CHAPTER FOUR

RESULTS AND DISCUSSION

In this chapter haplotype and Southern blot analyses of five South African FSHD families are presented. The haplotypes were constructed by genotyping short tandem repeat polymorphism (STRP) markers located at chromosome 4q35. Southern blot analysis was performed to determine the DNA rearrangements at the D4Z4 locus. Results presented in this chapter have been presented at several national and international conferences and selected abstracts have been published in international peer-reviewed journals (Appendix A).

4.1 SHORT TANDEM REPEAT POLYMORPHISM (STRP) ANALYSIS

Nine STRP markers were genotyped in five FSHD families. Primer sets were optimised with regard to the annealing temperature and $MgCl_2$ concentration. Annealing temperatures were calculated for each of the two primers in a primer set, with the formula reported by Thein and Wallace (1986). Table 4.1 lists the calculated and optimised annealing temperature and $MgCl_2$ concentration for all the markers. The primer sequences and other relevant data for these markers were presented in Table 3.3 and in paragraph 3.3.1.

Table 4.1: Optimised conditions for nine short tandem repeat polymorphism markers located on chromosome 4q35

Locus	DNA marker	Calculated T_a of forward primer	Calculated T_a of reverse primer	Optimised T_a	Optimised $MgCl_2$ concentration
D4S1523F	UT1366	60	58	55	1.5
D4S1652F	GATA5B02	62	64	55	1.5
D4S2930F	AFMa224xh1	70	58	55	1.5
D4S2390F	ATA22F02	58	60	55	1.5
D4S2299F	UT5785	56	56	56	1.0
D4S2283F	UT2219	60	58	58	1.5
D4S2688F	UT7694	54	58	55	1.5
D4S2921F	AFMa190zf5	52	60	55	1.5
D4S426F	AFM238ve3	68	66	55	1.5

T_a = annealing temperature.

A representative autoradiograph of each of the nine STRP markers utilised in this study is presented in subsequent paragraphs. The STRP markers are discussed in order of their genetic map position from the most telomeric to the most centromeric (Table 3.2). Size standards, consisting of the A, C, G and T sequencing reaction mixture of bacteriophage M13mp18, were loaded on each gel to enable precise sizing of marker alleles. Two of the sequencing reaction mixtures were mixed prior to loading on the gel (A with G and C with T). Allele size standards were also generated by mixing a portion of the PCR products of ca. six unrelated individuals. The allele size standard was loaded three or four times on the gel as a standardised reference throughout the gel to facilitate in the scoring of the alleles.

4.1.1 Short tandem repeat marker UT1366 at locus D4S1523

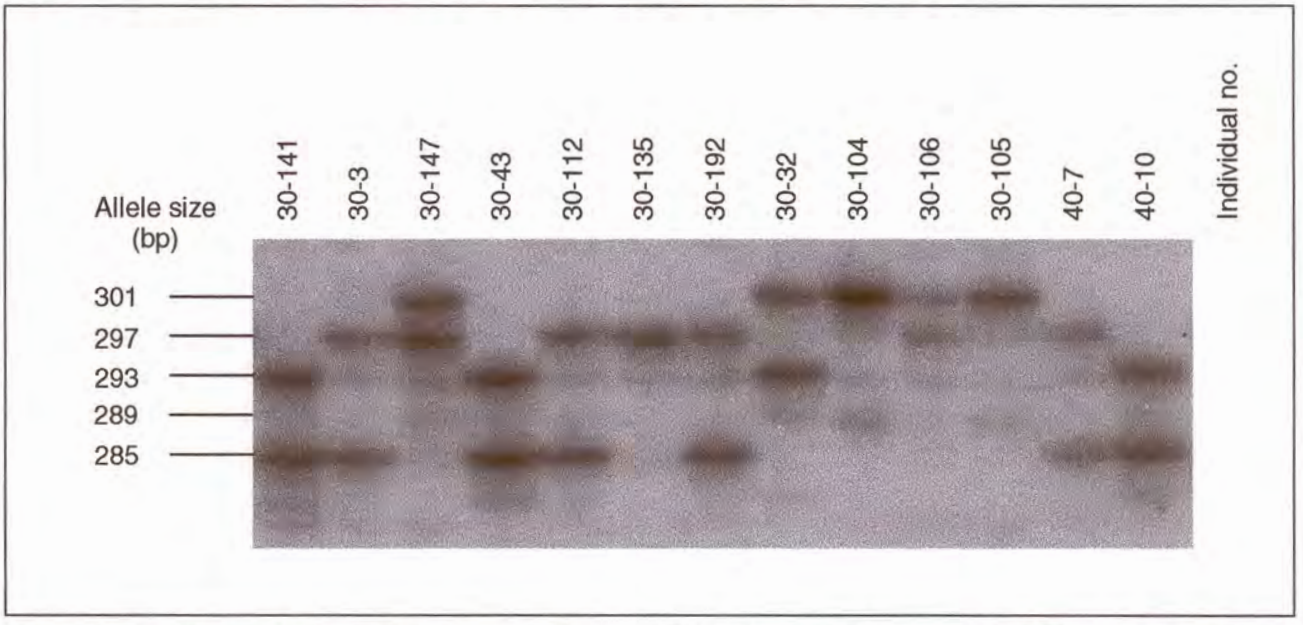
The primers of this tetranucleotide STRP marker were annealed at 55°C with the reverse primer selected for end labelling. Six alleles were observed in this study. The allele sizes of the seven reported alleles have never been published. The allele frequencies of only five of the alleles have, however, been reported (as discussed in paragraph 3.3.1.1). It was therefore not possible to correlate the six alleles obtained with the seven reported alleles. The allele sizes of marker UT1366 were thus determined for the first time through this investigation. Once the allele sizes have been reported on the CEPH databases, CEPH samples, genotyped with this marker, can be obtained to correlate the allele sizes generated in this study with those reported.

