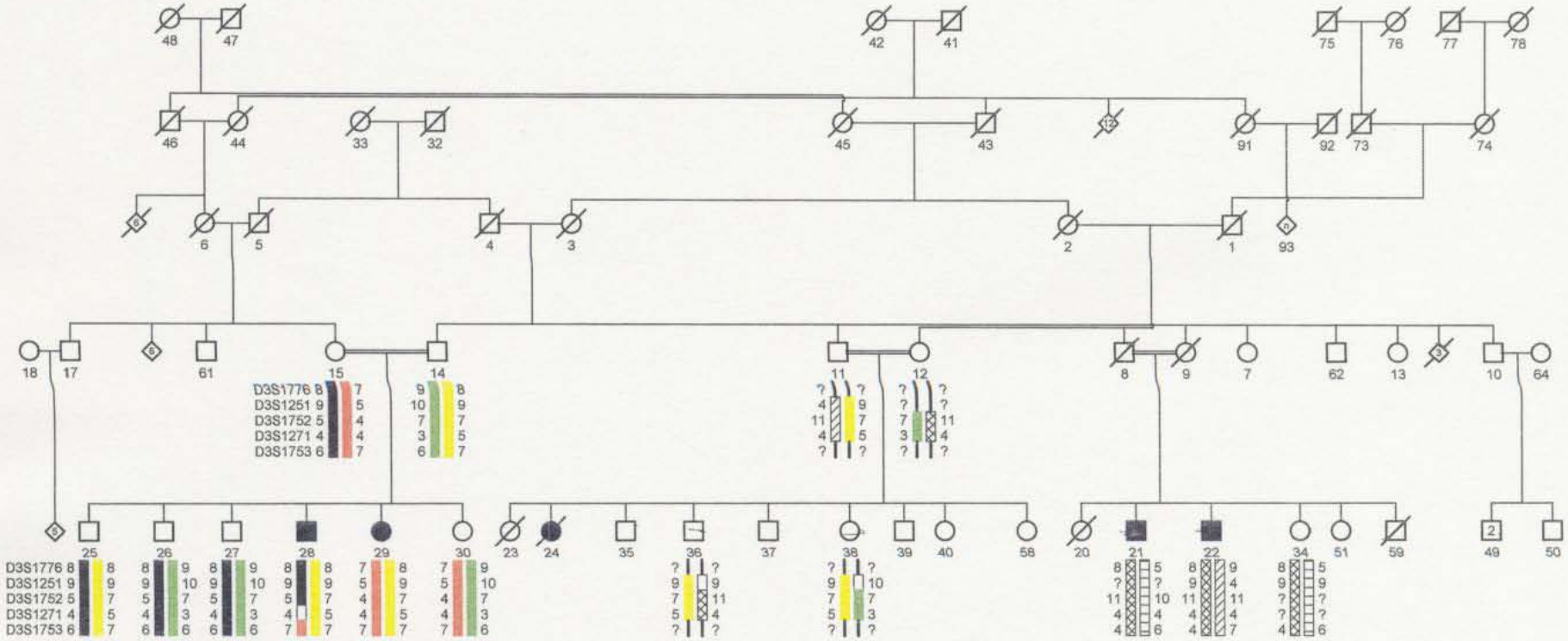
Chromosome 15
Number : B9

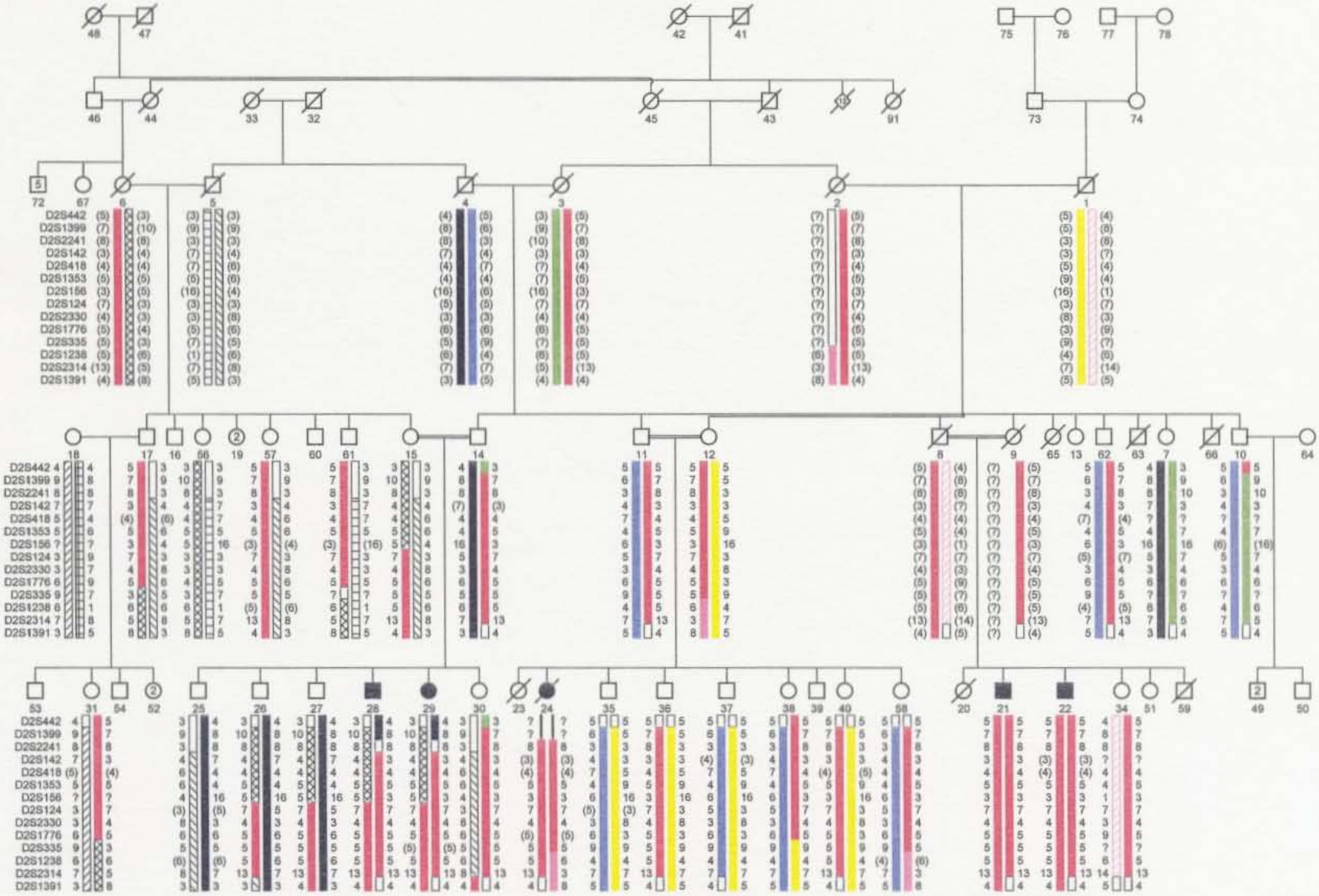


Chromosome 11
Number : B9



Chromosome 3
Number : B9




Chromosome 2
Number : B9
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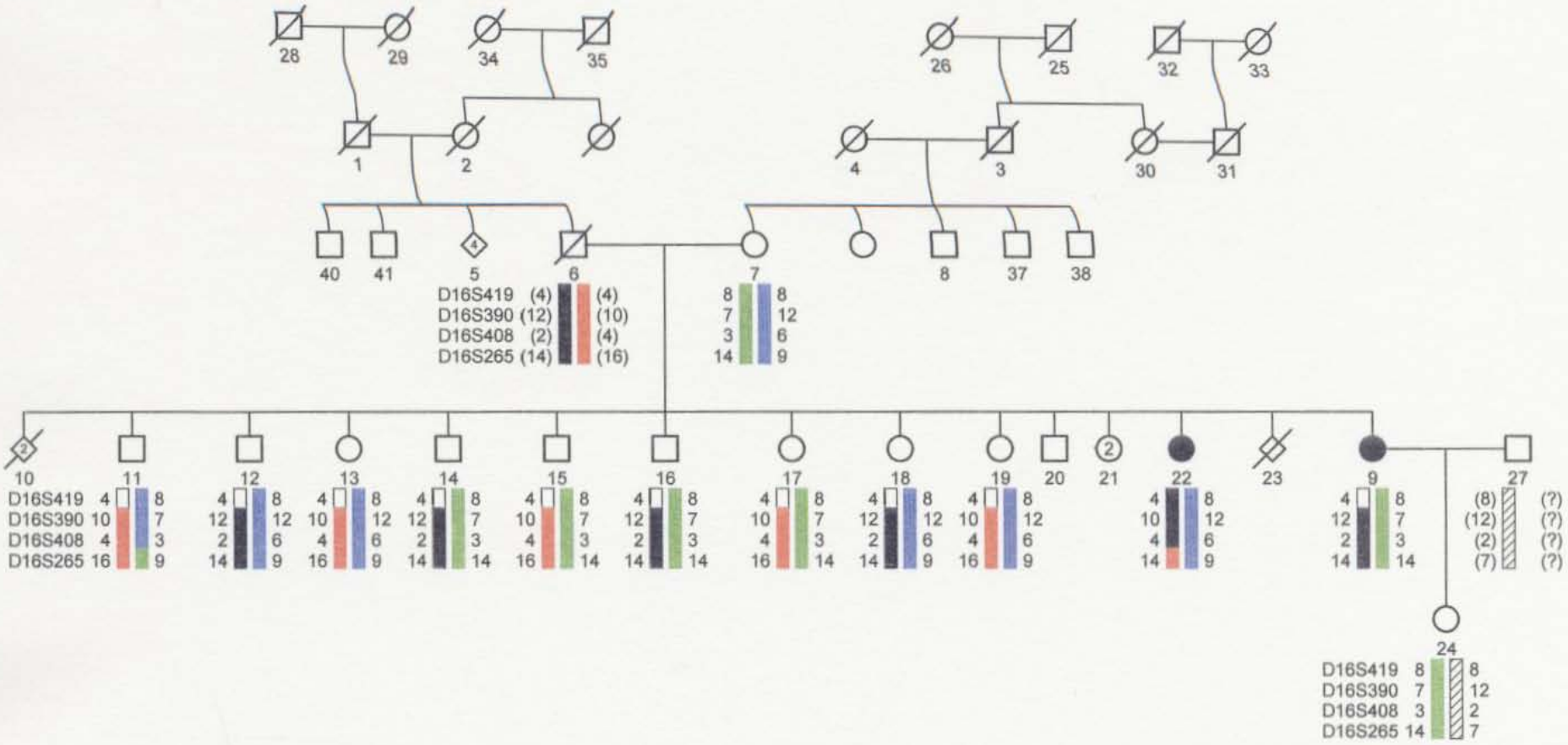
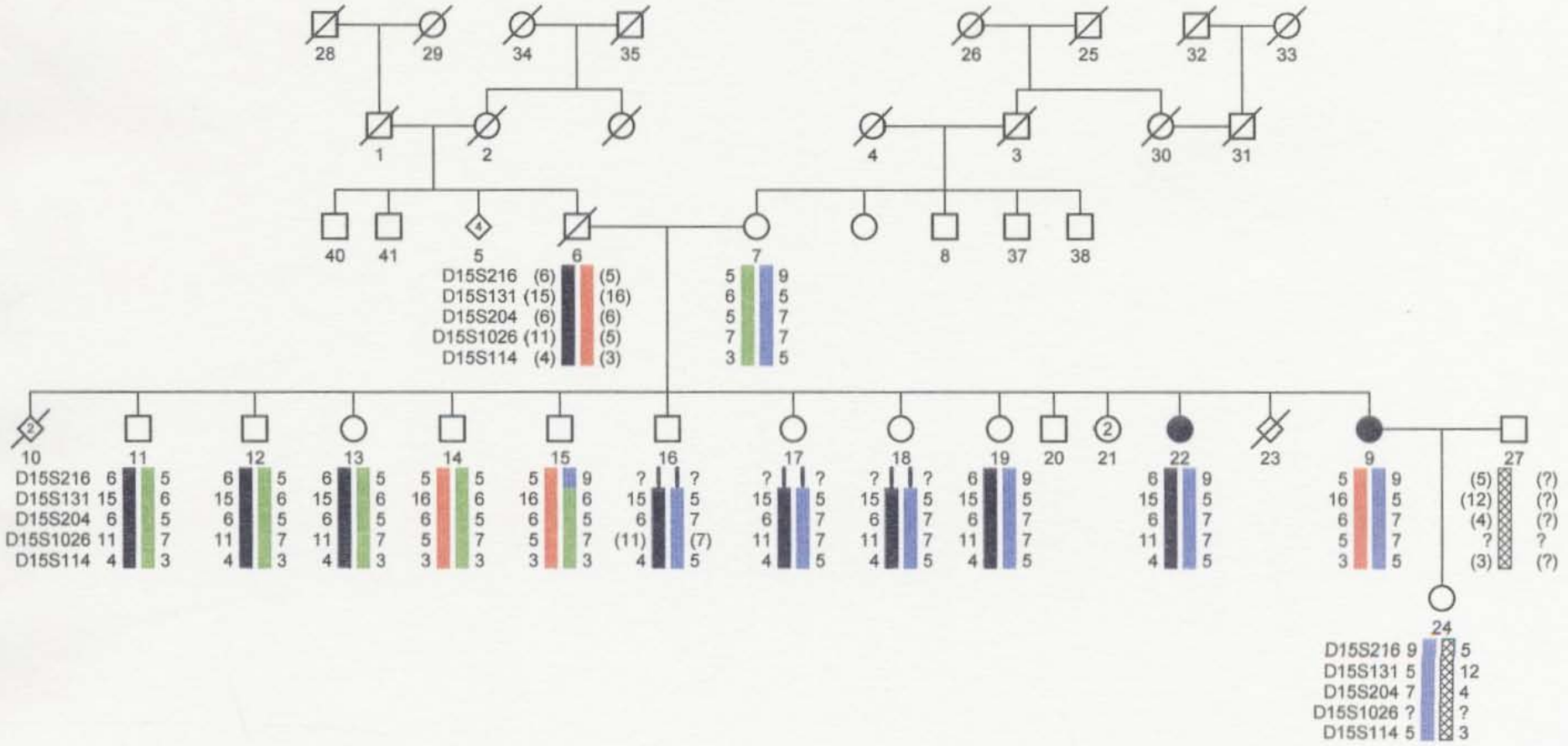
D2S1399
D2S2241
D2S142
D2S418
D2S1353
D2S156
D2S124
D2S2330
D2S1776
D2S335
D2S1238
D2S2314
D2S1391