A representative autoradiograph of the amplification products generated with this marker is presented in Figure 4.1. Marker UT1366 generated strand slippage products that differ in size by four base pairs from the true allele. The secondary amplification products did not complicate scoring, as the allele motif of this marker could be discerned, and the alleles could be identified with ease.

A constant secondary fragment of 293 bp can be observed for all the individuals presented in Figure 4.1. Individuals 30-104 and 30-105 were scored to be homozygous for the 301 bp allele. A less intense fragment of 289 bp was also observed for both individuals. However, this was not scored as a true allele, since it was observed that this fragment

(289 bp) was of equal intensity to the 301 bp allele in heterozygous individuals (not presented in Figure 4.1).

Figure 4.1: Representative autoradiograph of marker UT1366



Autoradiograph of the amplification products generated with marker UT1366 described by Gerken *et al.* (1993a). The reverse primer was end labelled with γ^{32}P-dATP and the products electrophoresed on a 6% polyacrylamide gel in 1X TBE buffer for 3.5 hours. Individuals genotyped are indicated at the top and allele sizes are indicated to the left of the autoradiograph.

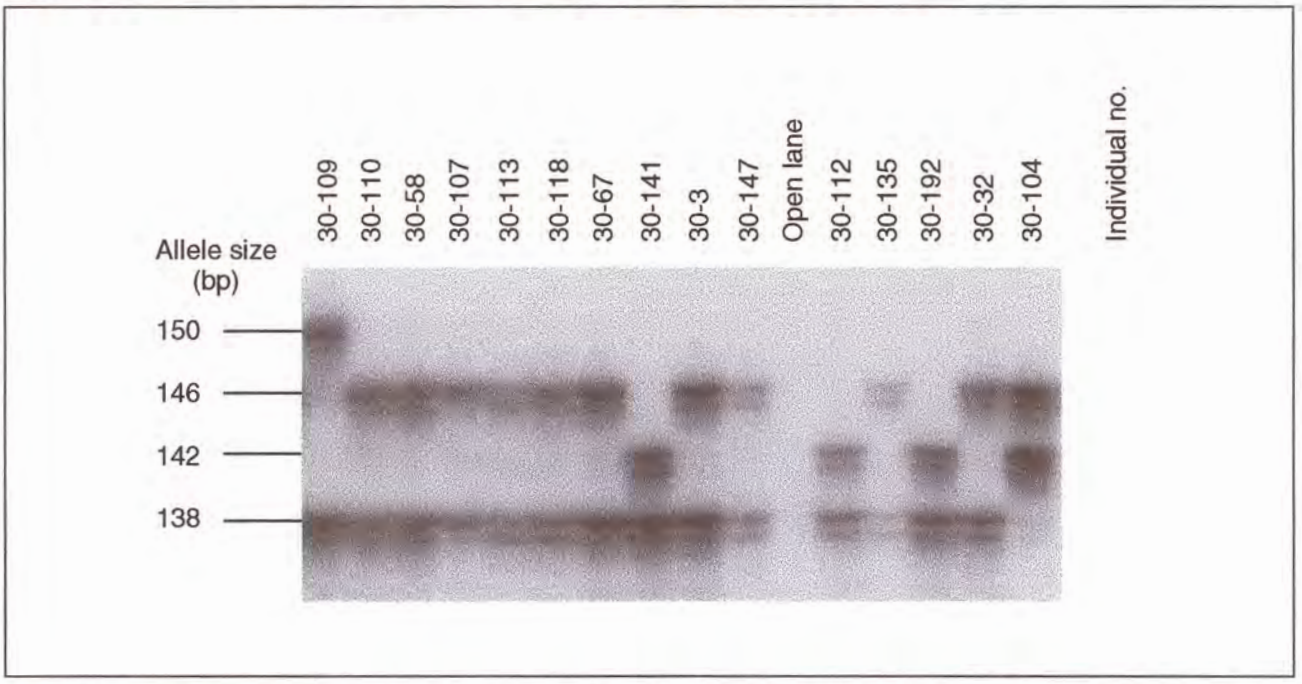
4.1.2 Short tandem repeat marker GATA5B02 at locus D4S1652

Marker GATA5B02 amplifies a tetranucleotide repeat $(CTAT)_n$ at locus D4S1652. This marker generates four alleles, ranging from 138 to 150 bp, and all four alleles were observed in this study (Figure 4.2). A heterozygosity value of 69 % was reported for this marker. As presented in Figure 4.2 most of the individuals investigated were heterozygous at this locus.

The primer set was annealed at 55°C, with the forward primer chosen for end labelling. It is evident from the autoradiograph presented here that a secondary amplification product, also referred to as "shadow bands" or "strand slippage", was generated for each allele, which migrated one base pair faster than the true allele. This marker therefore generates two fragments per allele. Hauge and Litt (1993) reported that the extra fragments are generated during PCR as a result of slipped strand mispairing. During slipped strand mispairing the primer-template complex misaligns therefore forming a heteroduplex DNA causing the two strands of DNA to differ in length by one or multiple repeat units. The extra fragments can complicate scoring when superimposed on true alleles. However, in

the case of this marker, strand slippage did not interfere with the scoring and the allele motif was discernible with ease.

Figure 4.2: Representative autoradiograph of marker GATA5B02



Autoradiograph of the amplification products generated with marker GATA5B02 described by Murray *et al.* (1995a). The forward primer was end labelled with γ^{32}P-dATP and the products electrophoresed on a 6% polyacrylamide gel in 1X TBE buffer for 2 hours. Individuals genotyped are indicated at the top and allele sizes are indicated to the left of the autoradiograph.

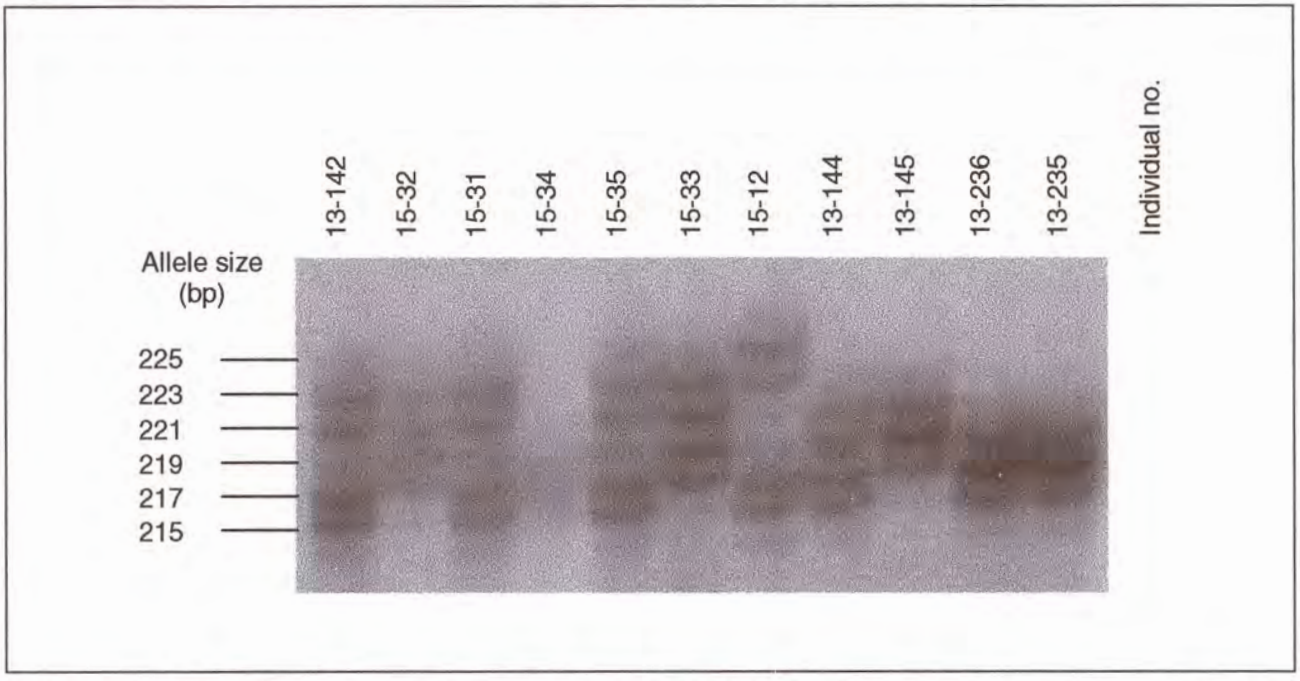
4.1.3 Short tandem repeat marker AFMa224xh1 at locus D4S2930

The primers of this marker were annealed at 55°C in a multiplex PCR reaction with the primers of markers AFMa190zf5 and ATA22F02. All nine of the reported alleles of marker AFMa224xh1, ranging from 217 to 233 bp, were observed in this study. Five of the alleles are presented in Figure 4.3, which shows a representative autoradiograph of the dinucleotide STRP at this locus.

Internal labelling of the amplification products was achieved by substituting one of the dNTPs in the PCR reaction mixture with an isotope labelled nucleotide. Both the strands are therefore visible on the gel, with the CA strand migrating faster than the GT strand, at a rate of one repeat (2 bp) difference between the two strands (Weber and May 1989). The scoring of the alleles could be obscured in this instance, as each allele is represented by two fragments. However, it did not obscure the results obtained with this marker.

The genotype (217-219) scored for individual 15-34 was confirmed on a longer exposure. The 219 bp allele for individual 13-145, and the 217 bp allele for individuals 13-236 and 13-235 appear darker than the other alleles. This is due to the strand slippage product of the GT strand being superimposed on the true allele generated from the CA strand. The strand slippage products did, however, not obscure the scoring of the alleles.