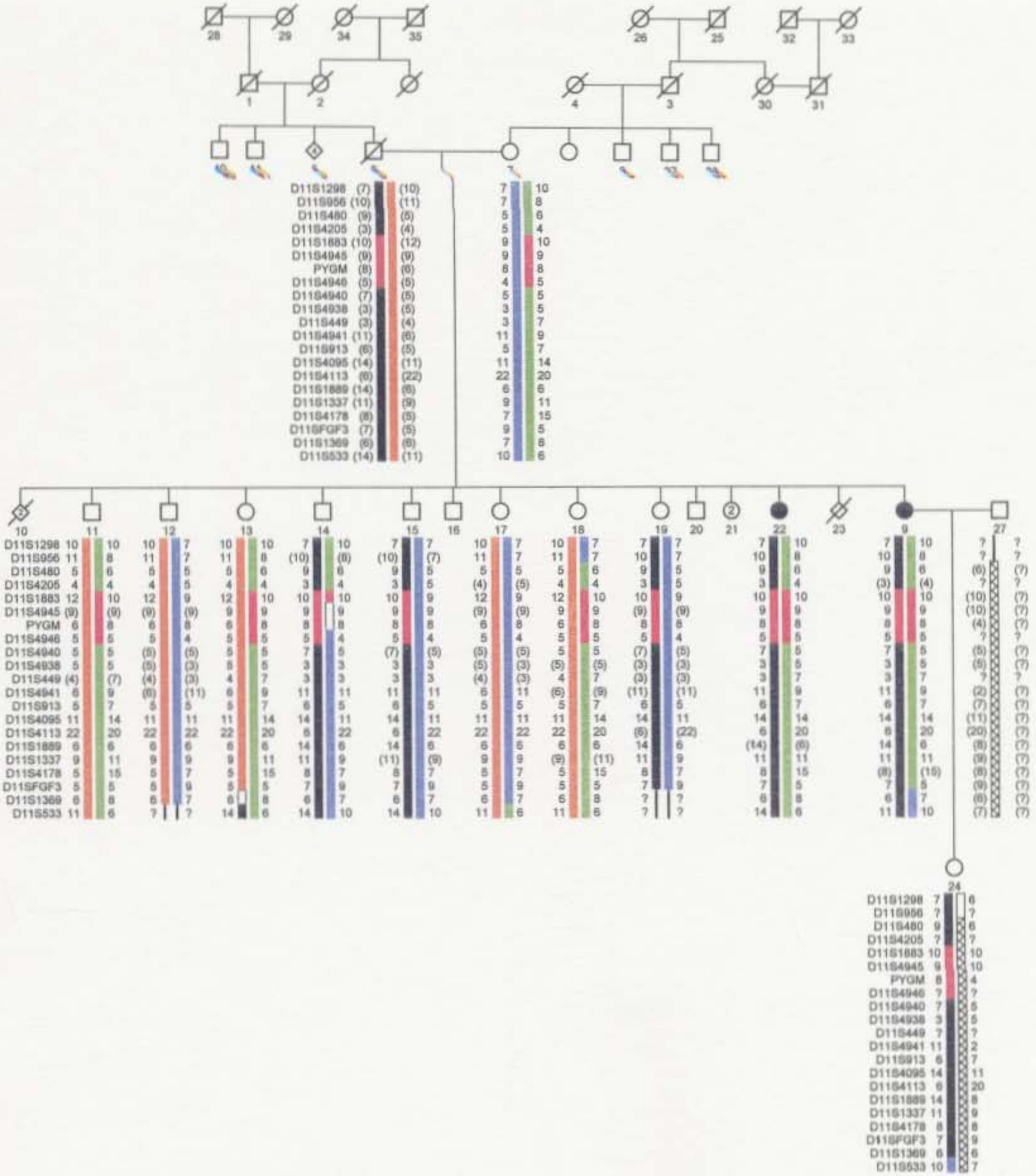
Chromosome 16
Number : B10



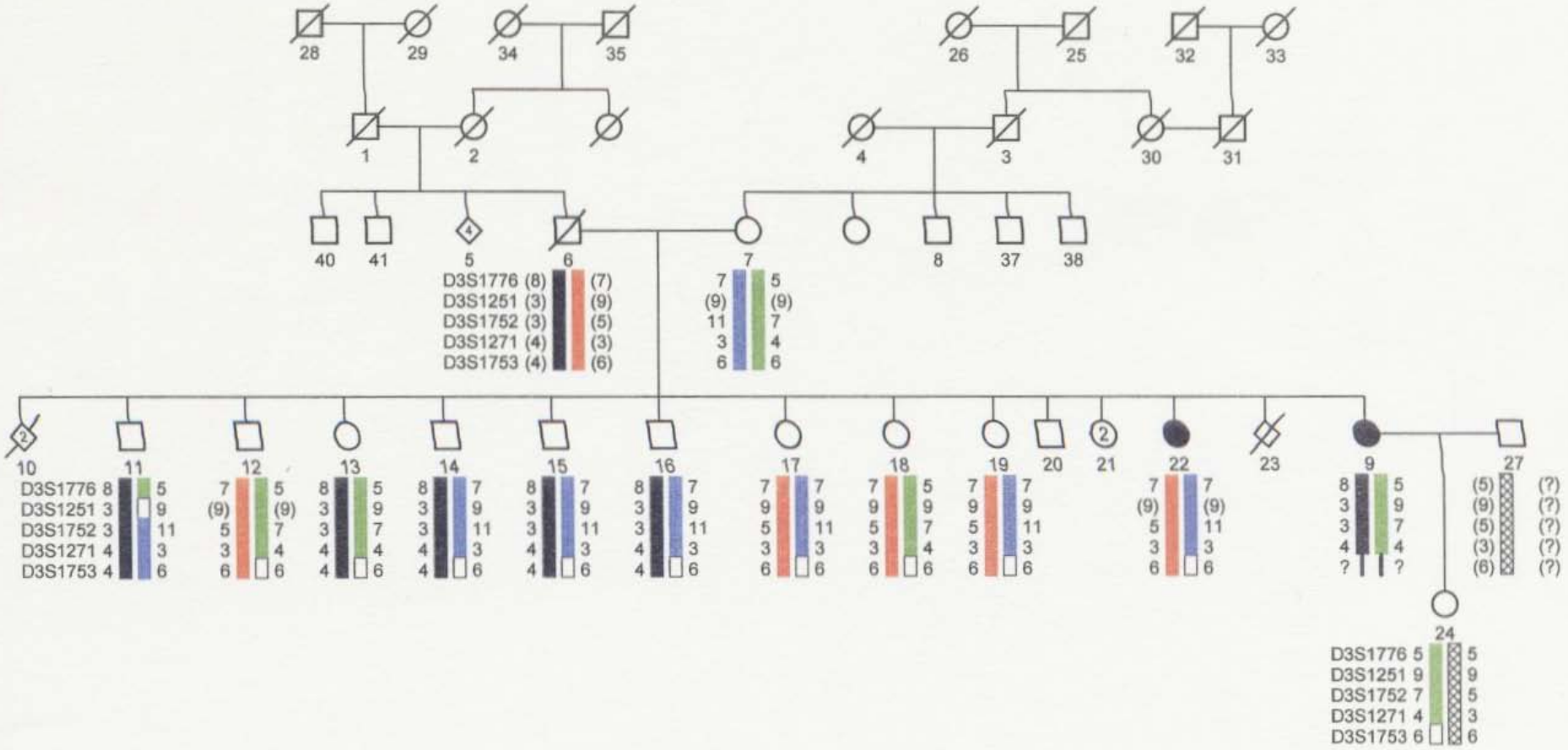
Chromosome 15
Number : B10



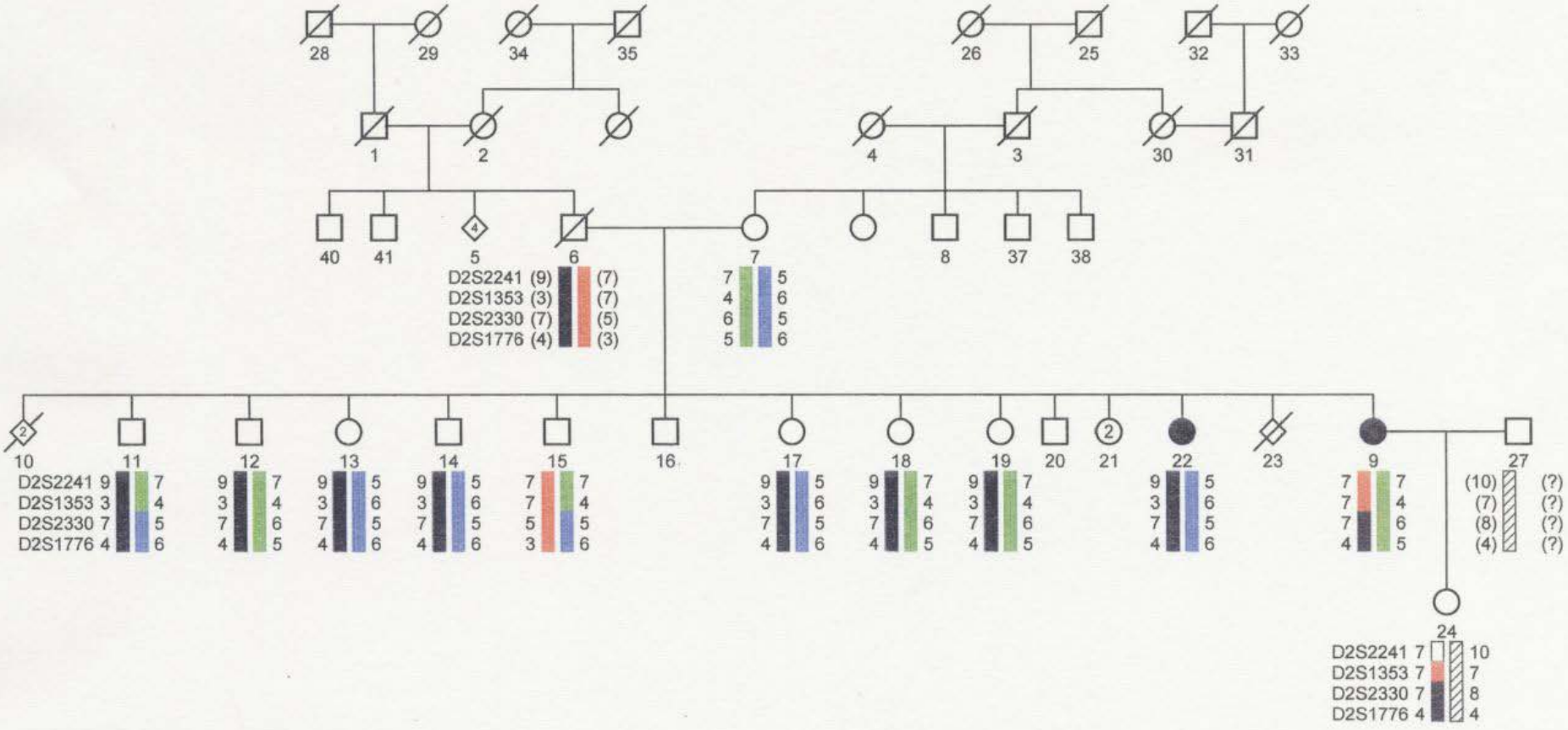
Chromosome 11
Number : B10



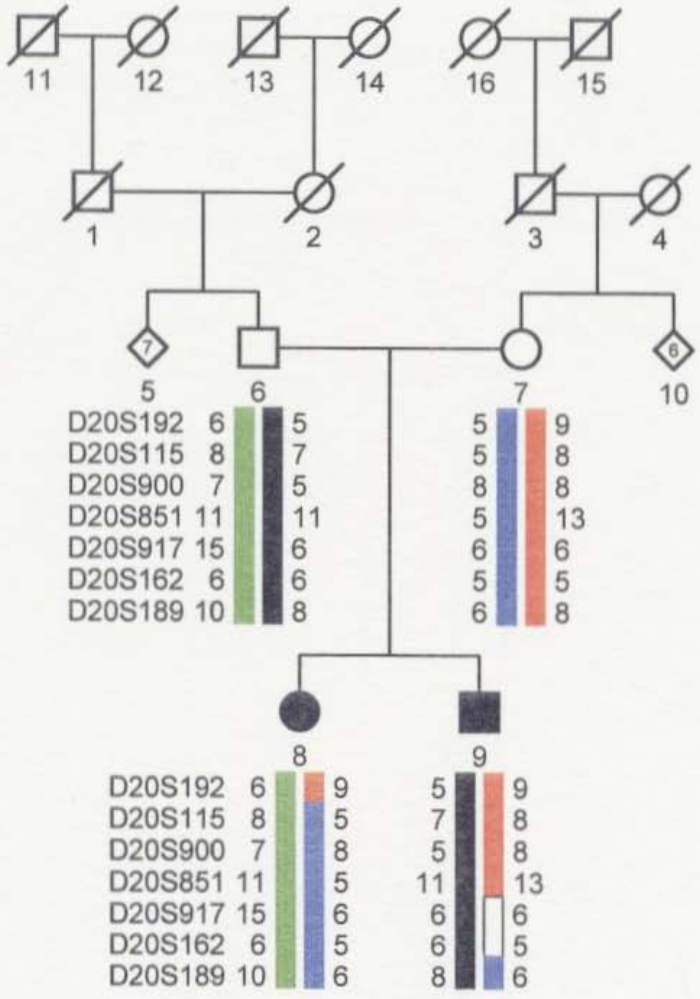
Chromosome 3
Number : B10



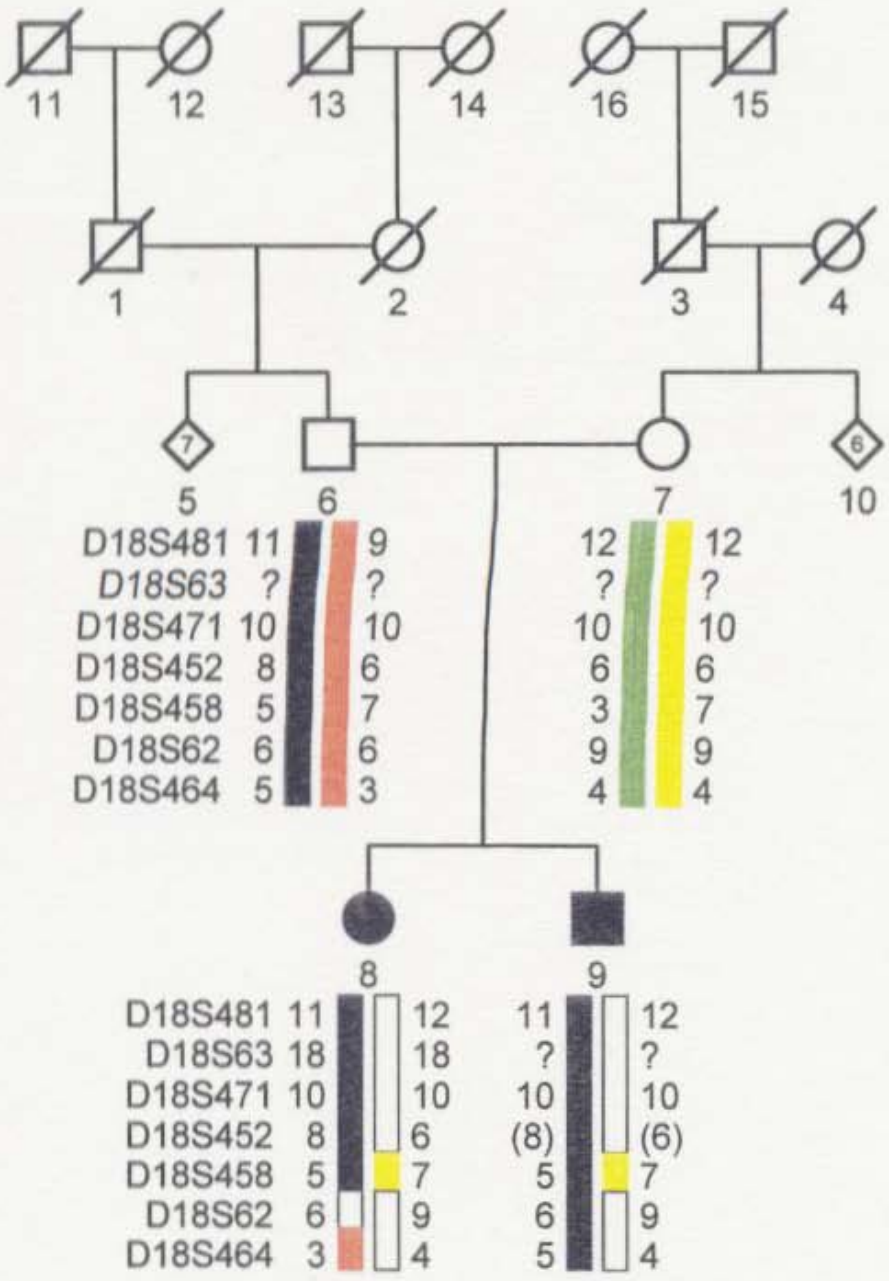
Chromosome 2
Number : B10



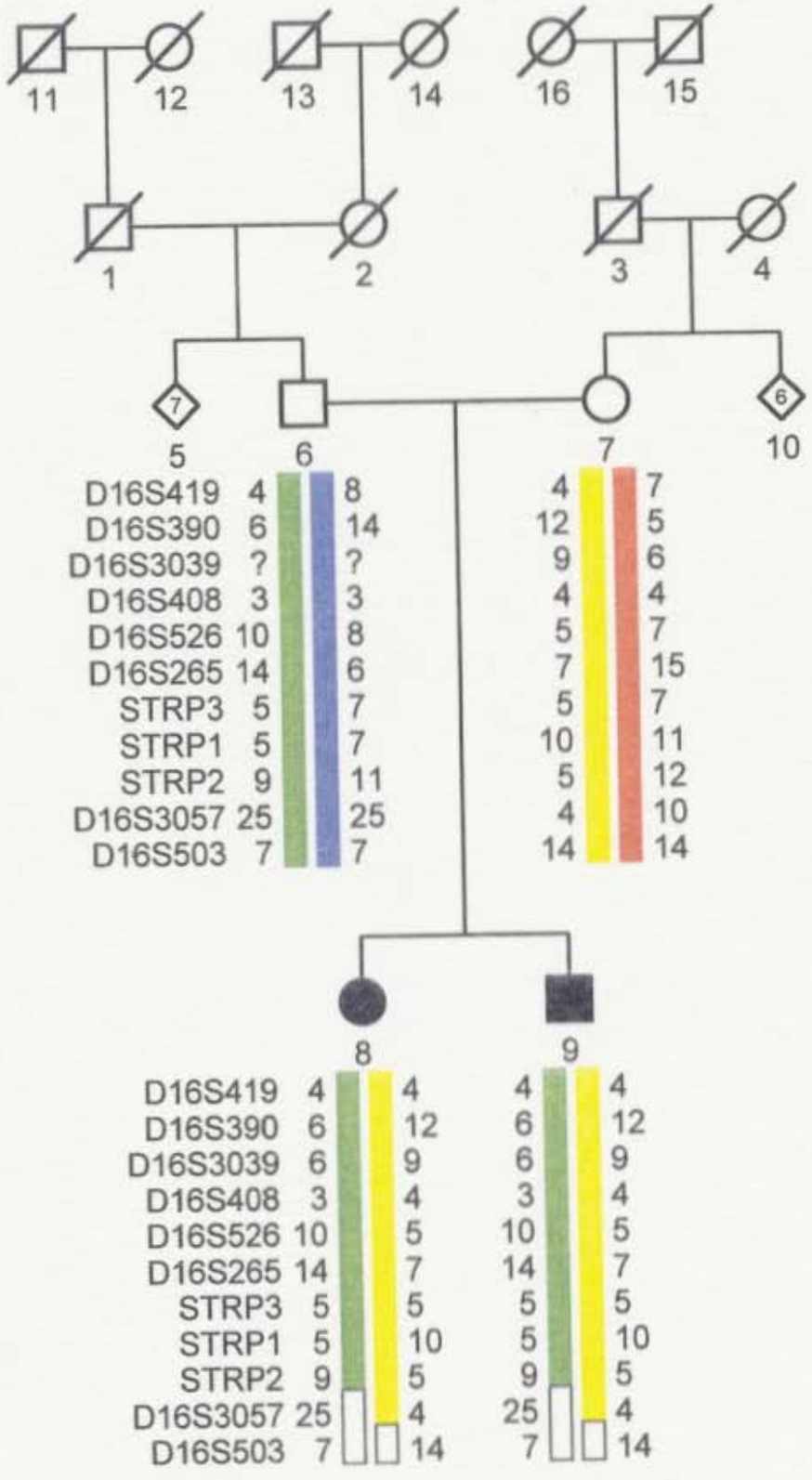
Chromosome 20
Number : B11



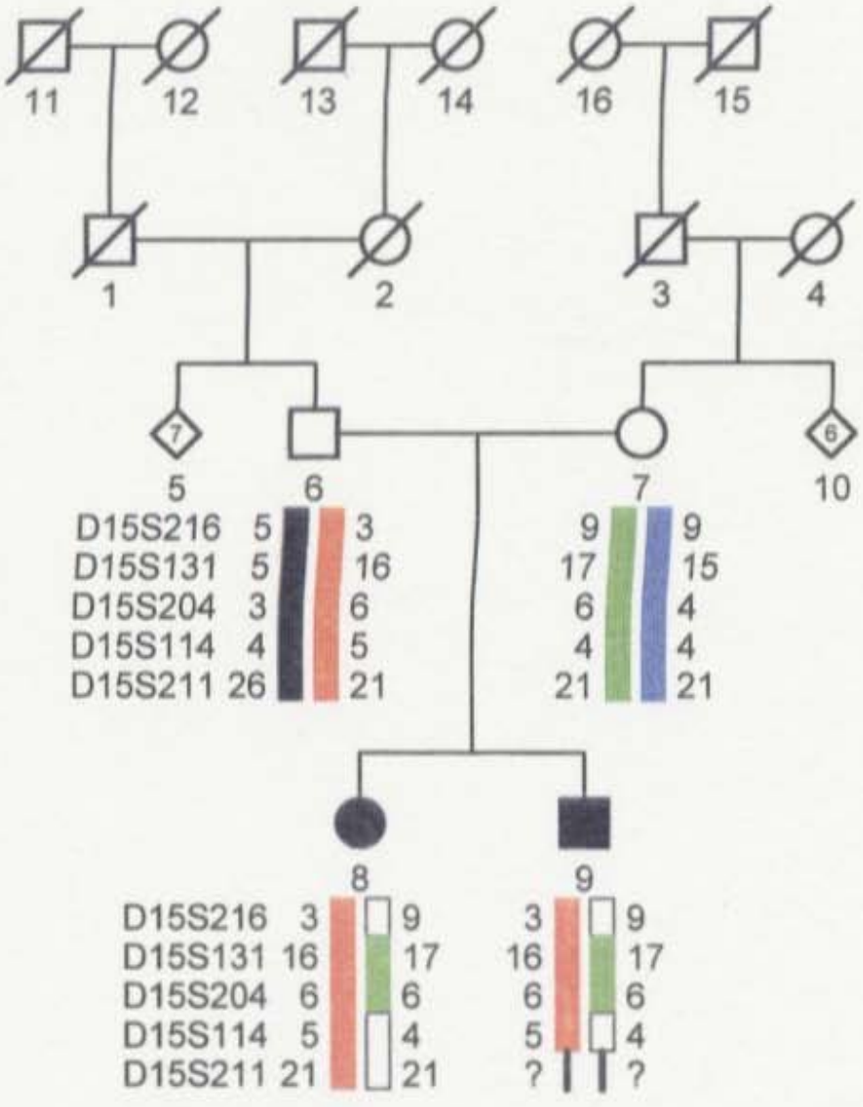
Chromosome 18
Number : B11



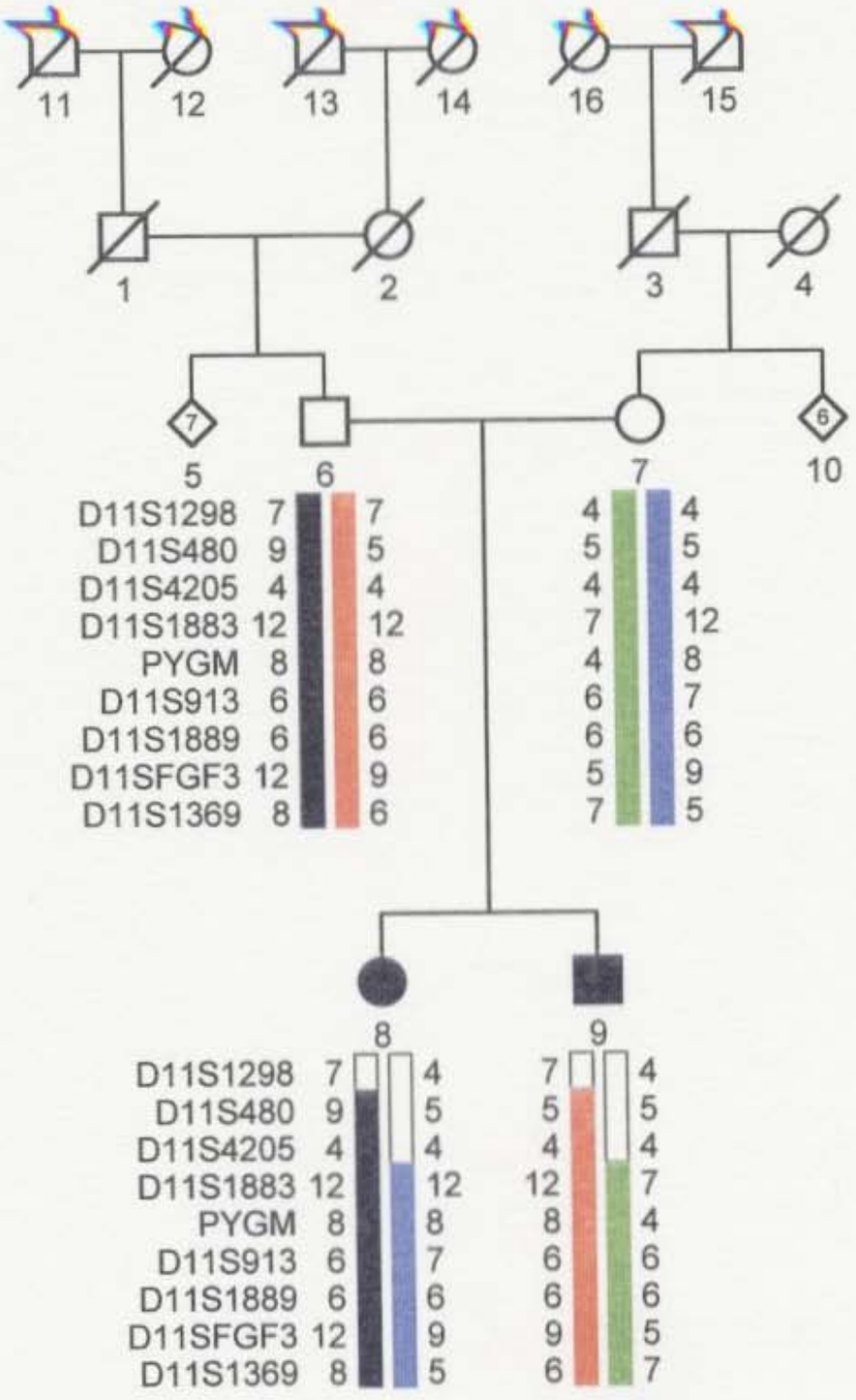
Chromosome 16
Number : B11



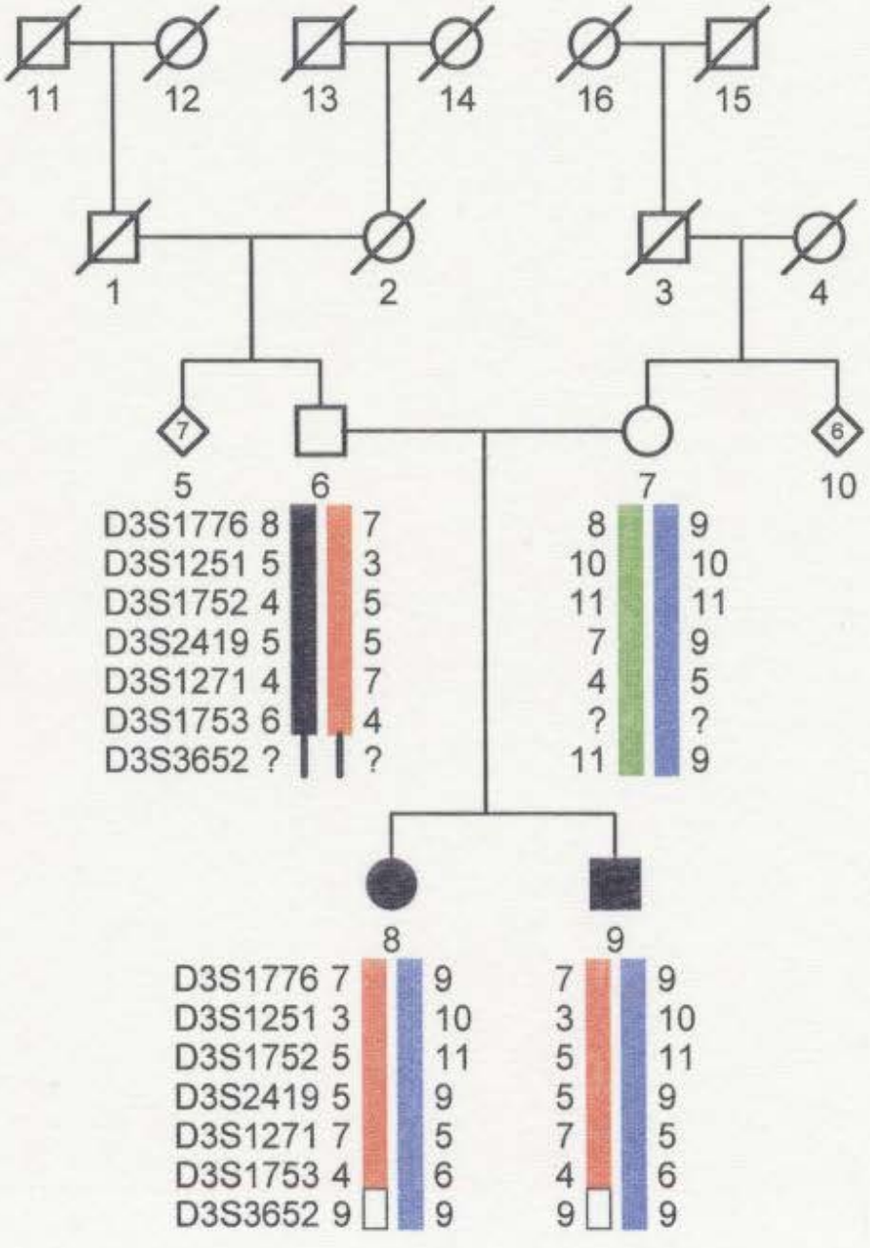
Chromosome 15
Number : B11



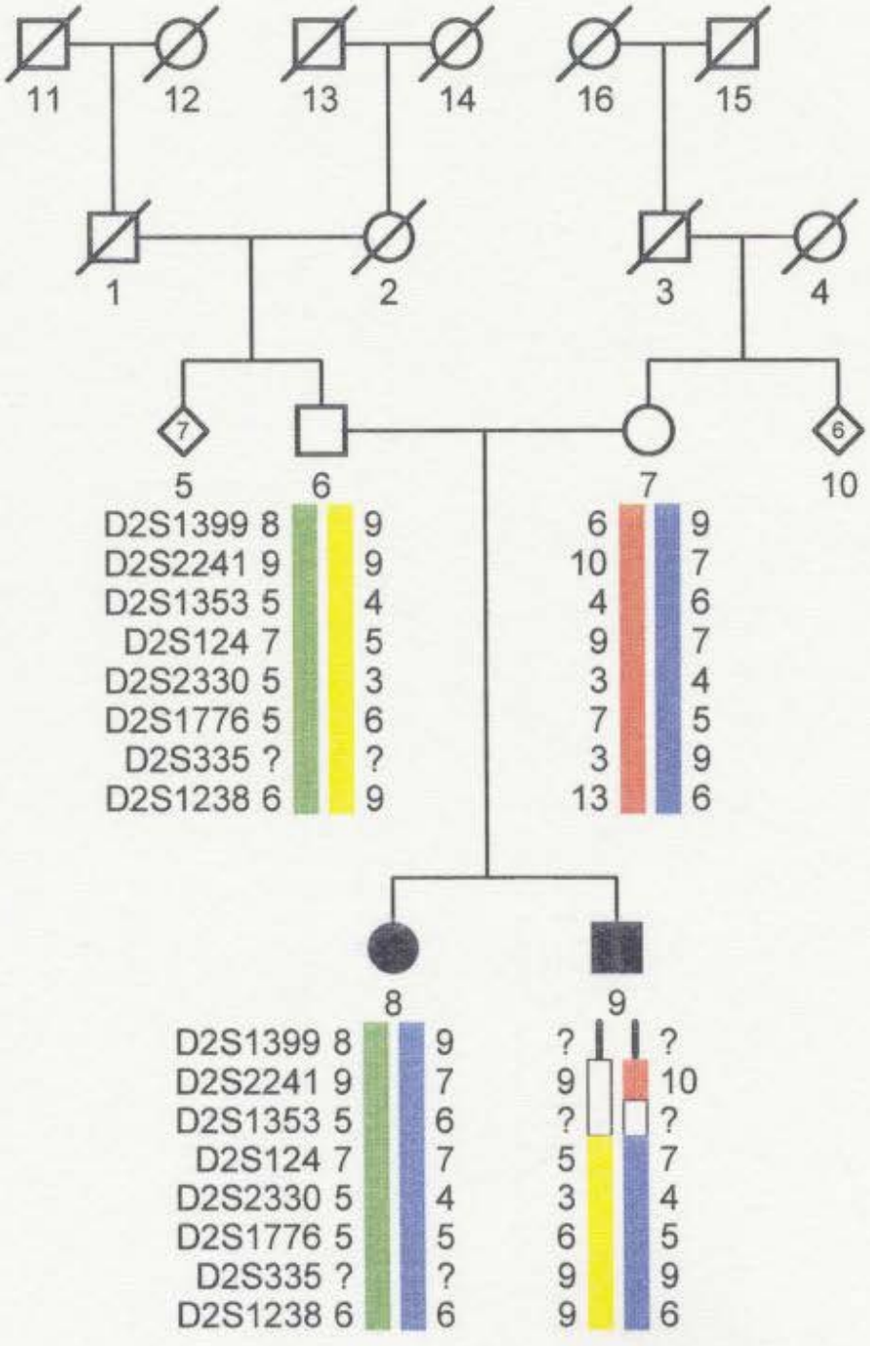
Chromosome 11
Number : B11



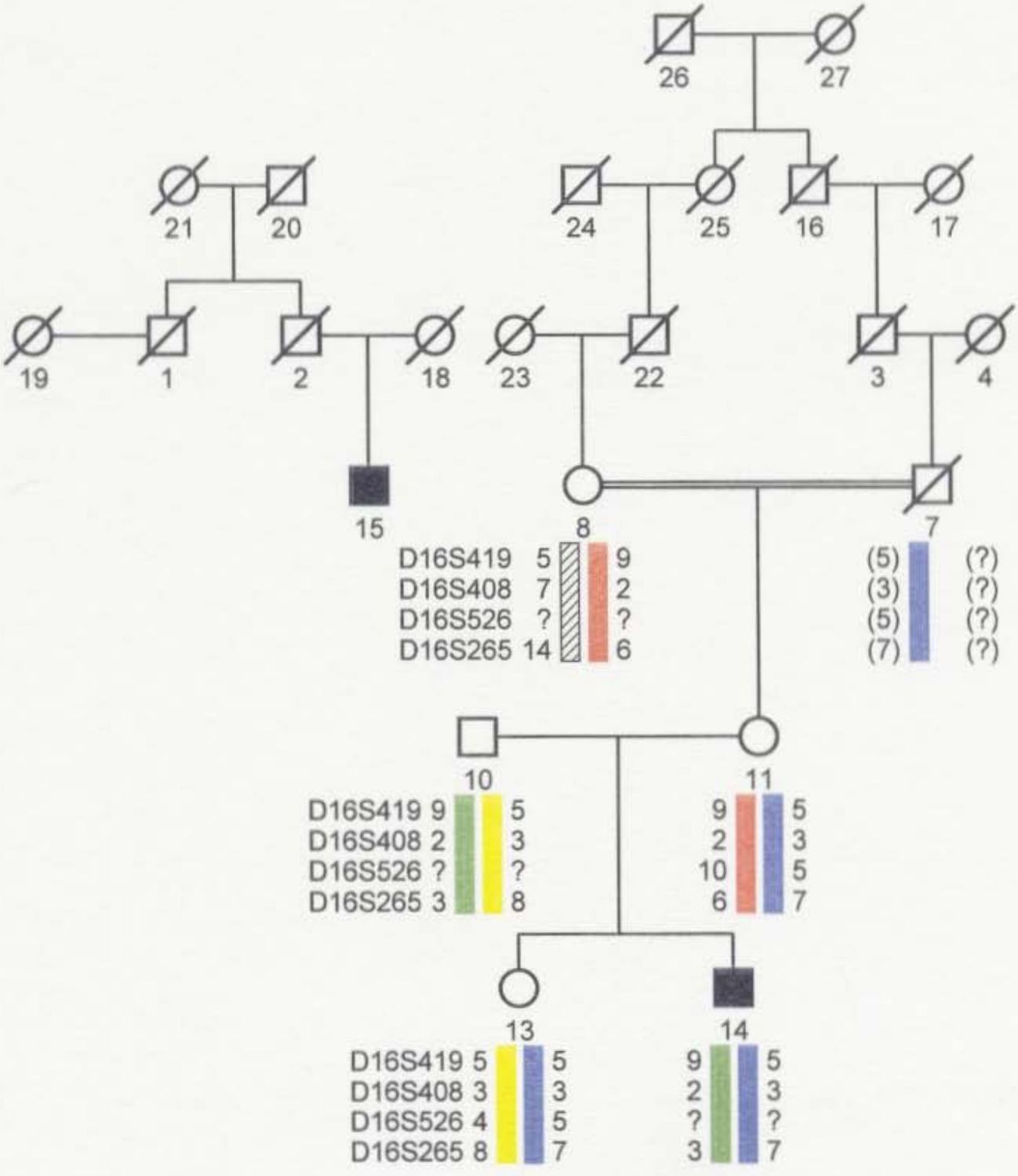
Chromosome 3
Number : B11



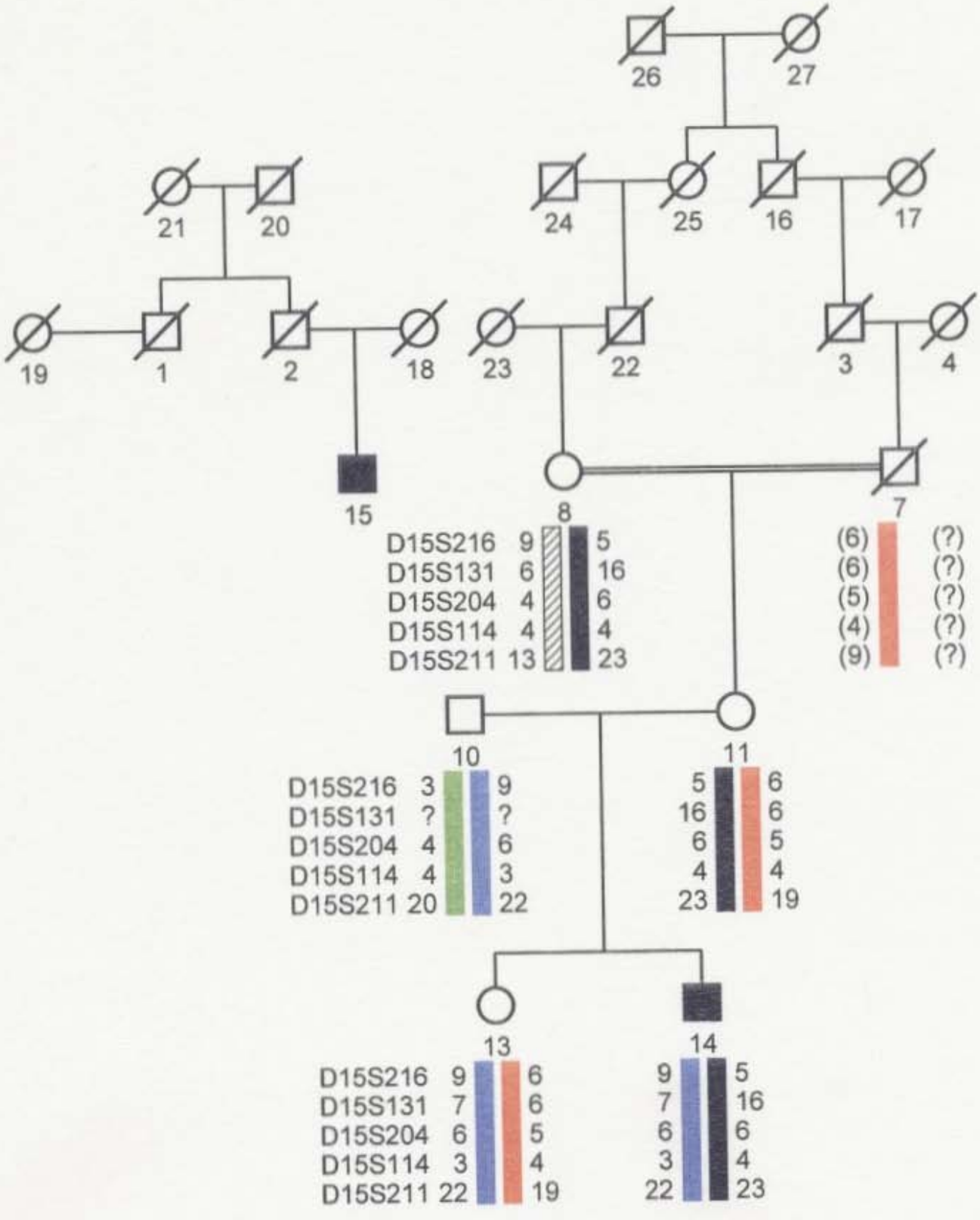
Chromosome 2
Number : B11