Figure 4.3: Representative autoradiograph of marker AFMa224xh1

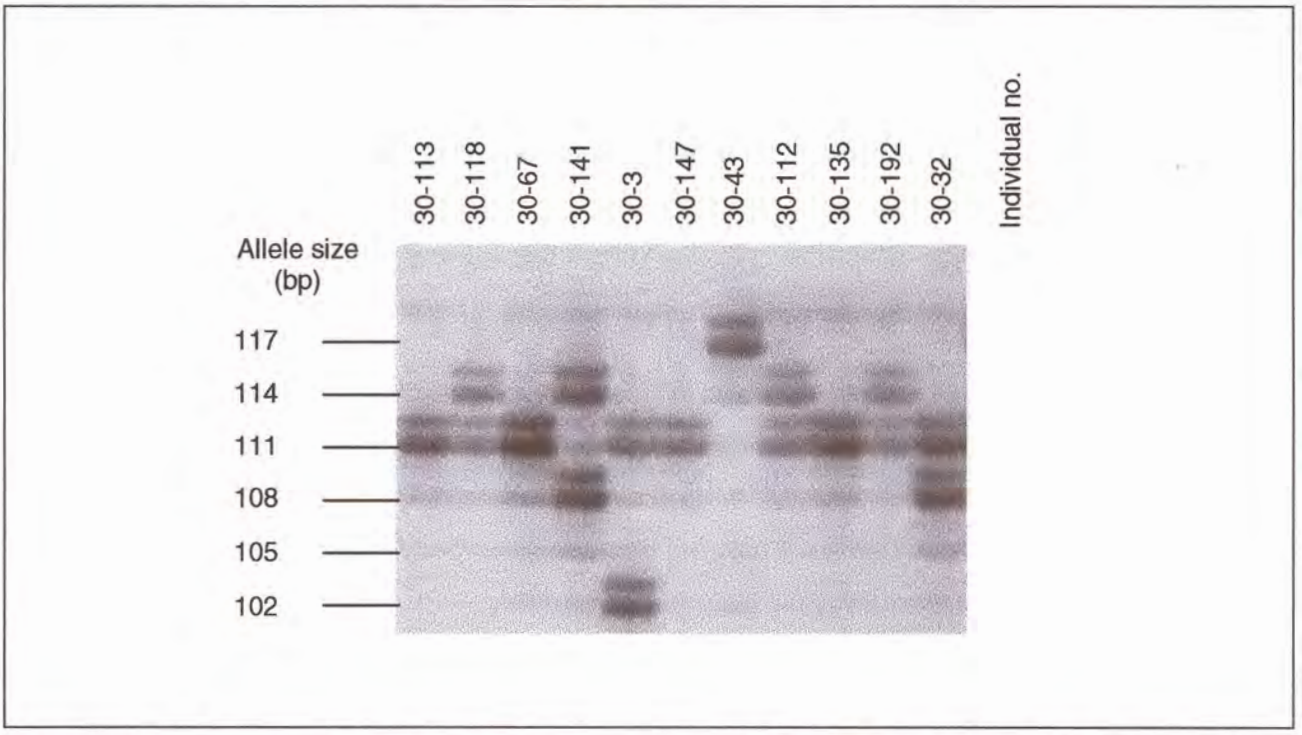


Autoradiograph of the amplification products generated with marker AFMa224xh1 described by Dib *et al.* (1996). Internal labelling was performed with α^{32}P-dCTP and the products electrophoresed on a 6% polyacrylamide gel in 1X TBE buffer for 2.5 hours. Individuals genotyped are indicated at the top and allele sizes are indicated to the left of the autoradiograph.

4.1.4 Short tandem repeat marker ATA22F02 at locus D4S2390

In this study all six of the previously reported alleles were observed and five of the alleles are presented in Figure 4.4. The amplification products were internally labelled in the multiplex reaction with markers AFMa224xh1 and AFMa190zf5 as described in paragraph 4.1.3. The sense strand, containing the $(ATA)_n$ repeat, migrated one base pair faster than the anti-sense strand, with the $(TAT)_n$ repeat. Two fragments per allele were therefore generated with this marker. It is also evident from Figure 4.4 that several constant secondary fragments were generated resulting in an allele motif of at least three fragments per allele. The sense strand was used for scoring of the alleles for this marker.

Figure 4.4: Representative autoradiograph of marker ATA22F02



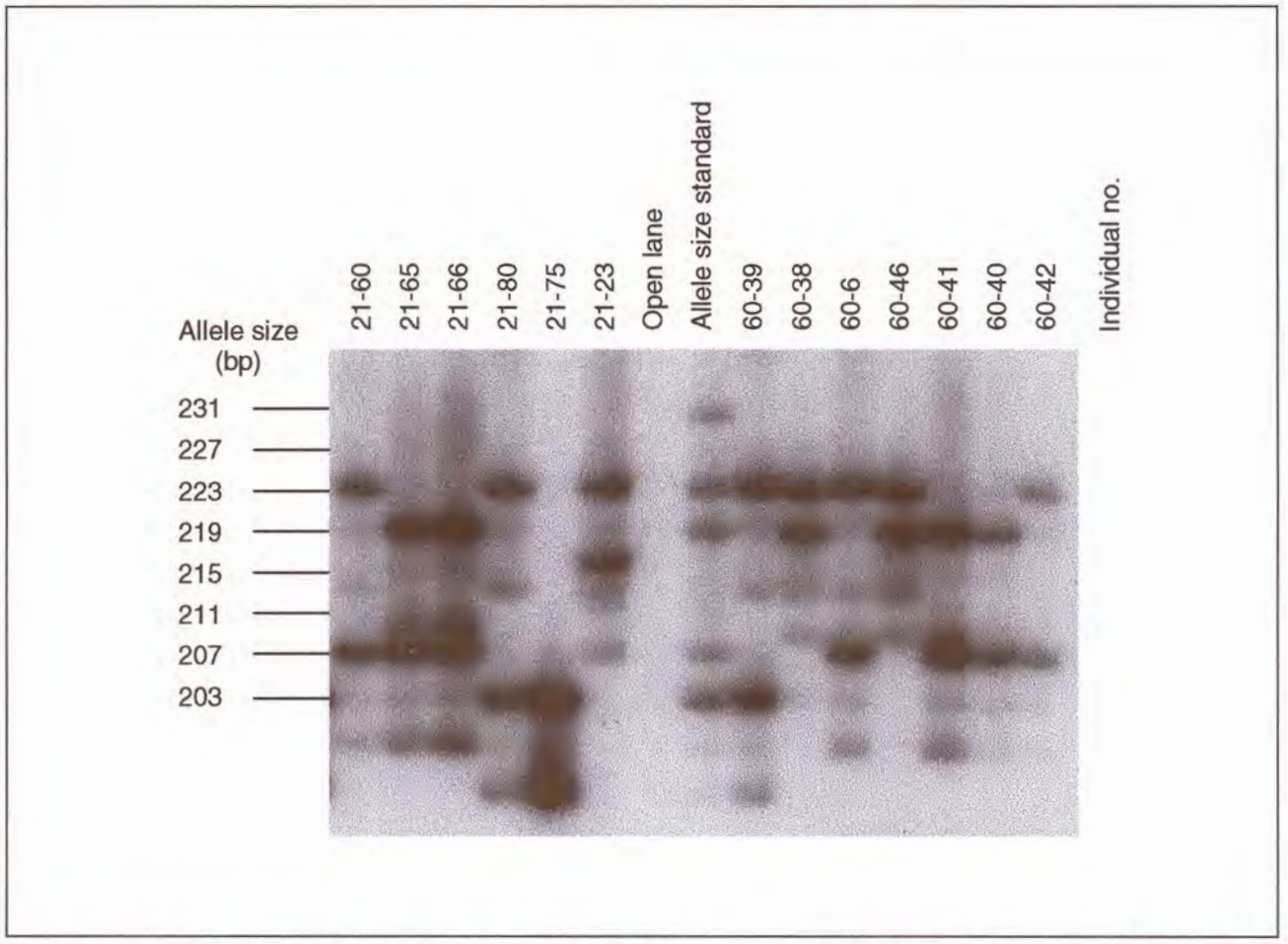
Autoradiograph of the amplification products generated with marker ATA22F02 described by Murray *et al.* (1995b). Internal labelling was performed with α^{32}P-dCTP and the products electrophoresed on a 6% polyacrylamide gel in 1X TBE buffer for 2.5 hours. Individuals genotyped are indicated at the top and allele sizes are indicated to the left of the autoradiograph.

4.1.5 Short tandem repeat marker UT5785 at locus D4S2299

All of the nine reported alleles, ranging from 203 to 235 bp, were observed in the five families included in this study. Figure 4.5 displays a representative autoradiograph of the amplification products generated with this marker. Six of the nine alleles are presented in Figure 4.5. The 231 bp allele was not observed in any of the individuals in Figure 4.5 but is presented in the allele size standard which consists of a mixture of PCR products of ca. six unrelated individuals.

The primer set of this marker was annealed at 56°C in the presence of 1 mM $MgCl_2$ with an end labelled reverse primer. Two secondary amplification products were generated for each allele, which migrated two base pairs faster than the true allele. This marker therefore generates three fragments per allele and the allele motif thus consisted of three distinct fragments, differing in size by 2 bp. Although not ideal, it did not obscure the scoring of the alleles.

Figure 4.5: Representative autoradiograph of marker UT5785

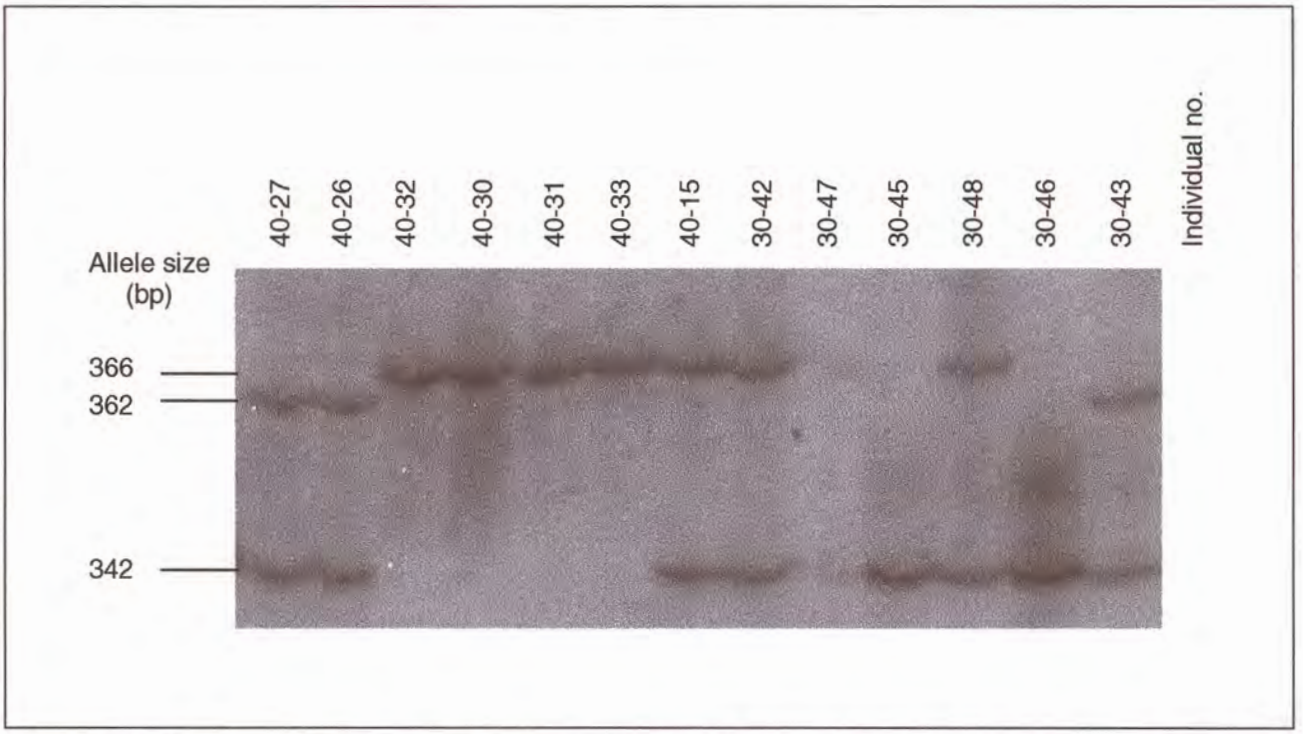


Autoradiograph of the amplification products generated with marker UT5785 described by Gerken *et al.* (1993b). The reverse primer was end labelled with γ^{32}P-dATP and the products electrophoresed on a 6% polyacrylamide gel in 1X TBE buffer for 3 hours. Individuals genotyped are indicated at the top and allele sizes are indicated to the left of the autoradiograph.