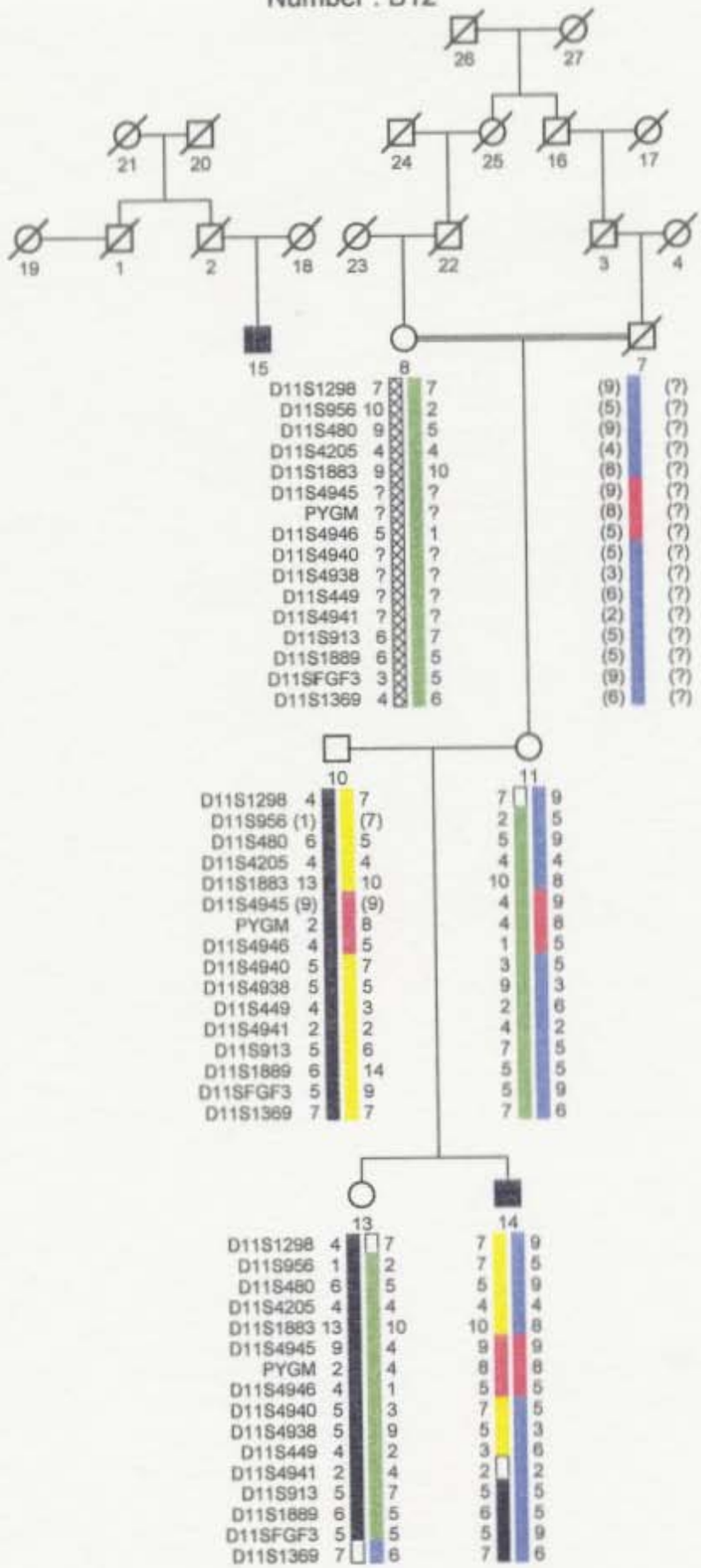
Chromosome 16
Number : B12



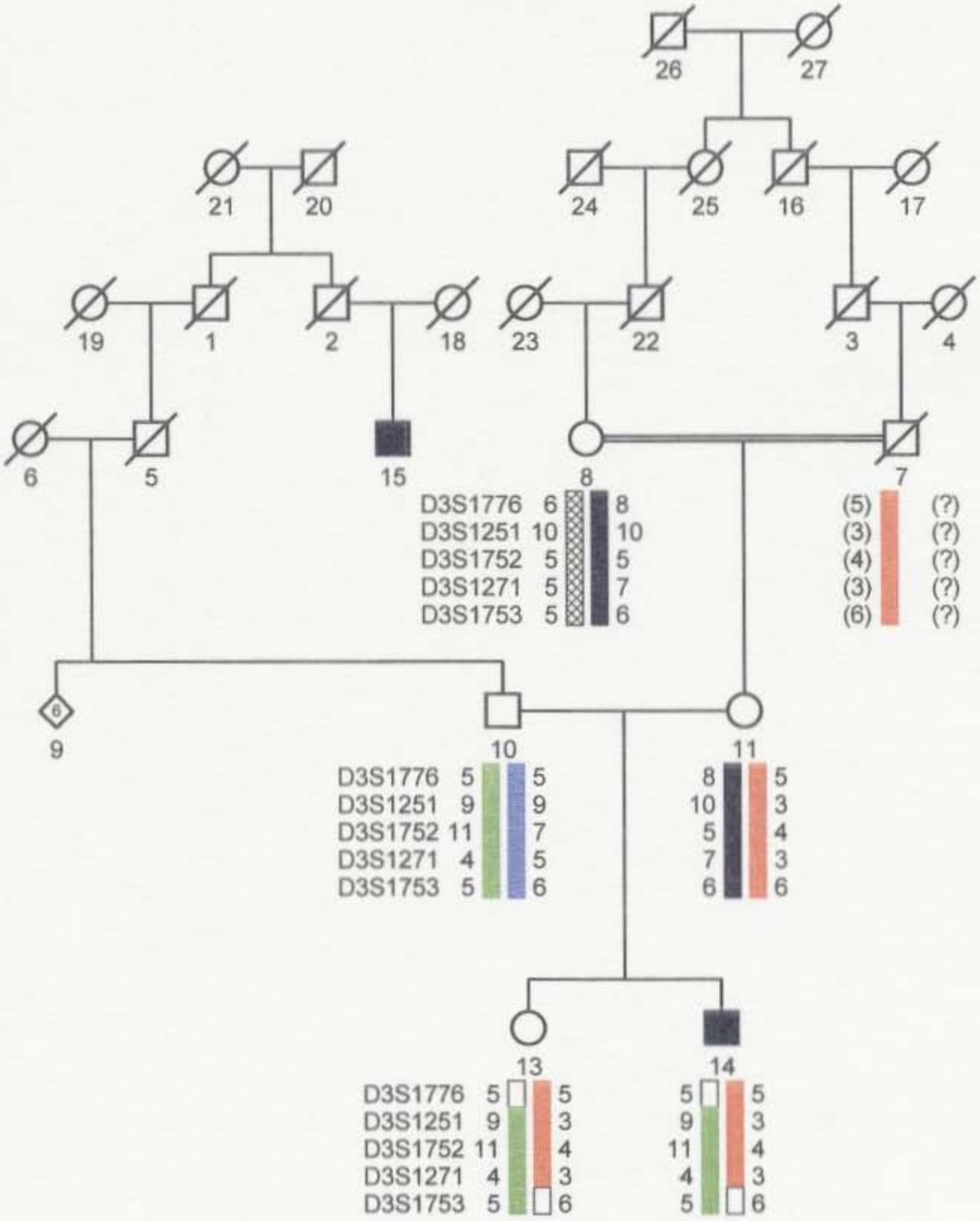
Chromosome 15
Number : B12

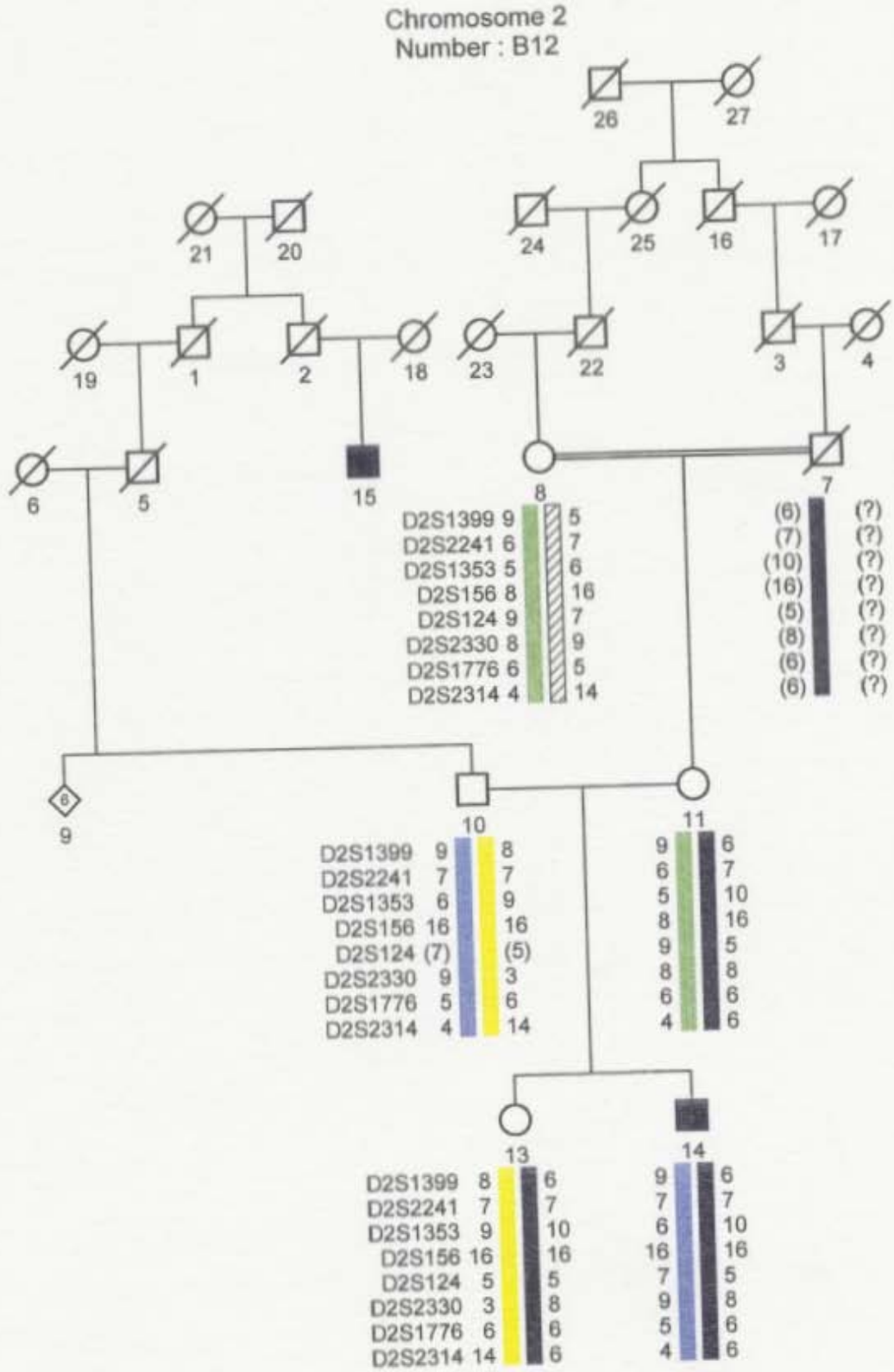


Chromosome 11
Number : B12



Chromosome 3
Number : B12




Chromosome 2
Number : B12
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6
5
15
8
D2S1399 9 5
D2S2241 6 7
D2S1353 5 6
D2S156 8 16
D2S124 9 7
D2S2330 8 9
D2S1776 6 5
D2S2314 4 14
7
(6) (?)
(7) (?)
(10) (?)
(16) (?)
(5) (?)
(8) (?)
(6) (?)
(6) (?)
9
10
D2S1399 9 8
D2S2241 7 7
D2S1353 6 9
D2S156 16 16
D2S124 (7) (5)
D2S2330 9 3
D2S1776 5 6
D2S2314 4 14
11
9 6
6 7
5 10
8 16
9 5
8 8
6 6
4 6
13
D2S1399 8 6
D2S2241 7 7
D2S1353 9 10
D2S156 16 16
D2S124 5 5
D2S2330 3 8
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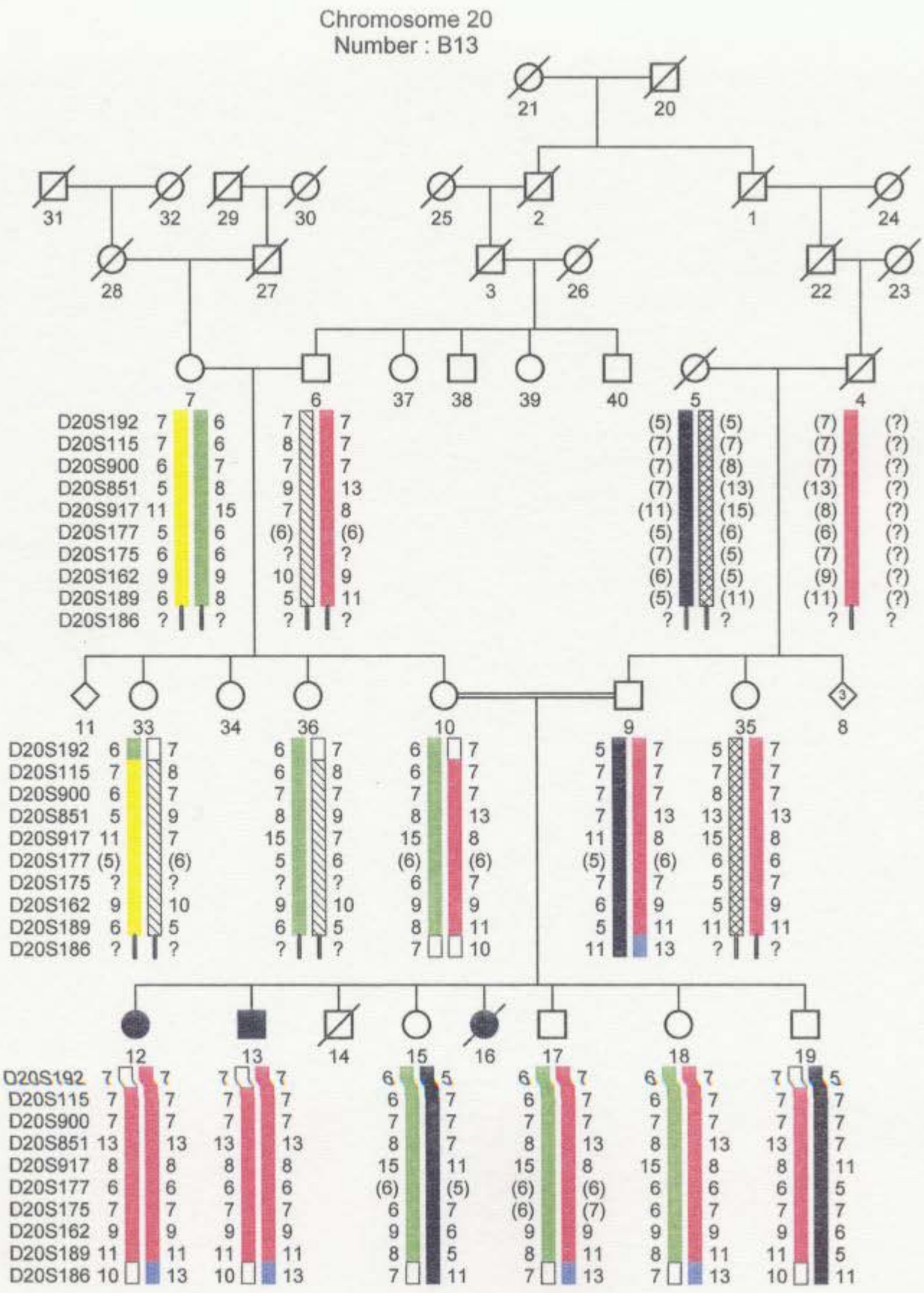
7 7
6 10
16 16
7 5
9 8
5 6
4 6