4.1.6 Short tandem repeat marker UT2219 at locus D4S2283

The reverse primer was selected for end labelling and the primers were annealed at 58°C. Four alleles have been reported for this marker but the allele sizes have not yet been reported (as mentioned in paragraph 3.3.1.6). Five alleles (342 bp and 358 to 370 bp) were generated with this marker in the five families investigated. Three of the alleles are presented in Figure 4.6, which displays a representative autoradiograph of this marker. The reported heterozygosity value for this marker is 59%. As presented in Figure 4.6, low levels of heterozygosity were also observed in the families investigated. The alleles generated with this marker were discernible with ease. The genotype for individual 30-47 was not scored from this autoradiograph, but from another autoradiograph that was exposed for a longer period of time.

Figure 4.6: Representative autoradiograph of marker UT2219



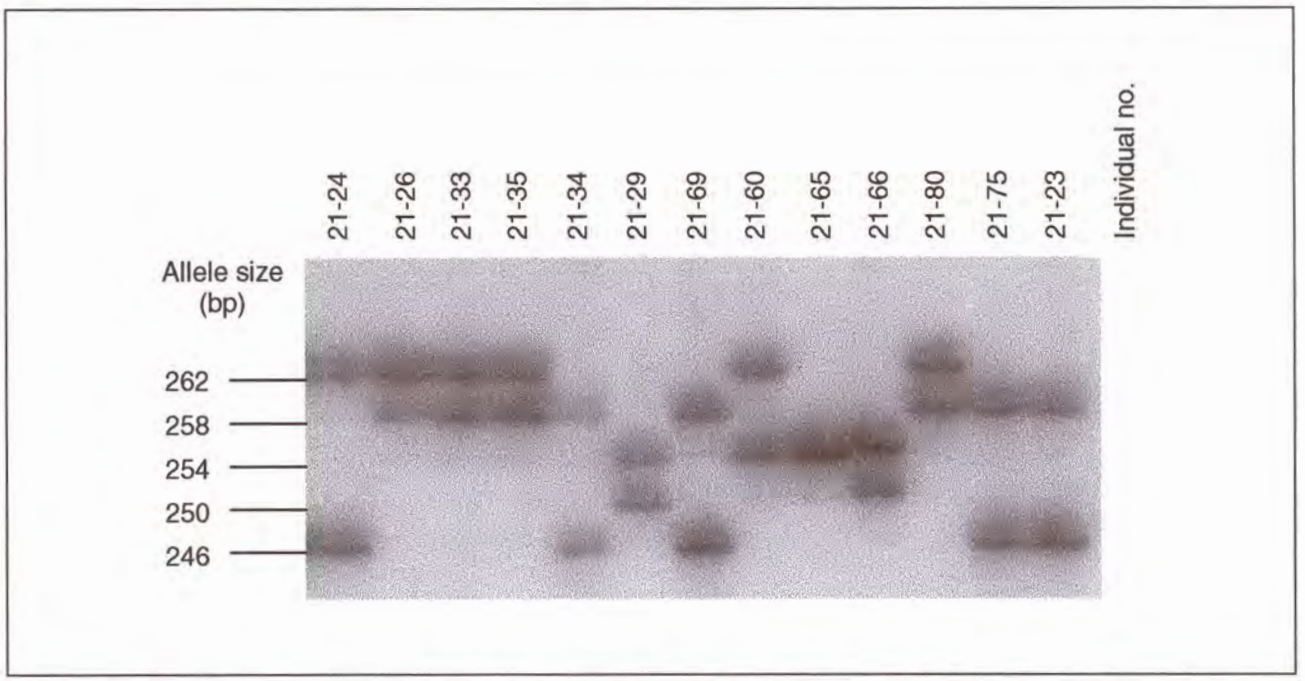
Autoradiograph of the amplification products generated with marker UT2219 described by Gerken *et al.* (1993c). The reverse primer was end labelled with γ^{32}P-dATP and the products electrophoresed on a 6% polyacrylamide gel in 1X TBE buffer for 5 hours. Individuals genotyped are indicated at the top and allele sizes are indicated to the left of the autoradiograph. The genotype for individual 30-47 was not scored from this autoradiograph.

4.1.7 Short tandem repeat marker UT7694 at locus D4S2688

Figure 4.7 displays a representative autoradiograph of the amplification products generated with this marker. All six alleles ranging from 246 to 266 bp were observed in this study of which five are presented in Figure 4.7. The primers of this marker were annealed at 55°C with the forward primer selected for end labelling.

A heterozygosity value of 52% was reported for marker UT7694. In this study, however, a relatively high level of heterozygosity was observed as displayed in Figure 4.7. No strand slippage products were generated with this marker and alleles could therefore be scored with ease.

Figure 4.7: Representative autoradiograph of marker UT7694



Autoradiograph of the amplification products generated with marker UT7694 described by Gerken *et al.* (1994). The forward primer was end labelled with γ^{32}P-dATP and the products electrophoresed on a 6% polyacrylamide gel in 1X TBE buffer for 3.5 hours. Individuals genotyped are indicated at the top and allele sizes are indicated to the left of the autoradiograph.