Chromosome 20
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23
37
38
39
40
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6
5
4
D20S192 7 6
D20S115 7 6
D20S900 6 7
D20S851 5 8
D20S917 11 15
D20S177 5 6
D20S175 6 6
D20S162 9 9
D20S189 6 8
D20S186 ? ?
7 7
8 7
7 7
9 13
7 8
(6) (6)
? ?
10 9
5 11
? ?
(5) (5)
(7) (7)
(7) (8)
(7) (13)
(11) (15)
(5) 6
(7) (5)
(6) (5)
(5) (11)
? ?
(7) (?)
(7) (?)
(7) (?)
(13) (?)
(8) (?)
(6) (?)
(7) (?)
(9) (?)
(11) (?)
? ?
11
33
34
36
10
9
35
8
D20S192 6 7
D20S115 7 8
D20S900 6 7
D20S851 5 9
D20S917 11 7
D20S177 (5) (6)
D20S175 ? ?
D20S162 9 10
D20S189 6 5
D20S186 ? ?
6 7
6 8
7 7
8 9
15 7
5 6
? ?
9 10
6 5
? ?
6 7
6 7
7 7
8 13
15 8
(6) (6)
6 7
9 9
8 11
7 10
5 7
7 7
7 7
7 13
11 8
(5) (6)
7 7
6 9
5 11
11 13
5 7
7 7
8 7
13 13
15 8
6 6
5 7
5 9
11 11
? ?
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D20S192 7 7
D20S115 7 7
D20S900 7 7
D20S851 13 13
D20S917 8 8
D20S177 6 6
D20S175 7 7
D20S162 9 9
D20S189 11 11
D20S186 10 13
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11 11
10 13
6 5
6 7
7 7
8 7
15 11
(6) (5)
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8 5
7 11
6 7
6 7
7 7
8 13
15 8
(6) (6)
(6) (7)
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7 13
6 7
6 7
7 7
8 13
15 8
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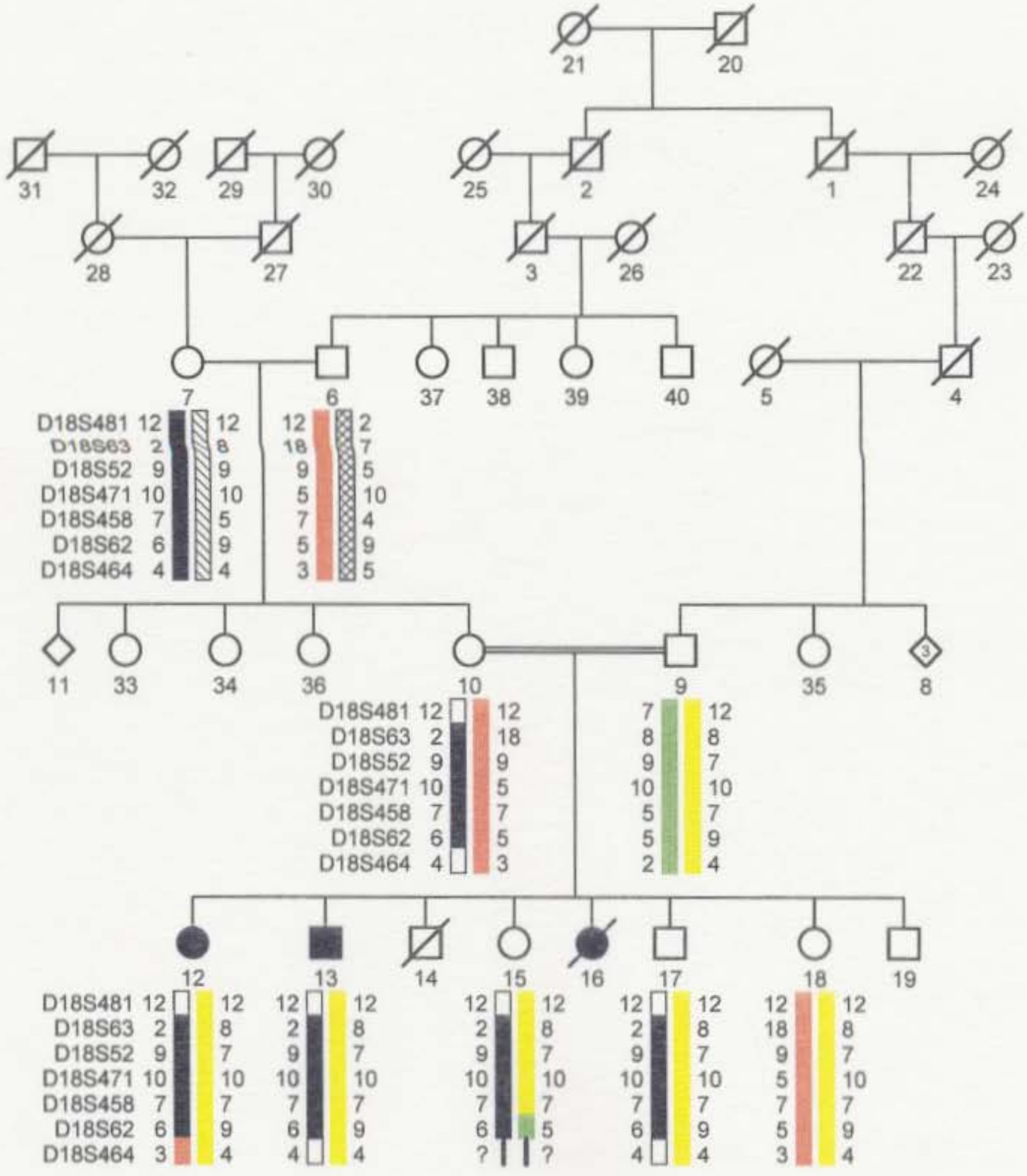
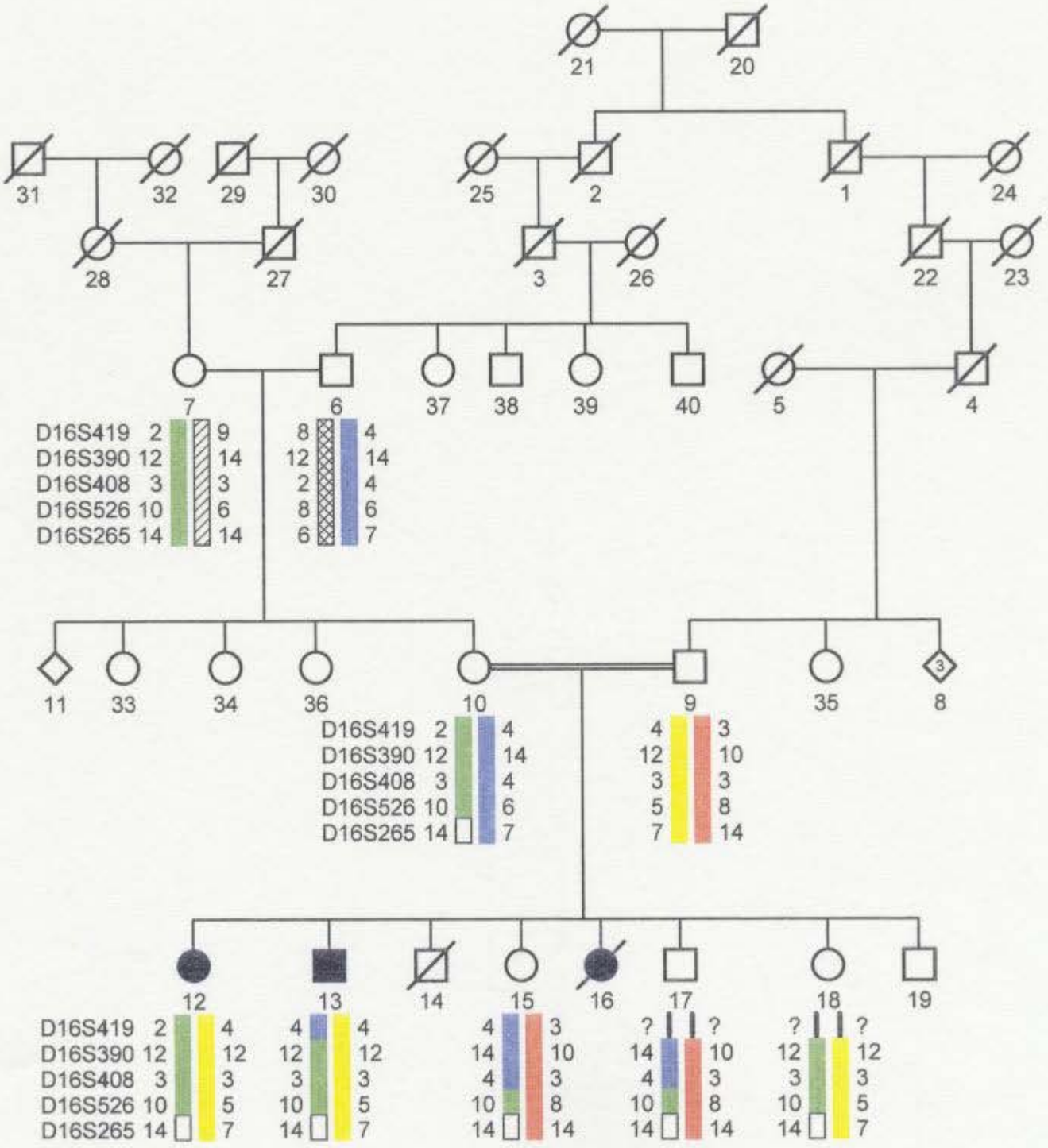
11 5
10 11

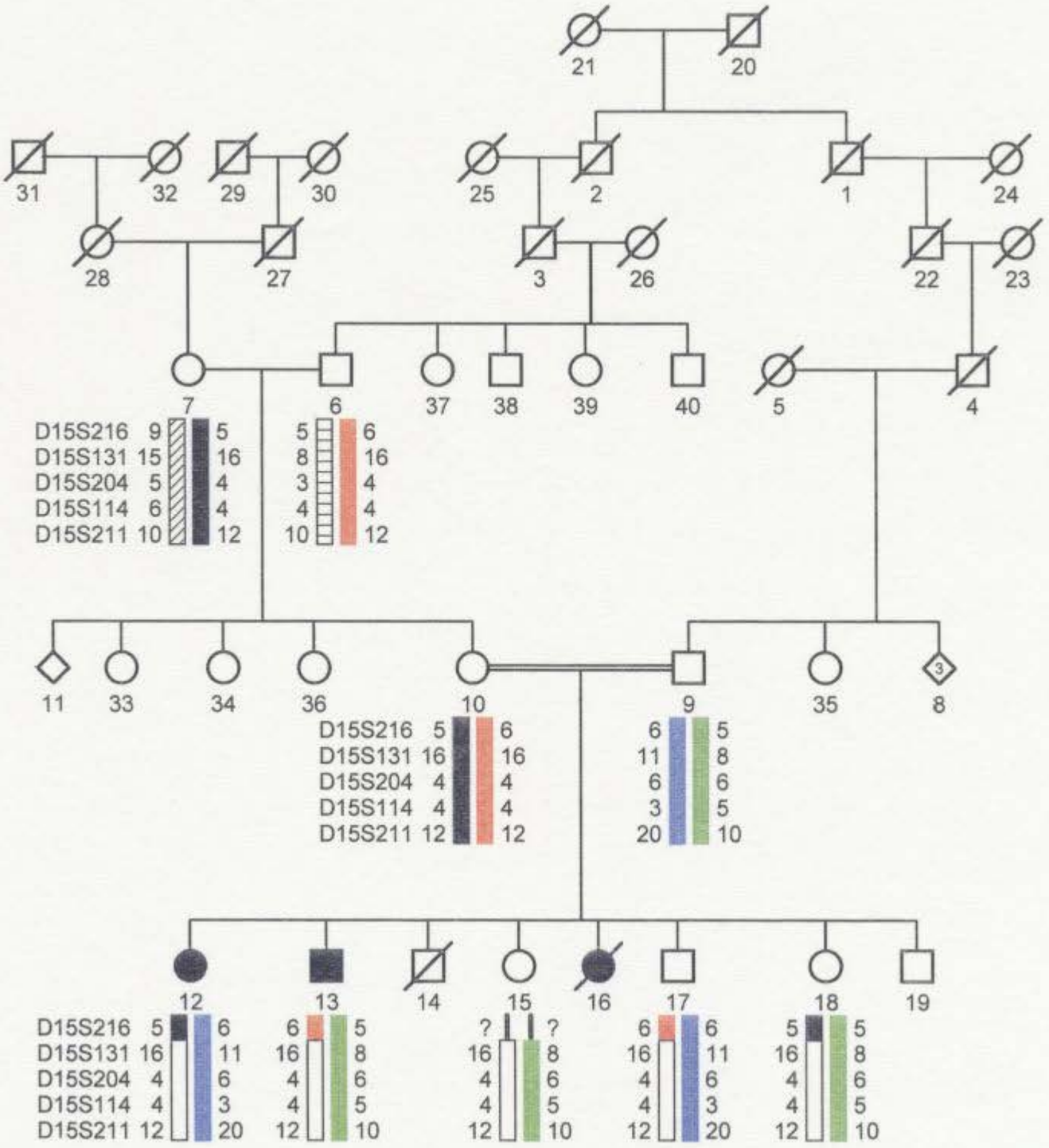
Chromosome 18
Number : B13



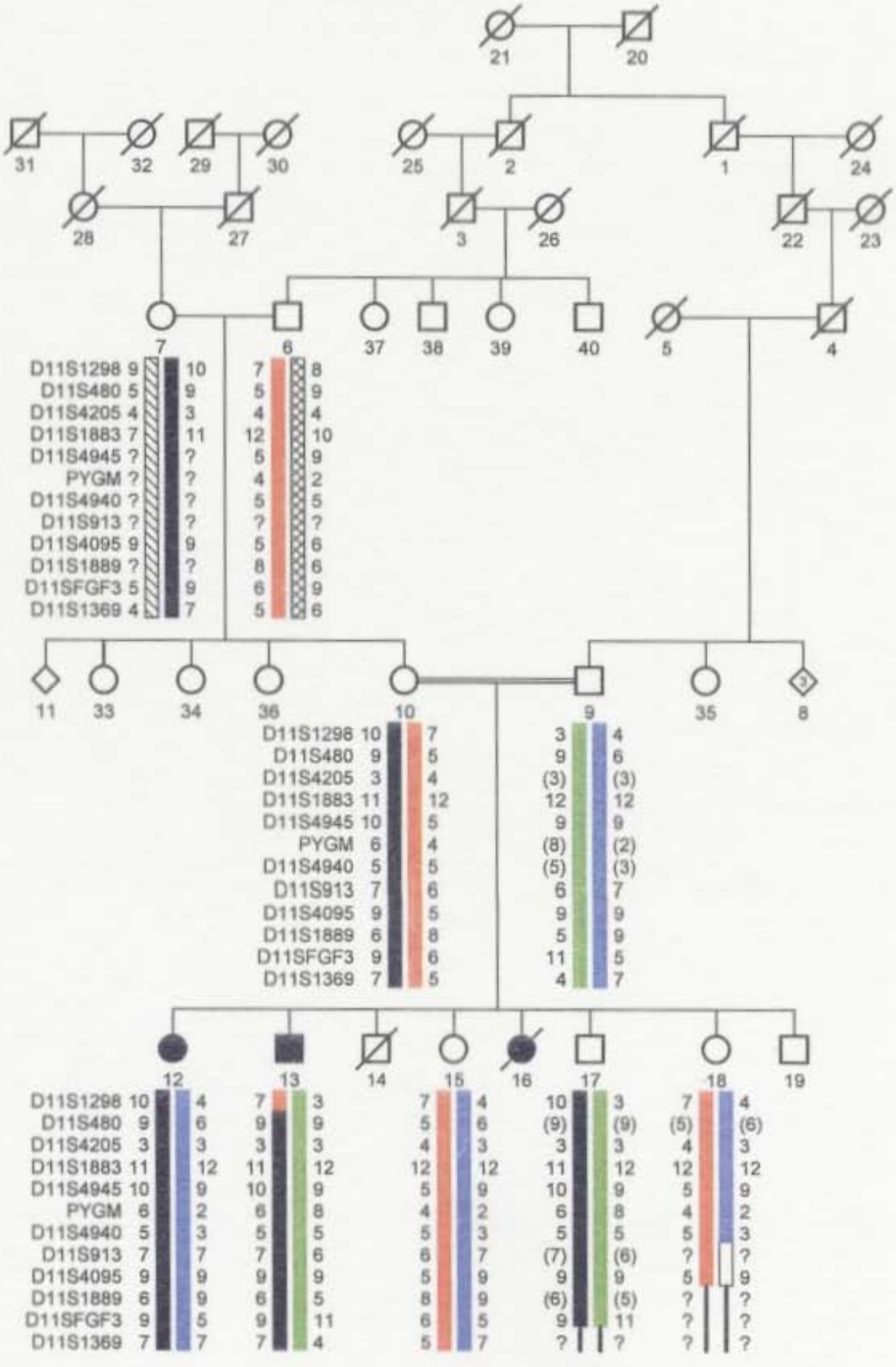
Chromosome 16
Number : B13



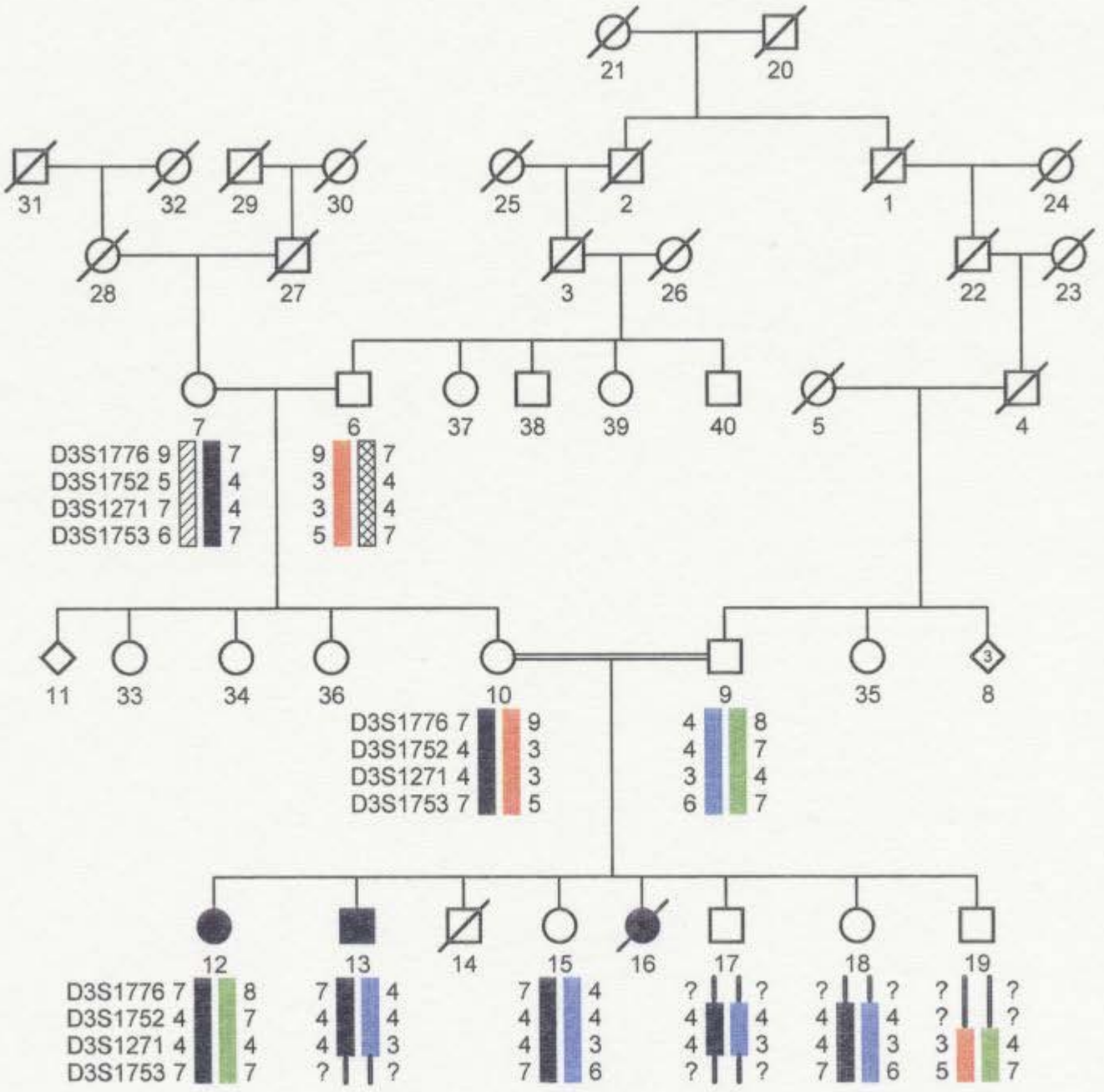
Chromosome 15
Number : B13



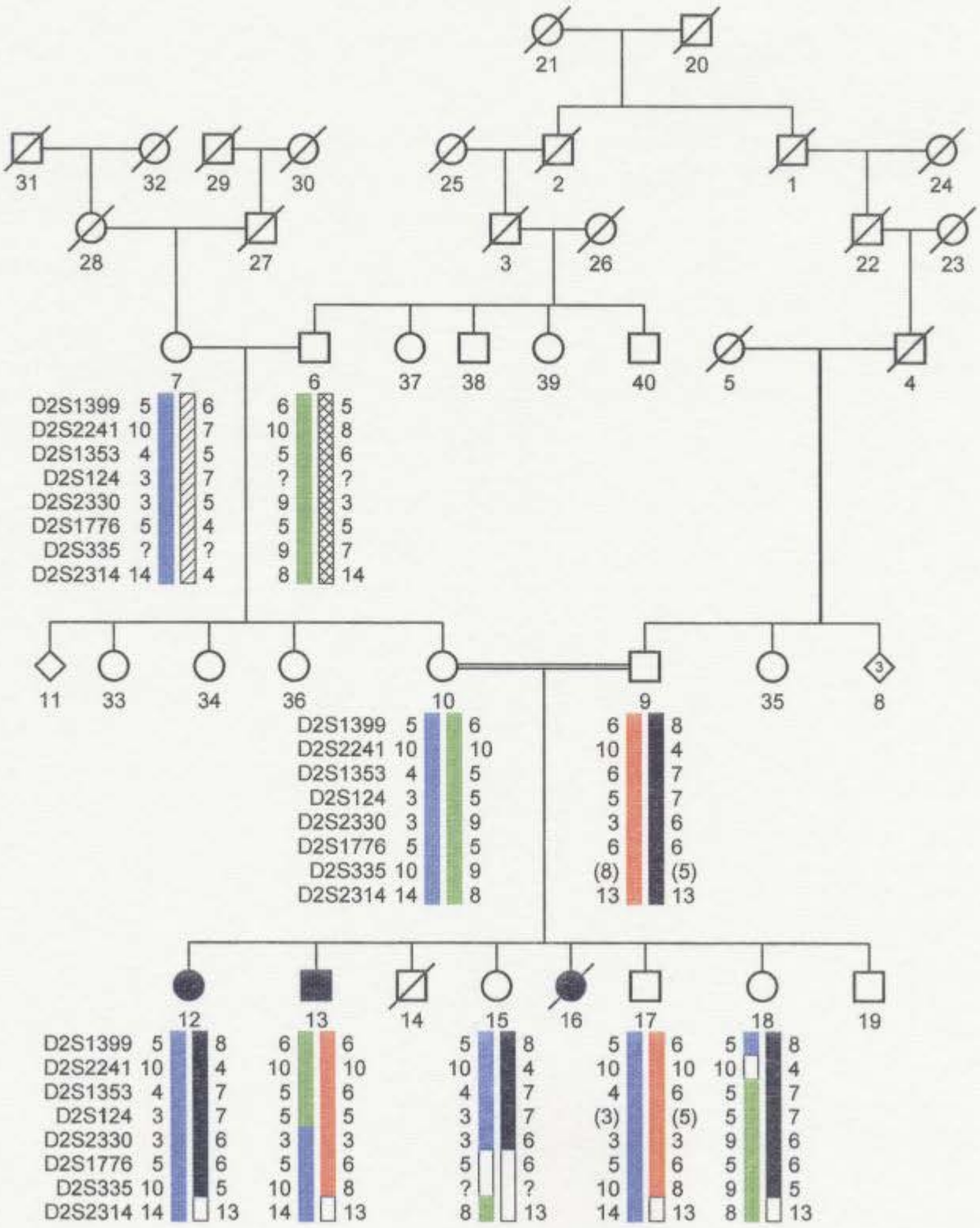
Chromosome 11
Number : B13



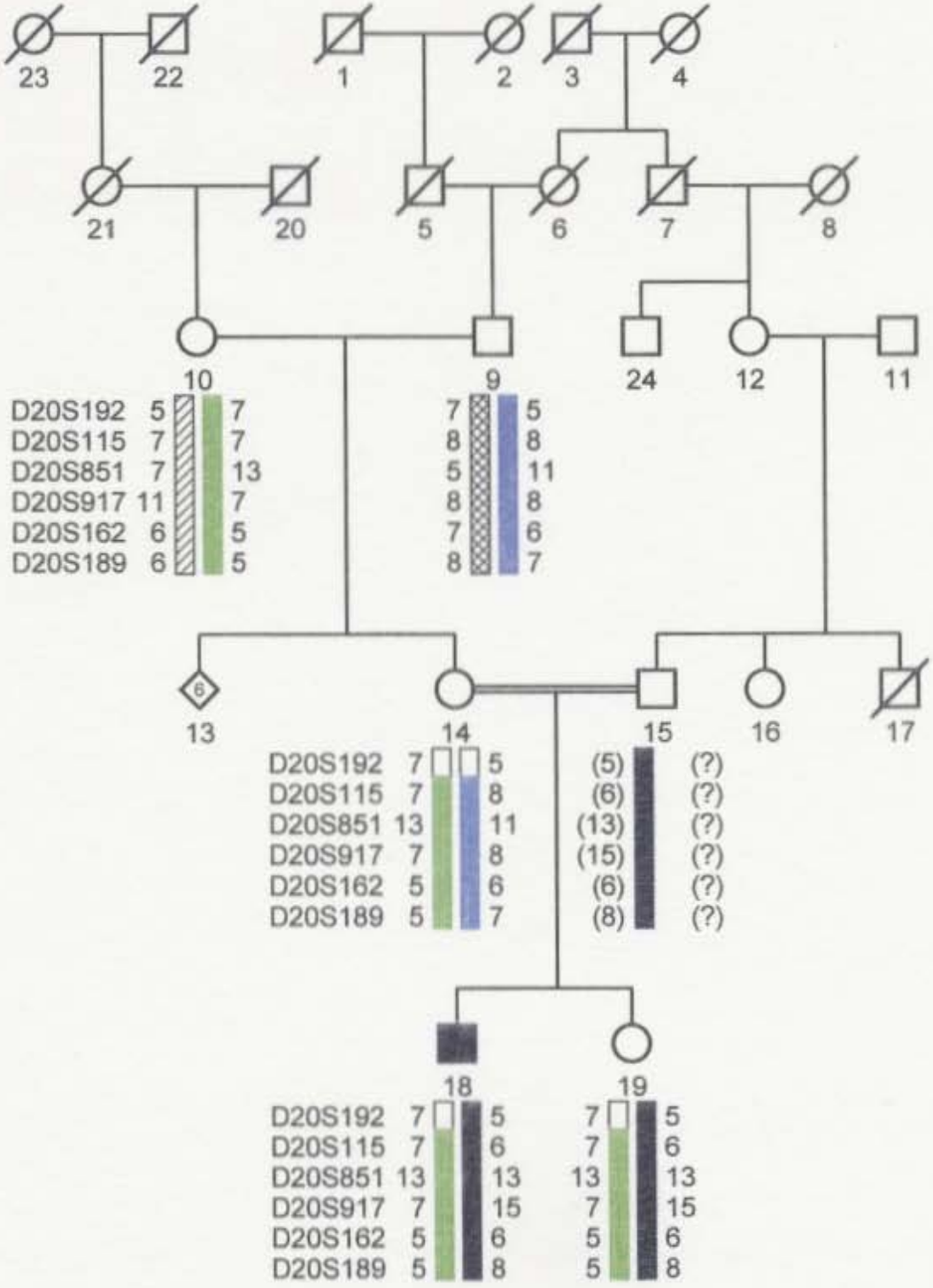
Chromosome 3
Number : B13



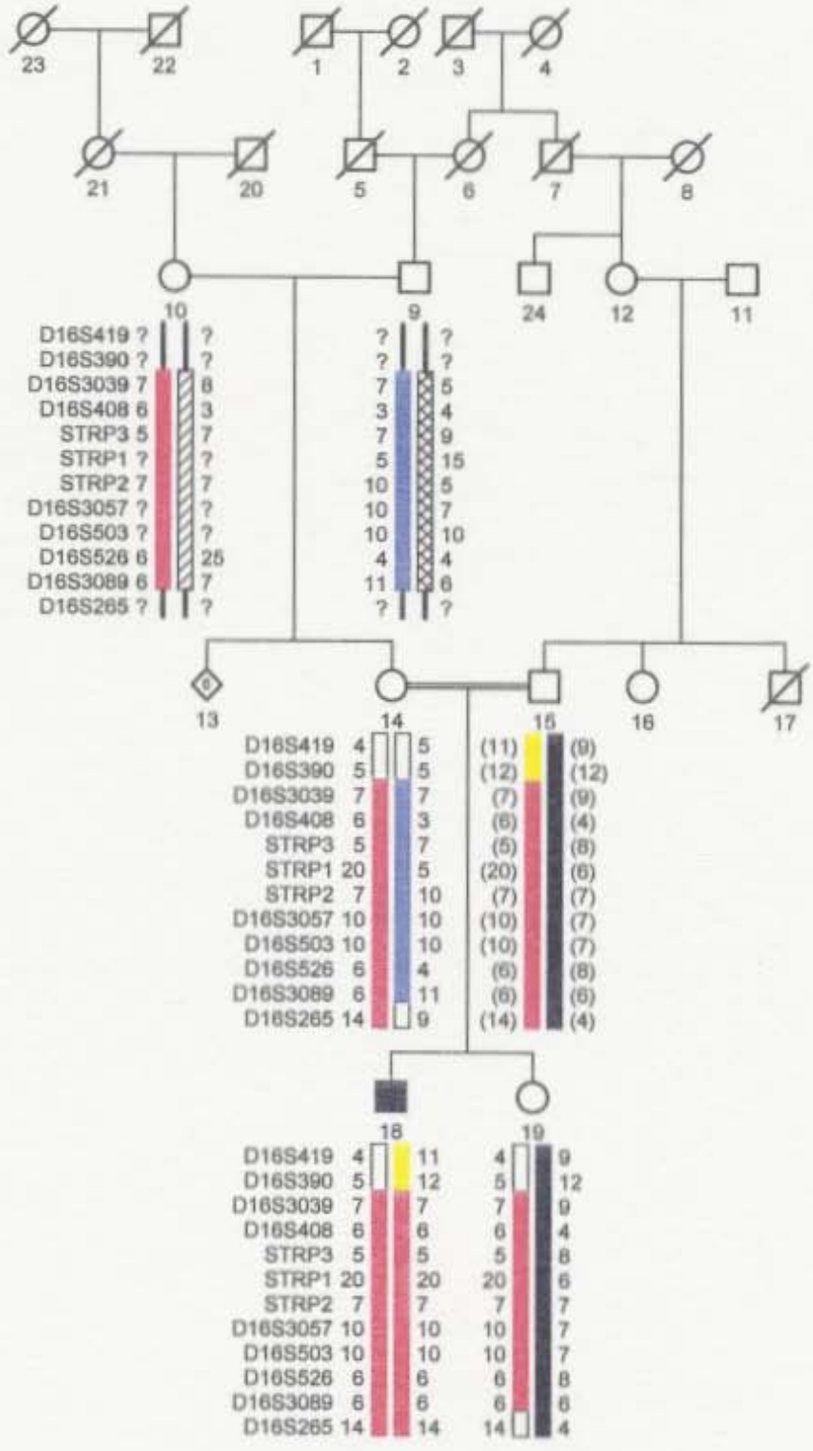
Chromosome 2
Number : B13

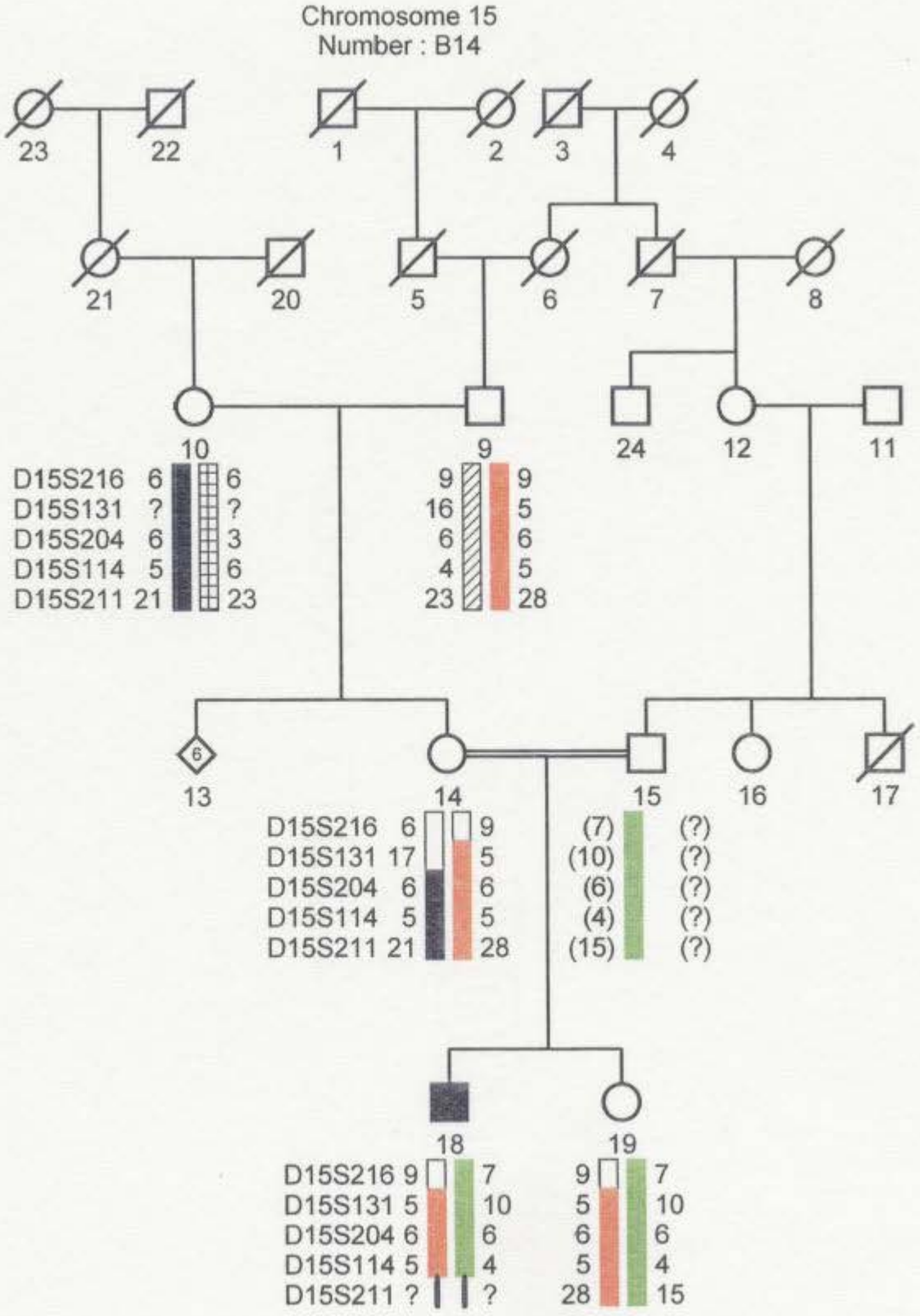


Chromosome 20
Number : B14



Chromosome 16
Number : B14




Chromosome 15
Number : B14
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22
1
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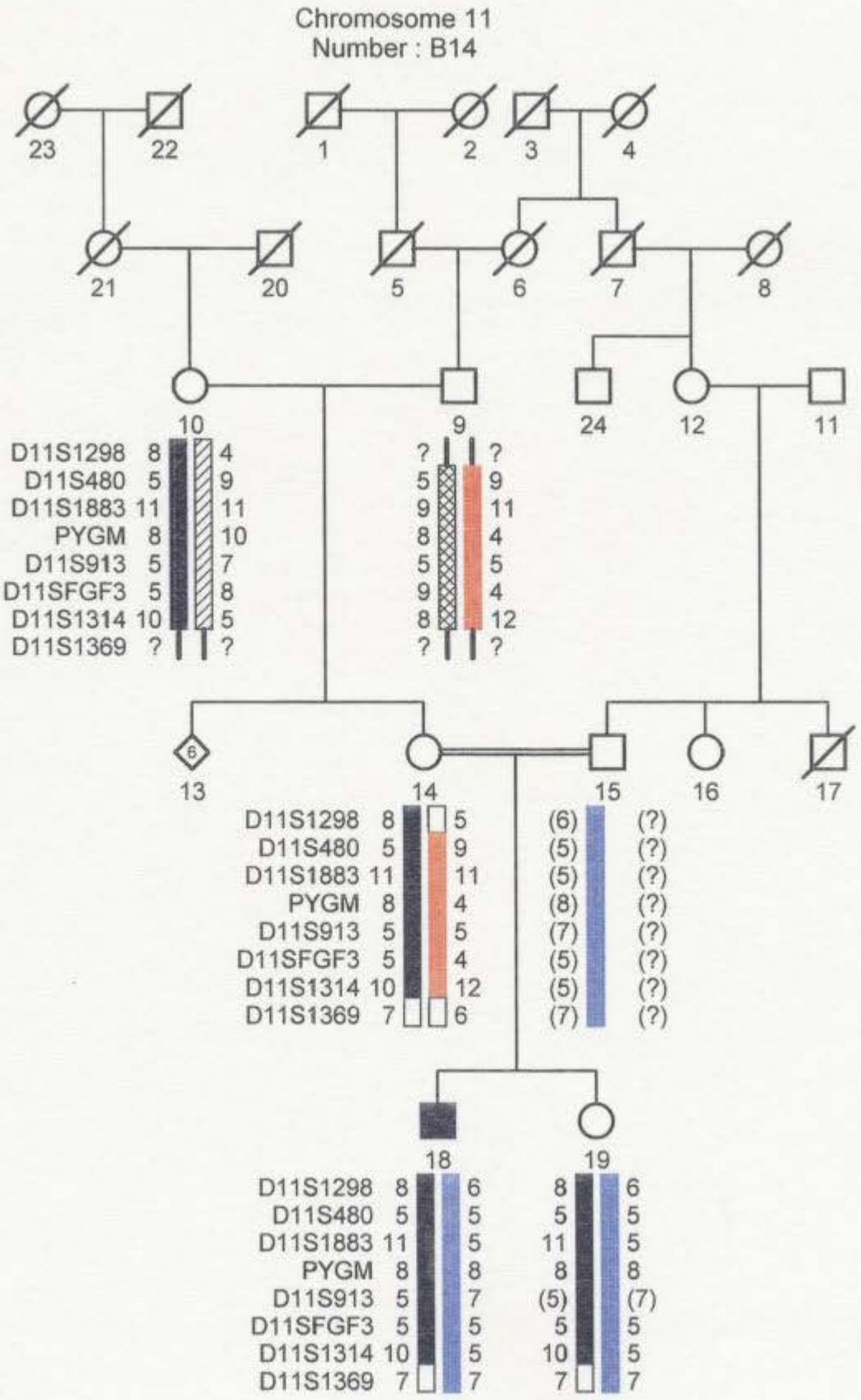
10
9
24
12
11
D15S216 6 6
D15S131 ? ?
D15S204 6 3
D15S114 5 6
D15S211 21 23
9 9
16 5
6 6
4 5
23 28
6
13
14
15
16
17
D15S216 6 9
D15S131 17 5
D15S204 6 6
D15S114 5 5
D15S211 21 28
(7) (?)
(10) (?)
(6) (?)
(4) (?)
(15) (?)
18
19
D15S216 9 7
D15S131 5 10
D15S204 6 6
D15S114 5 4
D15S211 ? ?
9 7
5 10
6 6
5 4
28 15


Chromosome 11
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D11S480 5 9
D11S1883 11 11
PYGM 8 10
D11S913 5 7
D11SFGF3 5 8
D11S1314 10 5
D11S1369 ? ?
? ?
5 9
9 11
8 4
5 5
9 4
8 12
? ?
13
14
15
16
17
D11S1298 8 5
D11S480 5 9
D11S1883 11 11
PYGM 8 4
D11S913 5 5
D11SFGF3 5 4
D11S1314 10 12
D11S1369 7 6
(6) (?)
(5) (?)
(5) (?)
(8) (?)
(7) (?)
(5) (?)
(5) (?)
(7) (?)
18
19
D11S1298 8 6
D11S480 5 5
D11S1883 11 5
PYGM 8 8
D11S913 5 7
D11SFGF3 5 5
D11S1314 10 5
D11S1369 7 7
8 6
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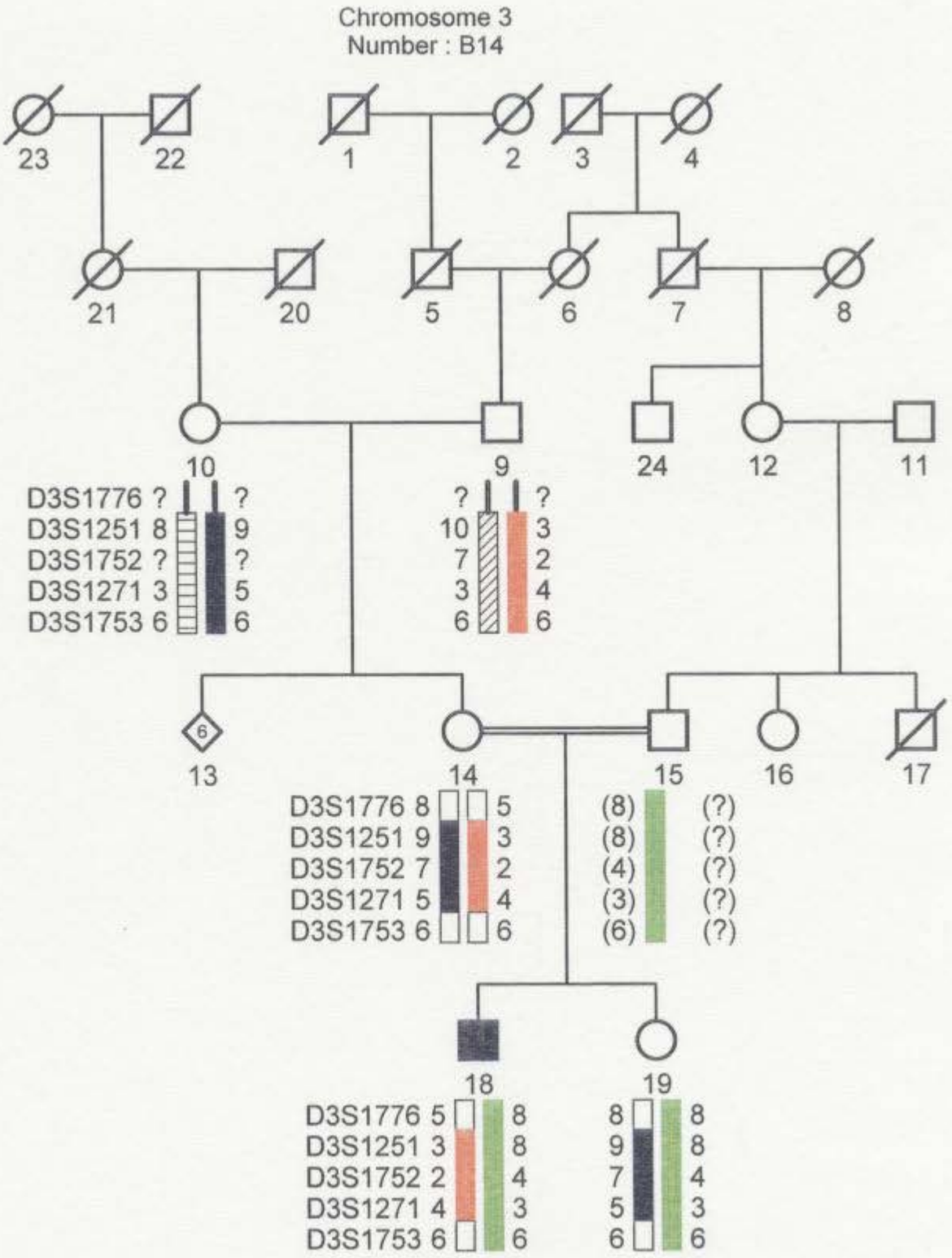
11 5
8 8
(5) (7)
5 5
10 5
7 7