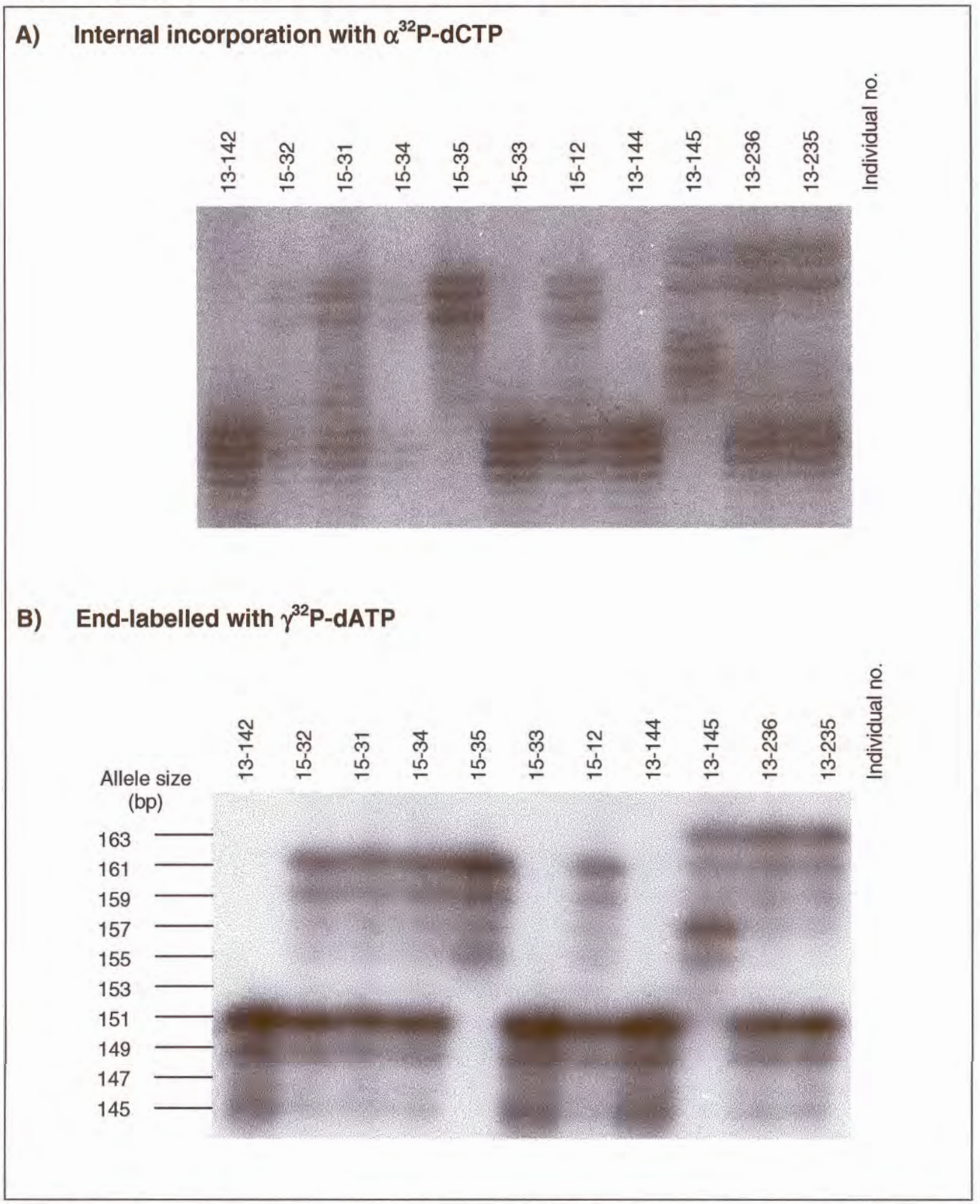
4.1.8 Short tandem repeat marker AFMa190zf5 at locus D4S2921

A representative autoradiograph of the alleles generated with this marker is displayed in Figure 4.8. As mentioned in paragraph 4.1.3 internal incorporation was at first utilised for the labelling of the amplification products and marker AFMa190zf5 was amplified in a multiplex PCR reaction with markers ATA22F02 and AFMa224xh1. Multiple strand slippage products were, however, observed which complicated the scoring of alleles (Figure 4.8A). For this reason end labelling of the primers was subsequently utilised for this marker.

The forward primer was end labelled and the marker was amplified in a standard PCR reaction. Although multiple strand slippage products were still visible after end labelling, these secondary products did not obscure the scoring of alleles. All eight of the reported alleles were observed, in the 100 individuals genotyped, of which four are presented in Figure 4.8B. Three additional alleles (147, 149 and 165 bp), not previously reported, were also observed in the families investigated but are not presented in Figure 4.8B. New markers are generally typed in a CEPH panel of individuals. It is therefore possible that alleles not previously reported in these panels, could be observed when the marker is

used in other populations, or in larger group of individuals. The possibility of observing new alleles in our unique population is therefore high.