Chromosome 3
Number : B14
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12
11
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D3S1251 8 9
D3S1752 ? ?
D3S1271 3 5
D3S1753 6 6
? ?
10 3
7 2
3 4
6 6
6
13
14
15
16
17
D3S1776 8 5
D3S1251 9 3
D3S1752 7 2
D3S1271 5 4
D3S1753 6 6
(8) (?)
(8) (?)
(4) (?)
(3) (?)
(6) (?)
18
19
D3S1776 5 8
D3S1251 3 8
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D3S1271 4 3
D3S1753 6 6
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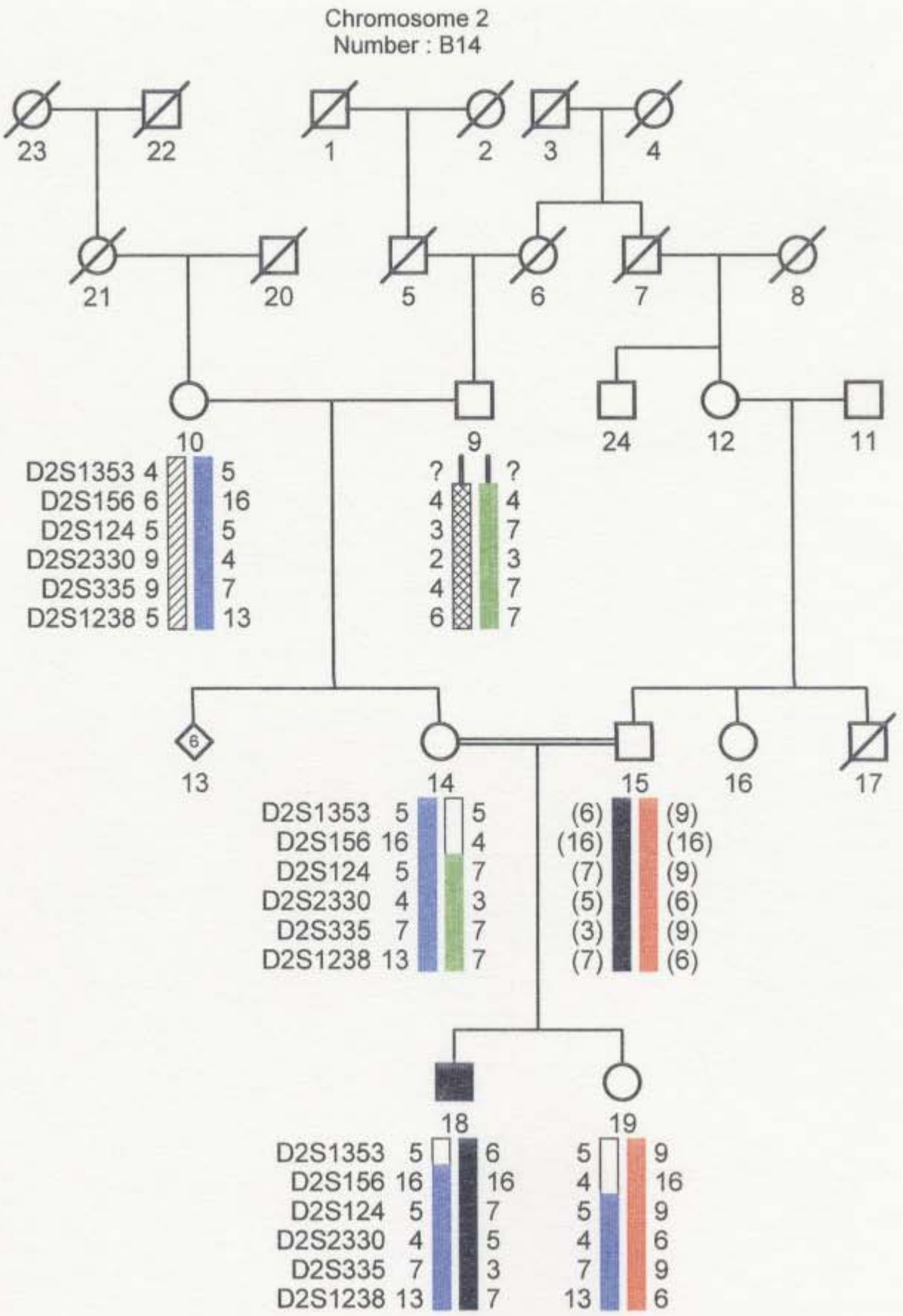
9 8
7 4
5 3
6 6


Chromosome 2
Number : B14
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22
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24
12
11
D2S1353 4 5
D2S156 6 16
D2S124 5 5
D2S2330 9 4
D2S335 9 7
D2S1238 5 13
? ?
4 4
3 7
2 3
4 7
6 7
6
13
14
15
16
17
D2S1353 5 5
D2S156 16 4
D2S124 5 7
D2S2330 4 3
D2S335 7 7
D2S1238 13 7
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(16) (16)
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D2S1353 5 6
D2S156 16 16
D2S124 5 7
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D2S335 7 3
D2S1238 13 7
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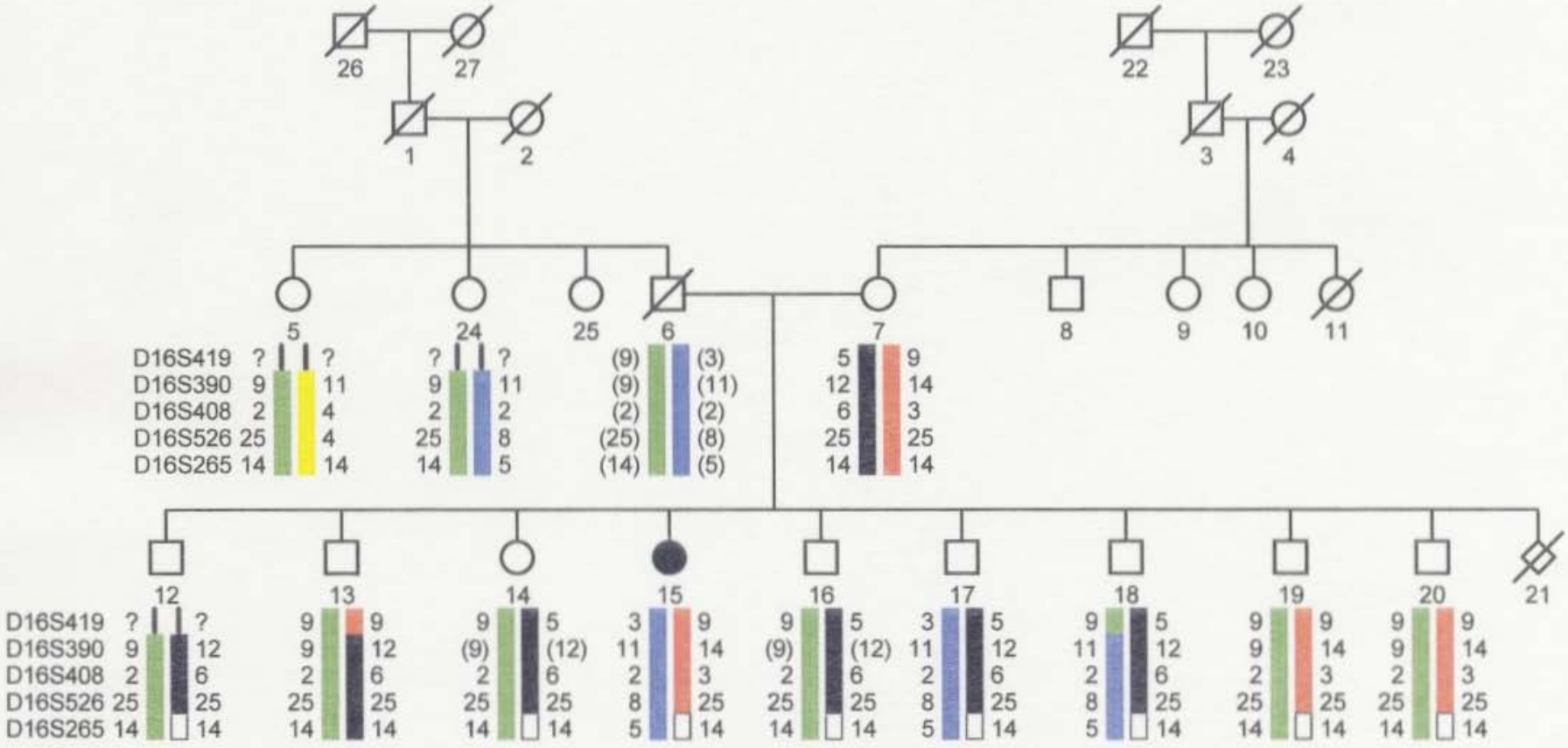
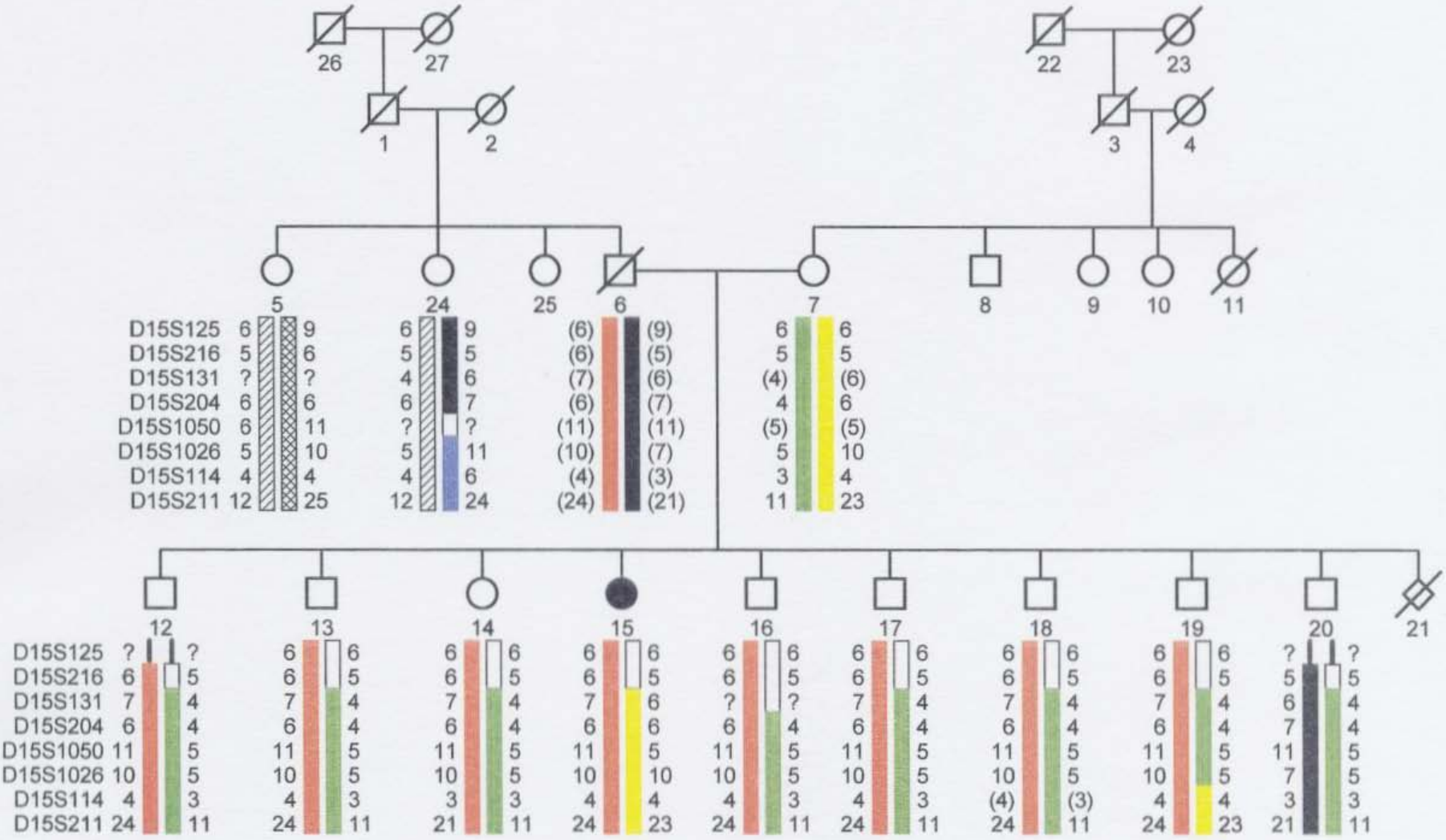
4 6
7 9
13 6

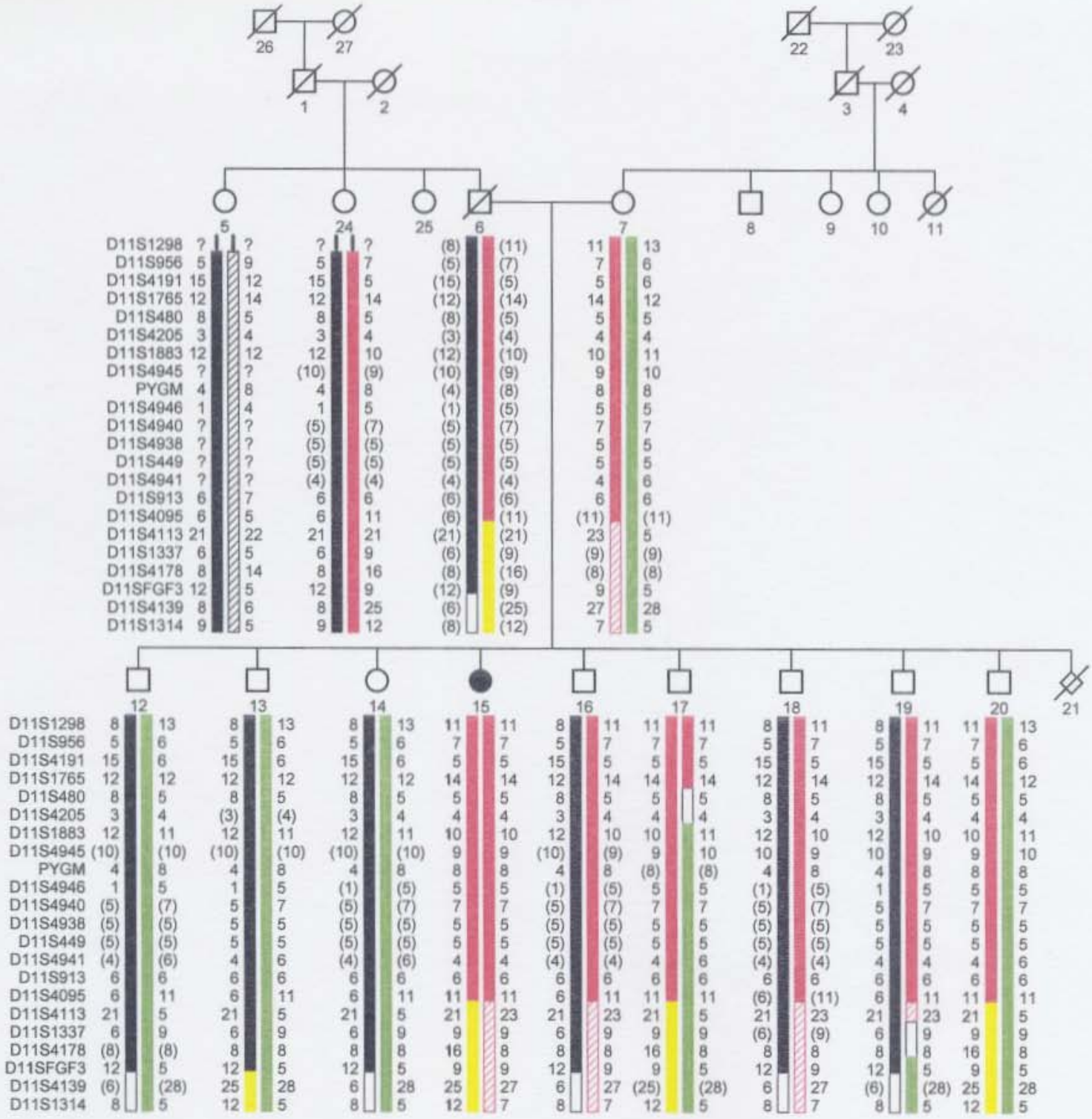
Chromosome 16
Number : B15



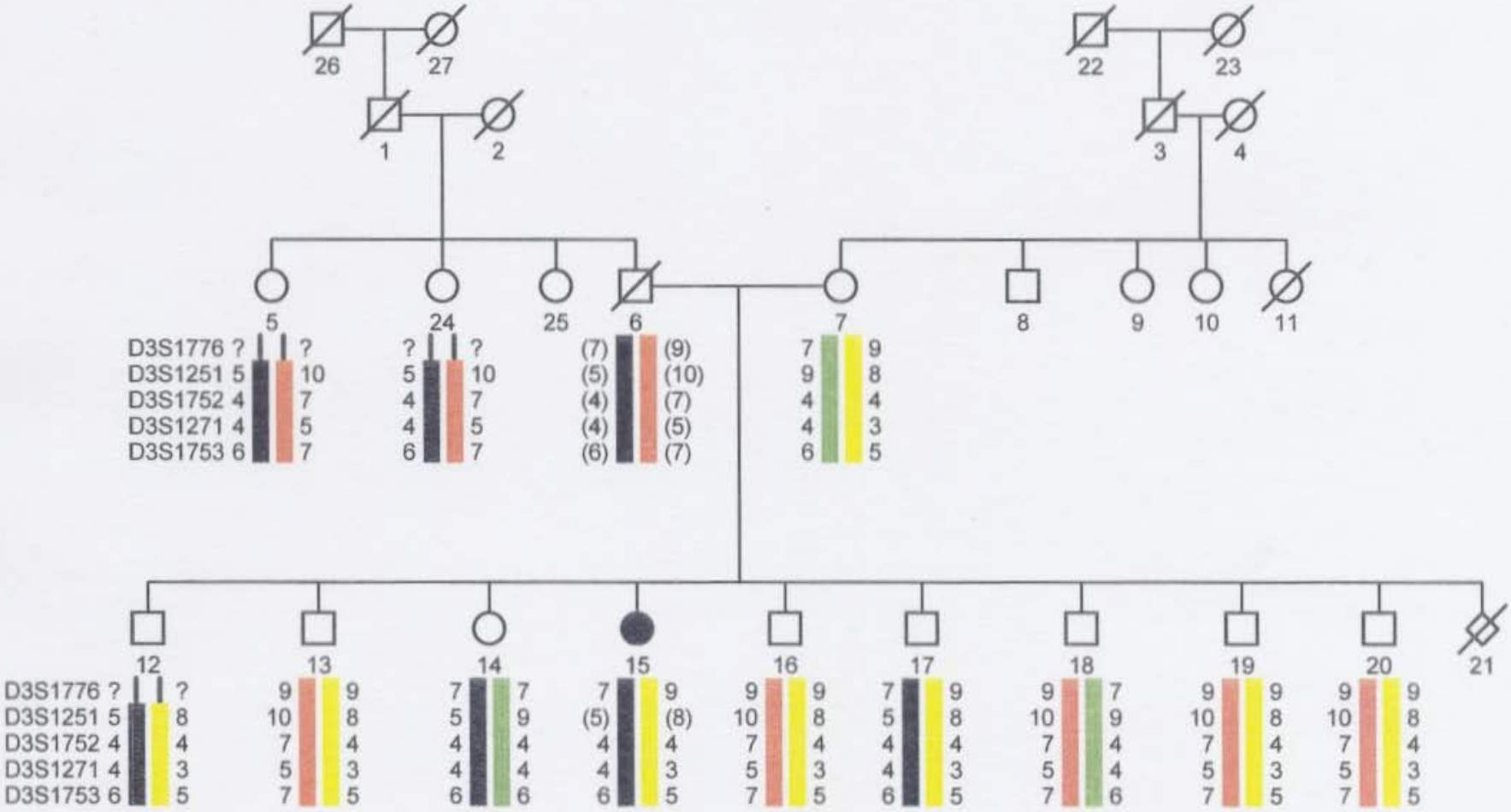
Chromosome 15
Number : B15



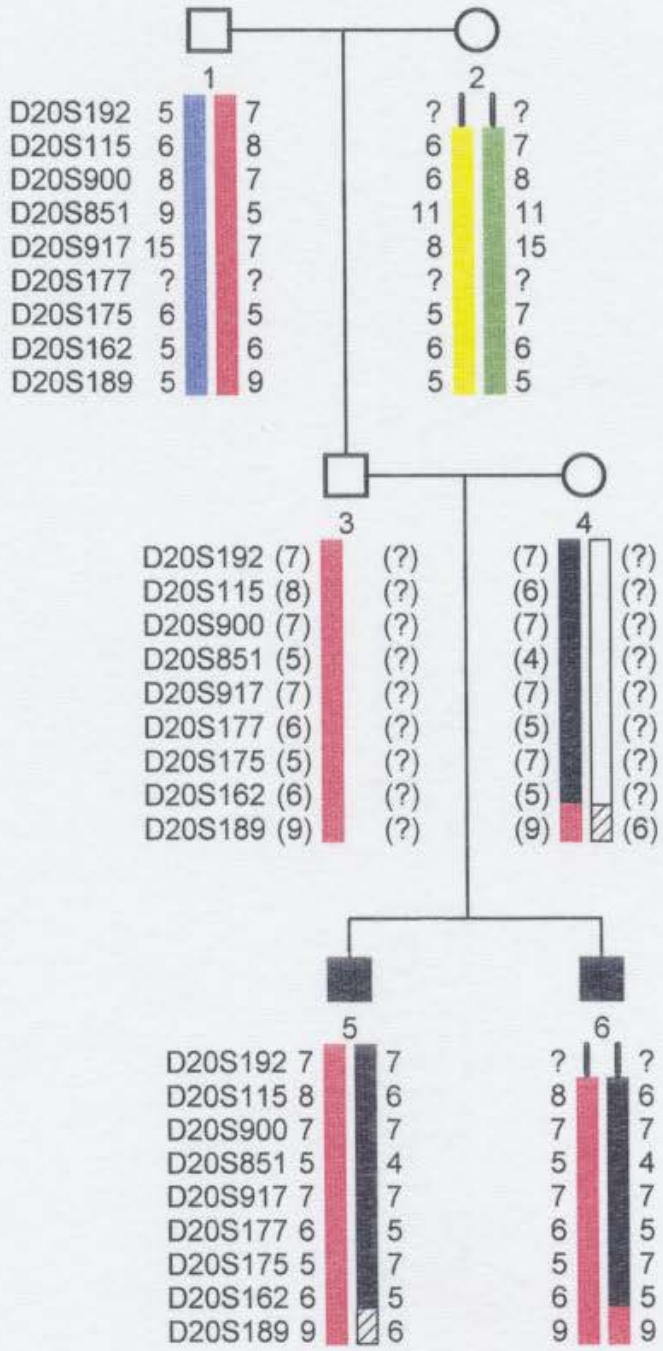
Chromosome 11
Number : B15

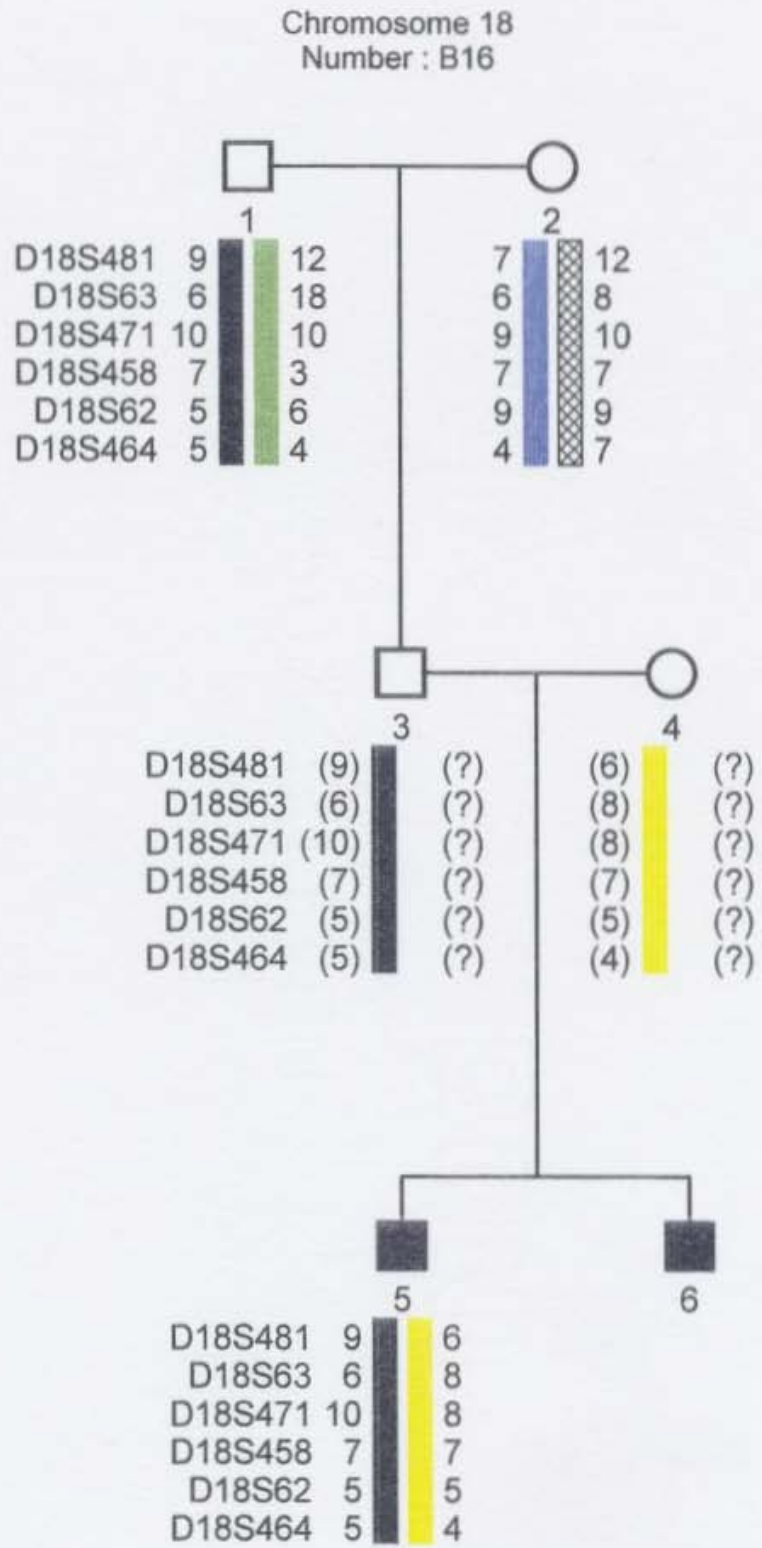


Chromosome 3
Number : B15



Chromosome 20
Number : B16




Chromosome 18
Number : B16
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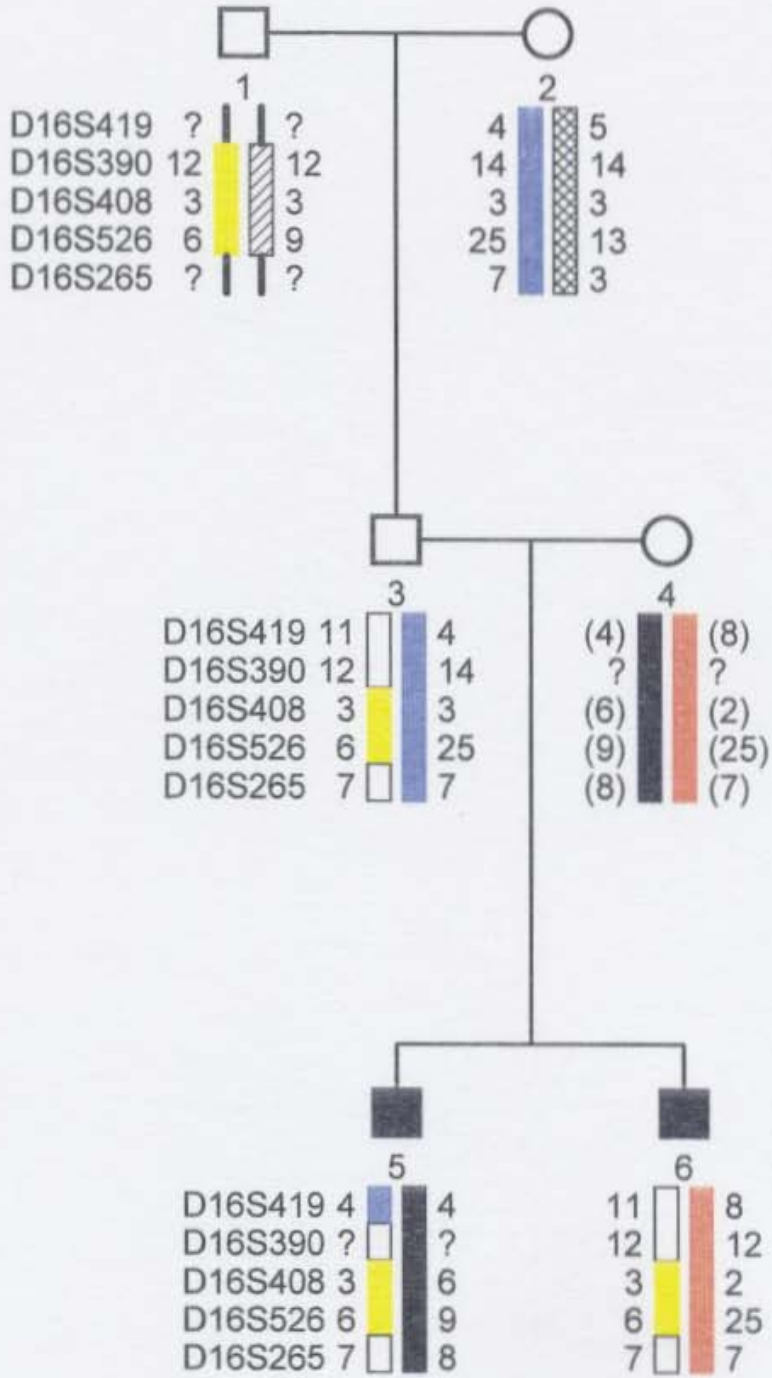
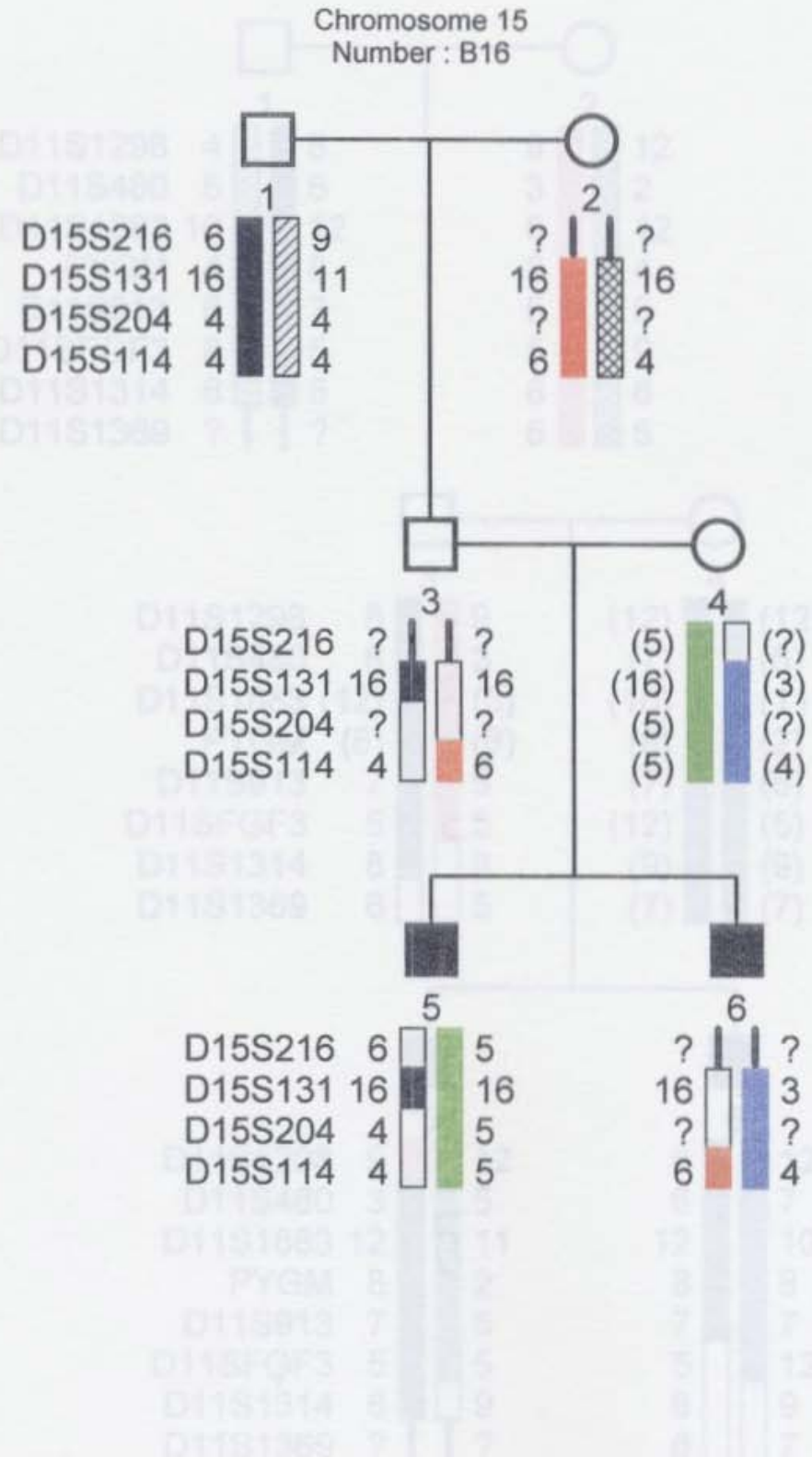
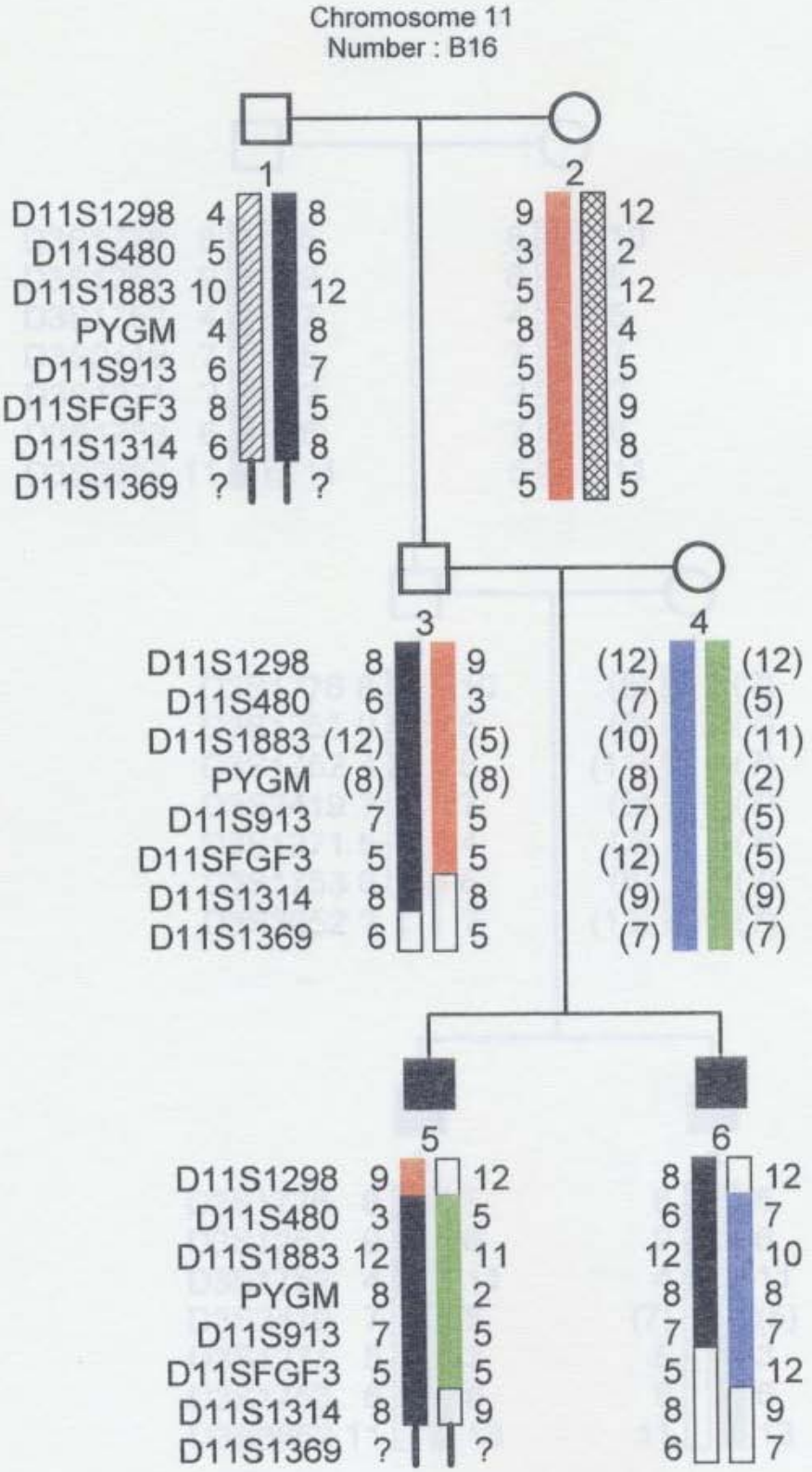
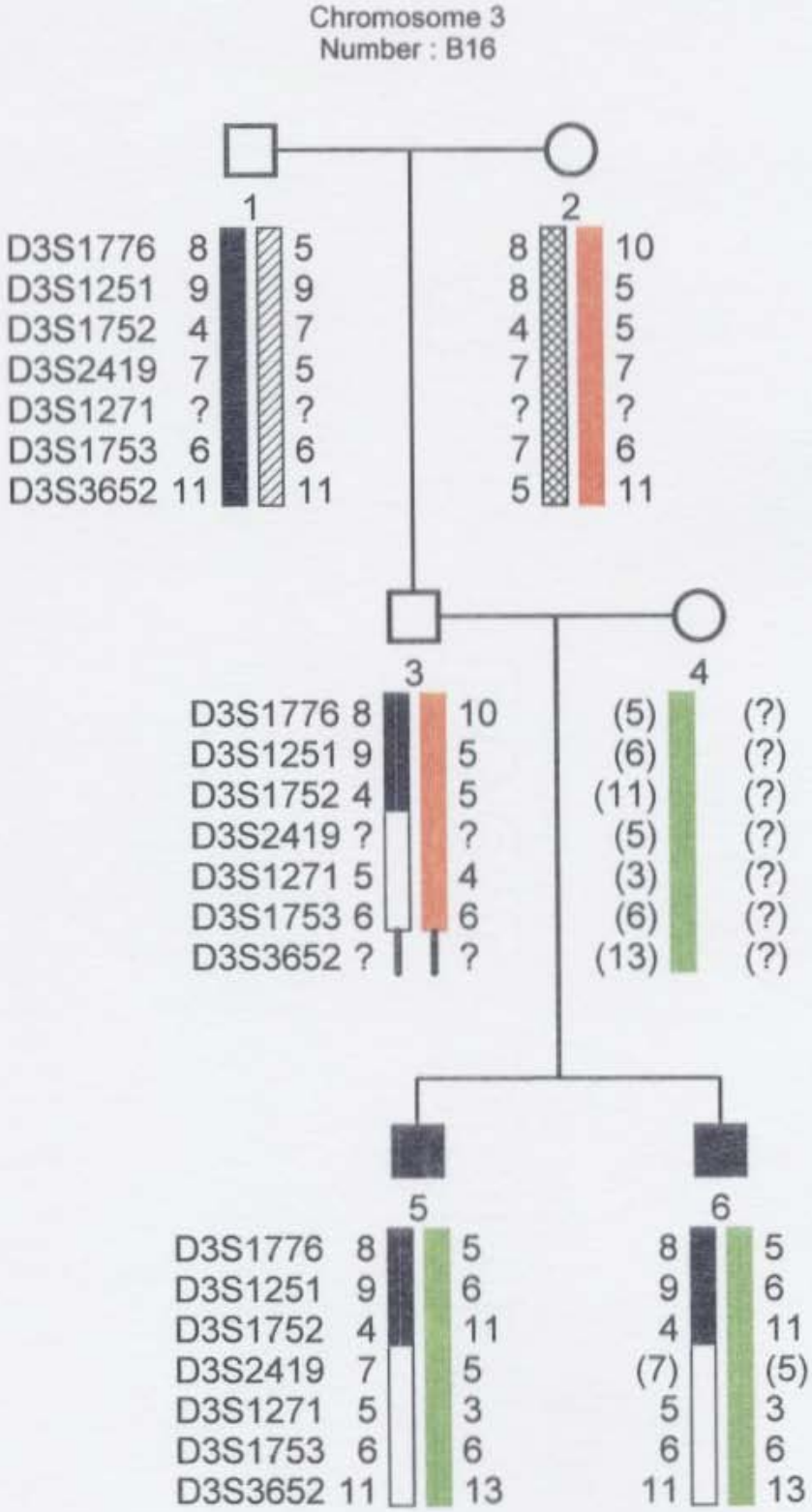
D18S481 9 12
D18S63 6 18
D18S471 10 10
D18S458 7 3
D18S62 5 6
D18S464 5 4
2
7 12
6 8
9 10
7 7
9 9
4 7
3
D18S481 (9) (?)
D18S63 (6) (?)
D18S471 (10) (?)
D18S458 (7) (?)
D18S62 (5) (?)
D18S464 (5) (?)
4
(6) (?)
(8) (?)
(8) (?)
(7) (?)
(5) (?)
(4) (?)
5
D18S481 9 6
D18S63 6 8
D18S471 10 8
D18S458 7 7
D18S62 5 5
D18S464 5 4
6

Chromosome 16
Number : B16

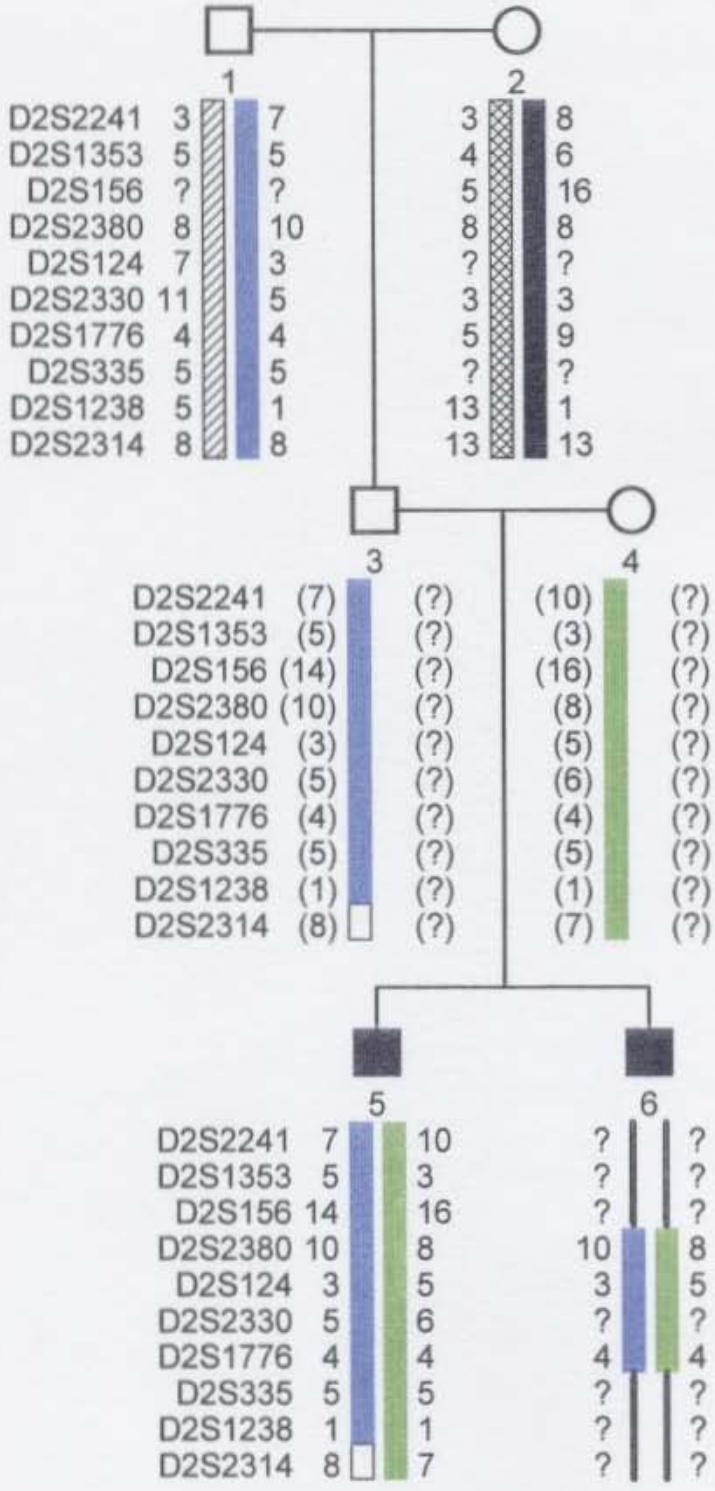



Chromosome 15
Number : B16
1
D15S216 6 9
D15S131 16 11
D15S204 4 4
D15S114 4 4
2
? ?
16 16
? ?
6 4
3
D15S216 ? ?
D15S131 16 16
D15S204 ? ?
D15S114 4 6
4
(5) (?)
(16) (3)
(5) (?)
(5) (4)
5
D15S216 6 5
D15S131 16 16
D15S204 4 5
D15S114 4 5
6
? ?
16 3
? ?
6 4

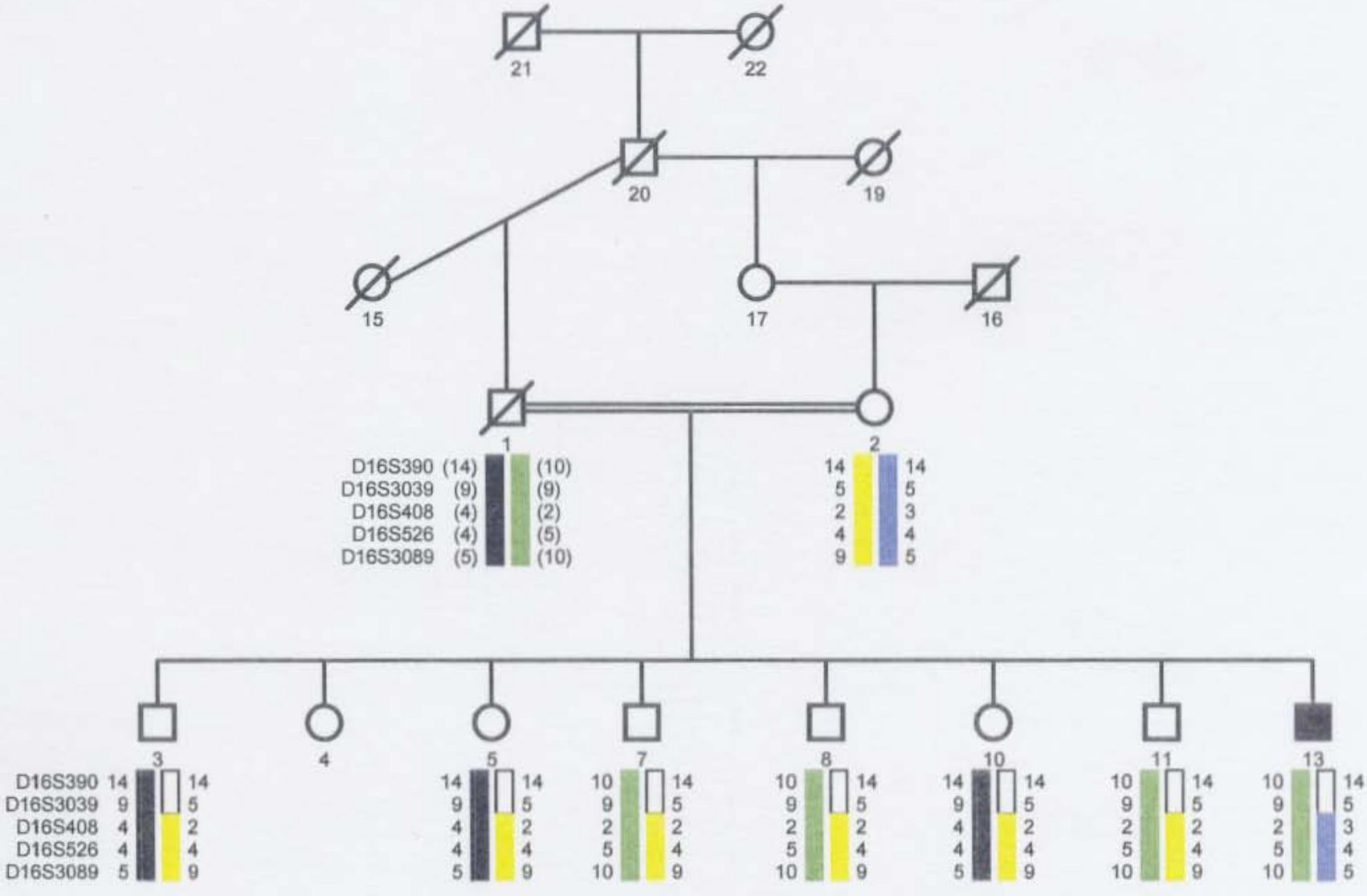

Chromosome 11
Number : B16
1
D11S1298 4 8
D11S480 5 6
D11S1883 10 12
PYGM 4 8
D11S913 6 7
D11SFGF3 8 5
D11S1314 6 8
D11S1369 ? ?
2
9 12
3 2
5 12
8 4
5 5
5 9
8 8
5 5
3
D11S1298 8 9
D11S480 6 3
D11S1883 (12) (5)
PYGM (8) (8)
D11S913 7 5
D11SFGF3 5 5
D11S1314 8 8
D11S1369 6 5
4
(12) (12)
(7) (5)
(10) (11)
(8) (2)
(7) (5)
(12) (5)
(9) (9)
(7) (7)
5
D11S1298 9 12
D11S480 3 5
D11S1883 12 11
PYGM 8 2
D11S913 7 5
D11SFGF3 5 5
D11S1314 8 9
D11S1369 ? ?
6
8 12
6 7
12 10
8 8
7 7
5 12
8 9
6 7

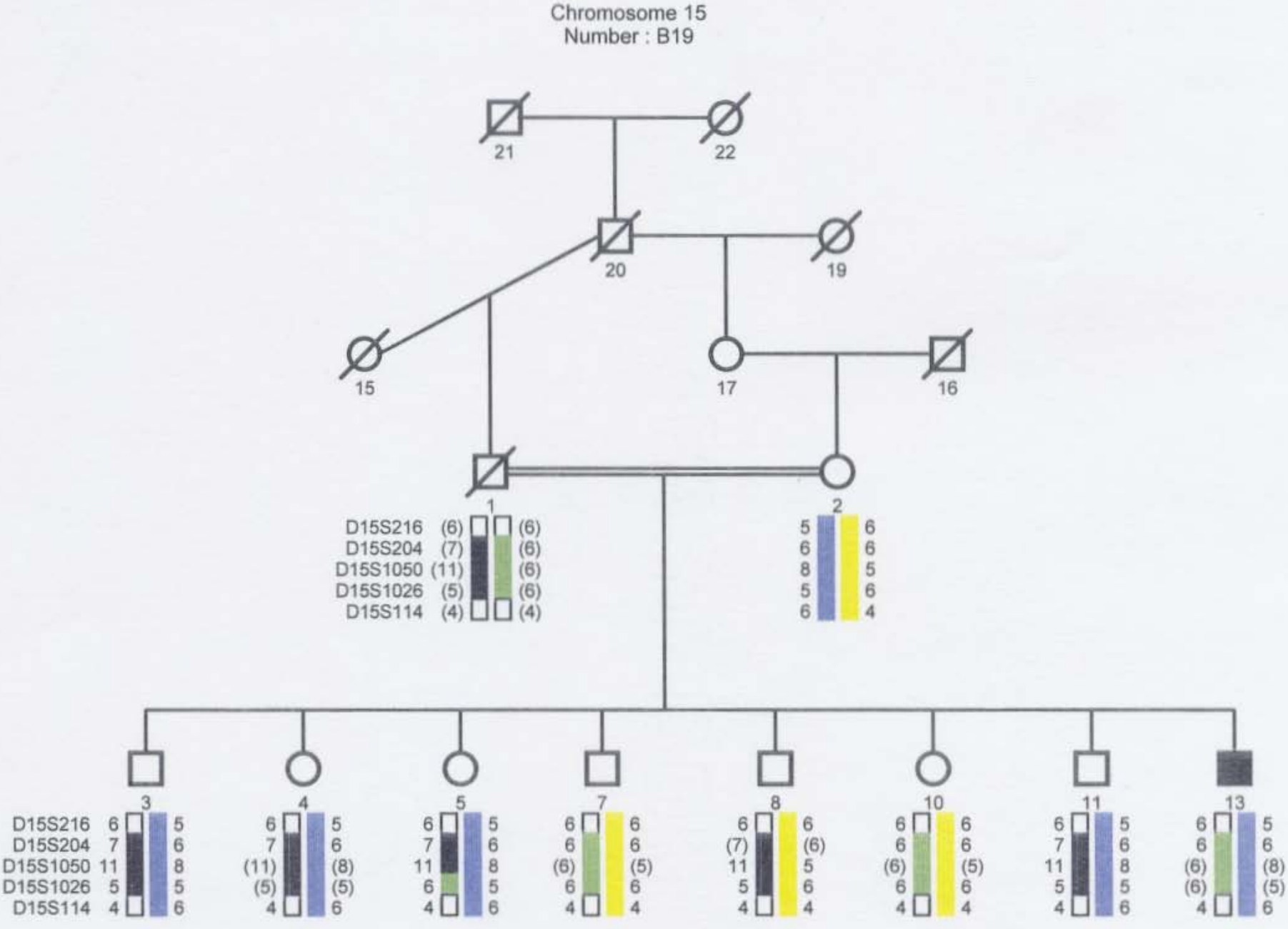

Chromosome 3
Number : B16
1
D3S1776 8 5
D3S1251 9 9
D3S1752 4 7
D3S2419 7 5
D3S1271 ? ?
D3S1753 6 6
D3S3652 11 11
2
8 10
8 5
4 5
7 7
? ?
7 6
5 11
3
D3S1776 8 10
D3S1251 9 5
D3S1752 4 5
D3S2419 ? ?
D3S1271 5 4
D3S1753 6 6
D3S3652 ? ?
4
(5) (?)
(6) (?)
(11) (?)
(5) (?)
(3) (?)
(6) (?)
(13) (?)
5
D3S1776 8 5
D3S1251 9 6
D3S1752 4 11
D3S2419 7 5
D3S1271 5 3
D3S1753 6 6
D3S3652 11 13
6
8 5
9 6
4 11
(7) (5)
5 3
6 6
11 13

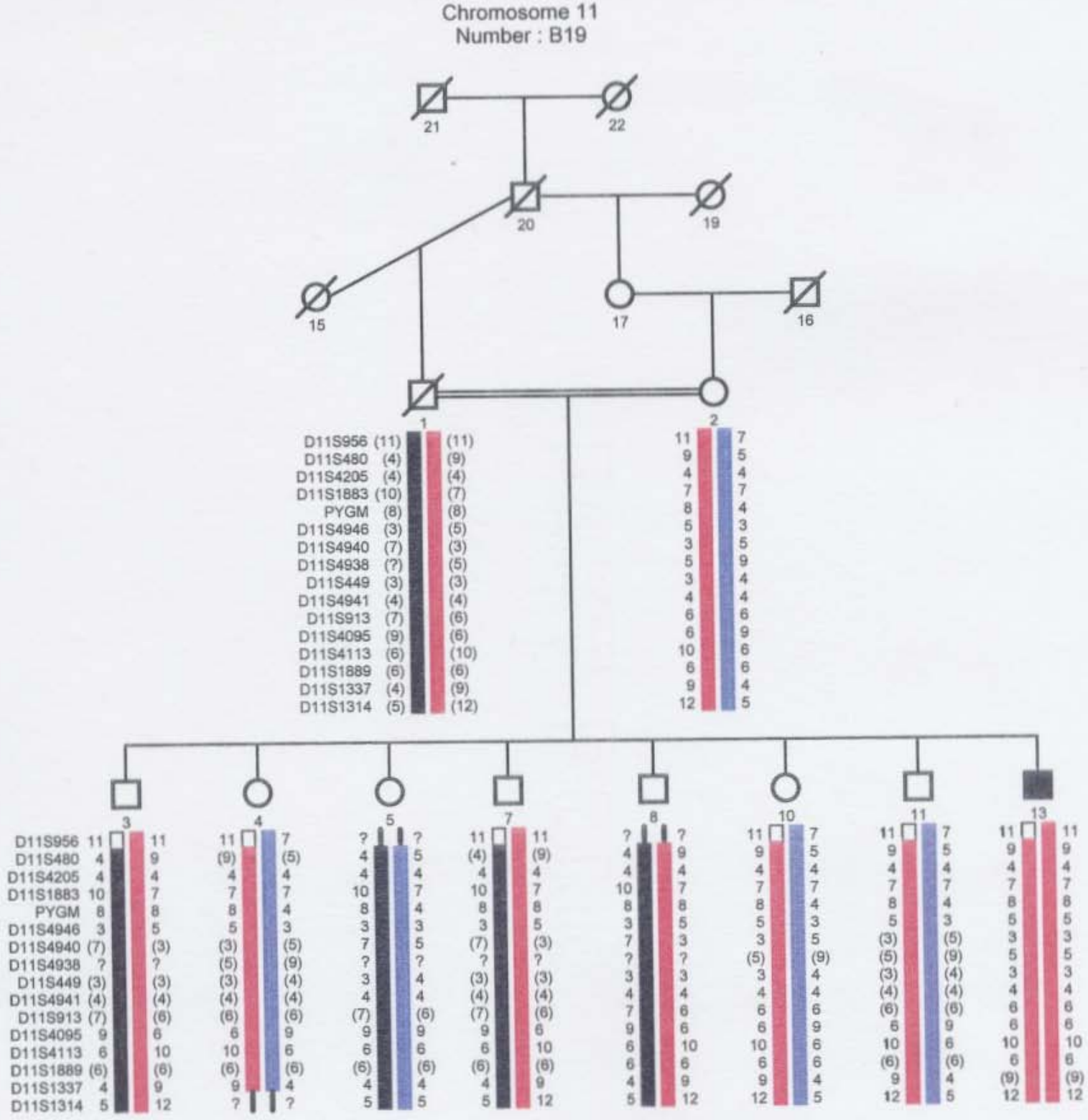
Chromosome 2
Number : B16



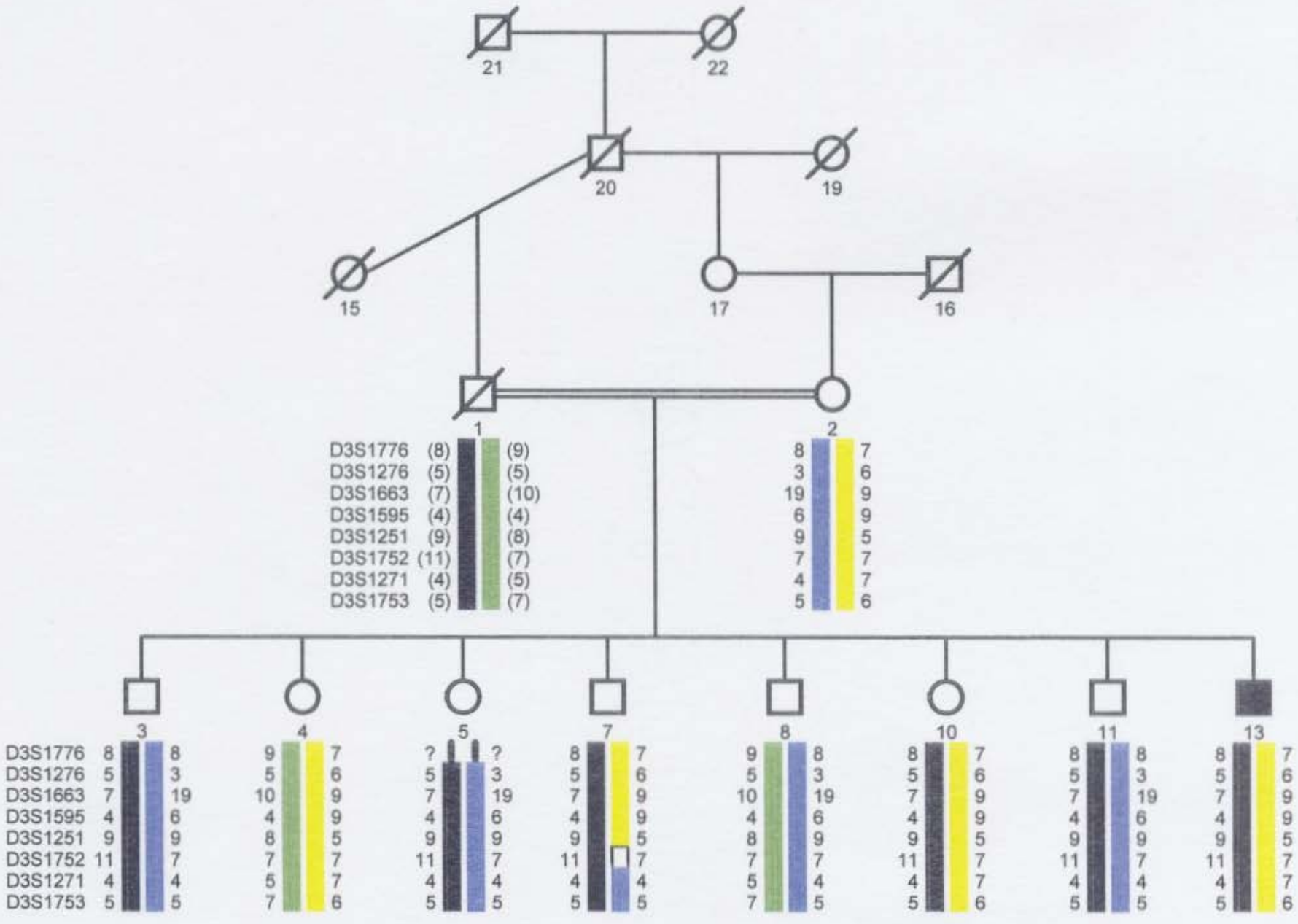
Chromosome 16
Number : B19




Chromosome 15
Number : B19
21
22
20
19
15
17
16
1
D15S216 (6) (6)
D15S204 (7) (6)
D15S1050 (11) (6)
D15S1026 (5) (6)
D15S114 (4) (4)
2
5 6
6 6
8 5
5 6
6 4
3
D15S216 6 5
D15S204 7 6
D15S1050 11 8
D15S1026 5 5
D15S114 4 6
4
6 5
7 6
(11) (8)
(5) (5)
4 6
5
6 5
7 6
11 8
6 5
4 6
7
6 6
6 6
(6) (5)
6 6
4 4
8
6 6
(7) (6)
11 5
5 6
4 4
10
6 6
6 6
(6) (5)
6 6
4 4
11
6 5
7 6
11 8
5 5
4 6
13
6 5
6 6
(6) (8)
(6) (5)
4 6


Chromosome 11
Number : B19
21
22
20
19
15
17
16
1
D11S956 (11) (11)
D11S480 (4) (9)
D11S4205 (4) (4)
D11S1883 (10) (7)
PYGM (8) (8)
D11S4946 (3) (5)
D11S4940 (7) (3)
D11S4938 (?) (5)
D11S449 (3) (3)
D11S4941 (4) (4)
D11S913 (7) (6)
D11S4095 (9) (6)
D11S4113 (6) (10)
D11S1889 (6) (6)
D11S1337 (4) (9)
D11S1314 (5) (12)
2
11 9 4 7 8 5 3 5 3 4 4 6 6 10 6 9 12
7 5 4 7 4 3 5 9 4 4 6 9 6 6 4 5
3
D11S956 11 11
D11S480 4 9
D11S4205 4 4
D11S1883 10 7
PYGM 8 8
D11S4946 3 5
D11S4940 (7) (3)
D11S4938 ? ?
D11S449 (3) (3)
D11S4941 (4) (4)
D11S913 (7) (6)
D11S4095 9 6
D11S4113 6 10
D11S1889 (6) (6)
D11S1337 4 9
D11S1314 5 12
4
11 (9) 4 7 8 5 (3) (5) (3) (4) (6) 6 10 (6) 9 ?
7 (5) 4 7 4 3 (5) (9) (4) (4) (6) 9 6 (6) 4 ?
5
? 4 4 10 8 3 7 ? 3 4 (7) 9 6 (6) 4 5
? 5 4 7 4 3 5 ? 4 4 (6) 9 6 (6) 4 5
7
11 (4) 4 10 8 3 (7) ? (3) (4) (7) 9 6 (6) 4 5
11 (9) 4 7 8 5 (3) ? (3) (4) (6) 6 10 (6) 9 12
8
? 4 4 10 8 3 7 ? 3 4 7 9 6 6 4 5
? 9 4 7 8 5 3 ? 3 4 6 6 6 10 6 9 12
10
11 9 4 7 8 5 3 (5) 3 4 6 6 10 6 9 12
7 5 4 7 4 3 5 (9) 4 4 6 9 6 6 4 5
11
11 9 4 7 8 5 (3) (5) (3) (4) (6) 6 10 (6) 9 12
7 5 4 7 4 3 (5) (9) (4) (4) (6) 9 6 (6) 4 5
13
11 9 4 7 8 5 3 5 3 4 6 6 10 6 (9) 12
11 9 4 7 8 5 3 5 3 4 6 6 10 6 (9) 12

Chromosome 3
Number : B19



Appendix B Microsatellite marker information for chromosome 2. All data were obtained from The Genome Database and Research Genetics, Inc.

Microsatellite Name	Repeat Type	Size (bp)	Heterozygosity
D2S442	Tetranucleotide	198 - ?	?
D2S1399	Tetranucletide	137 - 173	?
D2S2241	Dinucleotide	76 - 92	0.77
D2S142	Dinucleotide	254 - 266	0.77
D2S418	Dinucleotide	216 - 226	0.53
D2S1353	Trinucleotide	155 - ?	?
D2S156	Dinucleotide	168 - 198	0.86
D2S2380	Dinucleotide	159 - 175	0.63
D2S124	Dinucleotide	157 - 163	0.69
D2S2330	Dinucleotide	153 - 171	0.81
D2S1776	Tetranucleotide	288 - 308	?
D2S335	Dinucleotide	98 - 114	0.79
D2S1238	Tetranucleotide	261 – ?	>0.95
D2S2314	Dinucleotide	102 - 118	0.86
D2S1391	Tetranucleotide	124 - ?	?

Appendix C Microsatellite marker information for chromosome 3. All data were obtained from The Genome Database and Research Genetics, Inc.

Microsatellite Name	Repeat Type	Size (bp)	Heterozygosity
D3S1776	Dinucleotide	205 - 217	0.79
D3S1276	Dinucleotide	190 - 202	0.72
D3S1663	Tetranucleotide	360 - ?	0.80
D3S1595	Dinucleotide	295 - 317	0.83
D3S1251	Dinucleotide	125 - 139	0.77
D3S1752	Trinucleotide	201 - ?	?
D3S2419	Trinucleotide	213 - 225	?
D3S1271	Dinucleotide	146 - 158	0.75
D3S1753	Tetranucleotide	297 - 309	?
D3S3652	Dinucleotide	152 -160	0.69

Appendix D Microsatellite marker information for chromosome 11. All data were obtained from The Genome Database and Manickam *et al.* (1997).

Microsatellite	Repeat Type	Size (bp)	Heterozygosity
D11S1298	Tetranucleotide	180 - 240	0.86
D11S956	Tetranucleotide	247 - 303	0.88
D11S4191	Dinucleotide	111 - 135	0.88
D11S1765	?	248 - ?	?
D11S480	Dinucleotide	189 - 201	0.77
D11S4205	Dinucleotide	194 - 200	0.31
D11S1883	Dinucleotide	250 - 266	0.74
D11S4945	Dinucleotide	223 - ?	0.58
PYGM	Dinucleotide	156 - 190	0.90
D11S4946	Dinucleotide	154 - ?	0.73
D11S4940	Tetranucleotide	199 - ?	0.63
D11S4938	Tetranucleotide	159 - ?	0.54
D11S4941	Tetranucleotide	185 - ?	0.89
D11S913	Dinucleotide	220 - ?	0.32
D11S4095	Dinucleotide	193 - 205	0.65
D11S4113	Dinucleotide	218 - 262	0.81
D11S1889	Dinucleotide	183 - 207	0.69
D11S1337	Dinucleotide	279 - 293	0.60
D11S4178	Dinucleotide	238 - 260	0.68
FGF3	Dinucleotide	161 - 177	0.82
D11S4139	Dinucleotide	151 - 195	0.87
D11S1314	Dinucleotide	209 - 227	0.78
D11S1369	Tetranucleotide	180 - ?	?

Appendix E Microsatellite marker information for chromosome 15. All data were obtained from The Genome Database and Research Genetics, Inc.

Microsatellite Name	Repeat Type	Size (bp)	Heterozygosity
D15S125	Dinucleotide	157 - 169	0.80
D15S216	Dinucleotide	225 - 233	0.64
D15S131	Dinucleotide	235 - 274	0.84
D15S204	Dinucleotide	116 - 134	0.79
D15S1050	Dinucleotide	278 - 292	0.69
D15S1026	Dinucleotide	201 - 215	0.74
D15S114	Dinucleotide	177 - 187	0.72
D15S211	Dinucleotide	207 - 259	0.96

Appendix F Microsatellite marker information for chromosome 16. All data were obtained from The Genome Database, Research Genetics, Inc and P. Beales (personal communication; in red).

Microsatellite Name	Repeat Type	Size (bp)	Heterozygosity
D16S419	Dinucleotide	146 - 164	0.77
D16S390	Dinucleotide	177 - 195	0.80
D16S3039	Dinucleotide	255 - 265	0.73
D16S408	Dinucleotide	241 - 251	0.69
STRP3	?	149 - 157	?
STRP1	?	308 - 337	?
STRP2	?	192 - 200	?
D16S3057	Dinucleotide	188 - 206	0.73
D16S503	Dinucleotide	221 - 235	0.81
D16S526	Dinucleotide	205 - ?	0.83
D16S3089	Dinucleotide	174 - 200	0.88
D16S265	Dinucleotide	89 - 117	0.77

Appendix G Microsatellite marker information for chromosome 18. All data were obtained from The Genome Database and Research Genetics, Inc.

Microsatellite Name	Repeat Type	Size (bp)	Heterozygosity
D18S481	Dinucleotide	183 - 203	0.76
D18S63	Dinucleotide	255 - 279	0.80
D18S52	Dinucleotide	116 - 130	0.77
D18S471	Dinucleotide	259 - 265	0.66
D18S452	Dinucleotide	123 - 141	0.83
D18S458	Dinucleotide	208 - 218	0.59
D18S62	Dinucleotide	187 - 195	0.67
D18S464	Dinucleotide	283 - 291	0.65

Appendix H Microsatellite marker information for chromosome 20. All data were obtained from The Genome Database and Research Genetics, Inc.

Microsatellite Name	Repeat Type	Size (bp)	Heterozygosity
D20S192	Dinucleotide	287 - 299	0.75
D20S900	Dinucleotide	135 - 145	0.63
D20S115	Dinucleotide	232 - 238	0.67
D20S851	Dinucleotide	128 - 150	0.74
D20S917	Dinucleotide	141 - 167	0.81
D20S177	Dinucleotide	94 - 102	0.59
D20S175	Dinucleotide	166 - 174	0.67
D20S162	Tetranucleotide	246 - ?	0.80
D20S189	Dinucleotide	295 - 309	0.75
D20S186	Dinucleotide	113 - 135	0.86

Appendix I Primers used for sequencing exons 3 through 6 of *MKKS*.

Primer Name	Primer Sequence (5'-3')
MKKS.x3aF	GATTTTATAGCCACAATGCT
MKKS.x3aR	ATGACAGTGGTGGGTGTCAA
MKKS.x3bF	TCTGGTGAGCATACAGGCAG
MKKS.x3bR	CGTTTGGAAGCTAAGAAGCC
MKKS.x3cF	GATCCTCCTTTGTTTGGTGC
MKKS.x3cR	GGTTAAGCAGCTGGTCCAAG
MKKS.x3dF	AATCAACTGCCCTCAAGGTG
MKKS.x3dR	CCTTTGCTGCCAGAAATGAT
MKKS.x4F	ATGCTTGTGGGGCTTTTATG
MKKS.x4R	AATGGCAACACATGCCAAAT
MKKS.x5F	GCACCACACAAGTTTTGTTC
MKKS.x5R	CCTATACATGCACCCCTGAA
MKKS.x6aF	GTGCCAGACCCCAAATTAAA
MKKS.x6aR	CCAGTTGAGTTCTTCCTGGC
MKKS.x6bF	GGCAGATTCTCCCTGTGTTG
MKKS.x6bR	GCATTTCCATTCACGAATCA