Figure 4.8: Representative autoradiograph of marker AFMa190zf5

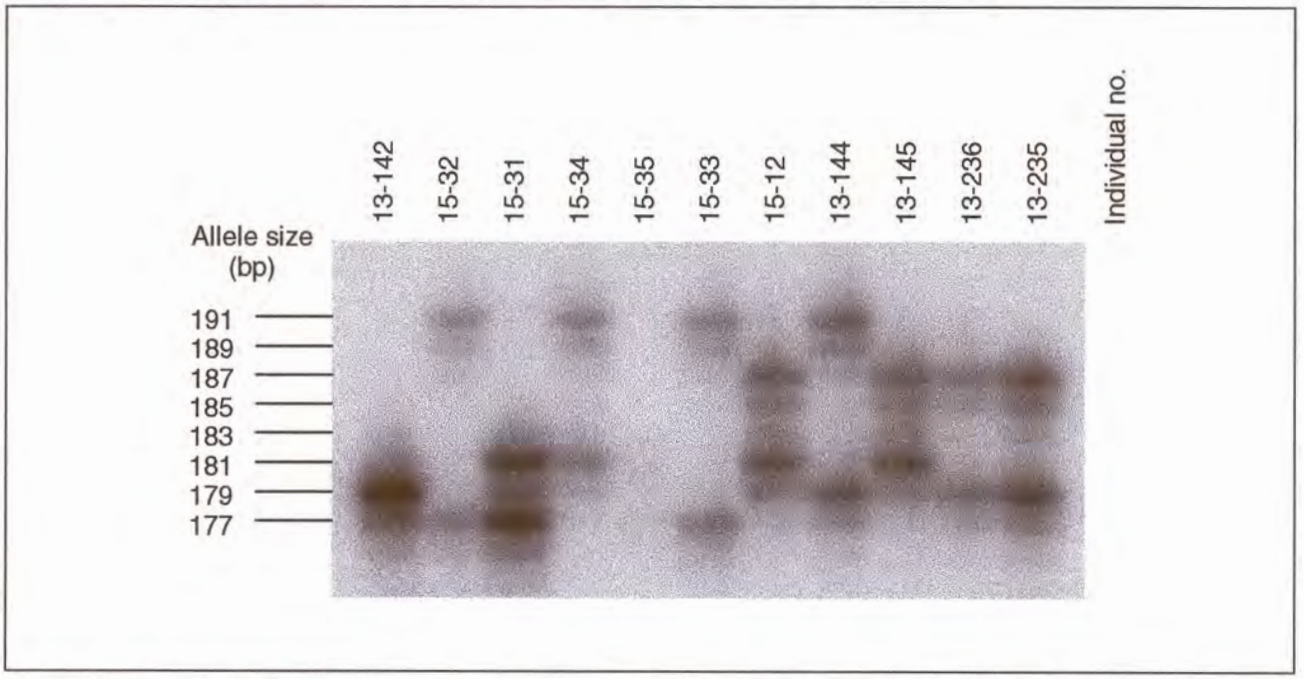


Autoradiographs of the amplification products generated with marker AFMa190zf5 described by Dib *et al.* (1996). A) = Internal labelling with α^{32}P-dCTP and the products electrophoresed on a 6% polyacrylamide gel in 1X TBE buffer for 2.5 hours. B) = The forward primer was end labelled with γ^{32}P-dATP and the products electrophoresed on a 6% polyacrylamide gel in 1X TBE buffer for 2.5 hours. Individuals genotyped are indicated at the top and allele sizes are indicated to the left of the autoradiograph.

4.1.9 Short tandem repeat marker AFM238ve3 at locus D4S426

The primers of this STRP marker were annealed at 55°C, with the forward primer selected for end labelling. Alleles generated with this marker are presented in Figure 4.9. This dinucleotide marker $(CA)_n$ generated six alleles ranging from 177-191 bp (excluding 183 bp and 185 bp). All six of the reported alleles were observed in the five families investigated. Five of the six alleles are presented in Figure 4.9, which display a representative autoradiograph for marker AFM238ve3. It is evident from the autoradiograph presented here that a secondary amplification product, 2 bp smaller than the true allele, was generated for each allele. This did, however, not obscure the scoring of the alleles. The genotype for individual 15-35 was scored from an autoradiograph that was exposed for a longer period of time.

Figure 4.9: Representative autoradiograph of marker AFM238ve3



Autoradiograph of the amplification products generated with marker AFM238ve3 described by Weissenbach *et al.* (1992). The forward primer was end labelled with γ^{32}P-dATP and the products electrophoresed on a 6% polyacrylamide gel in 1X TBE buffer for 2.5 hours. Individuals genotyped are indicated at the top and allele sizes are indicated to the left of the autoradiograph. The genotype for individual 15-35 was not scored from this autoradiograph.

Genotypes generated with the nine STRP markers could be scored with ease. Even though all the markers did not have high heterozygosity values they were all sufficiently informative for the construction of haplotypes in the group of families selected for this study.

4.2 SOUTHERN BLOT ANALYSIS

Southern blotting was performed to determine whether DNA rearrangements at the D4Z4 locus were present in the South African FSHD population. A Southern blot protocol was provided by Dr. Silvère van der Maarel (Department of Human Genetics, Leiden University Medical Centre, the Netherlands) [Lemmers *et al.*, 1998 and Church and Gilbert, 1984]. Previously, molecular studies had been performed for the proband of family F30 and two of his children at the Leiden University Medical Centre. This proband was utilised as a positive control for the optimisation and standardisation of Southern blot analysis.

The restriction endonucleases (*Eco* RI and *Bln* I) utilised for the whole genome digest did not have compatible buffers (Table 4.2) and two subsequent reactions had to be performed for each enzyme in its corresponding buffer. A protocol for a restriction enzyme buffer that was compatible with both enzymes was provided by Dr. van der Maarel for the double restriction endonuclease reaction and consisted of 5 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 10 mM NaCl supplemented with 3.3 mM spermidine. Ca. 75-85% complete digestion was achieved with this buffer. The preparation of the buffer, however, presented technical difficulties. A large volume of buffer had to be prepared to ensure measurable amounts of reagents, and resulted in the storage of large amounts of buffer, which would expire before it could be utilised. The preparation of this buffer was therefore not time or cost effective.

Table 4.2: Relative enzyme activity in Amersham buffers

Amersham buffer	L	M	H	K	T (+ BSA)
Eco RI	20[a]	100	100	120[a]	80[a]
Bln I	<20	20	40	100	20

L = low salt buffer, M = medium salt buffer, H = high salt buffer, K = potassium buffer, T = Tris acetate buffer, BSA = bovine serum albumin, a = weak star activity is detected. Adapted from restriction endonuclease product information sheet, Amersham (1996).

Subsequently the one-phor-all buffer supplied with restriction endonucleases from Amersham was utilised. The compatibility and an optimal 2X buffer concentration, to prevent star activity, with the use of *Eco* RI digestion reactions were previously determined by Amersham. The compatibility and concentration of the buffer for use with *Bln* I had, however, not previously been determined. Utilising the one-phor-all buffer at a 2X final

concentration with *Eco* RI and *Bln* I in a single restriction endonuclease reaction resulted in 100% complete digestion.

Very faint or even no fragments were visible on the autoradiograph after utilising the original Southern blot protocol (Lemmers *et al.,* 1998). The fragments of the molecular weight markers utilised to determine the sizes of the fragments were, however, visible on the autoradiograph. It was therefore evident that some conditions had to be adapted to enable the visualisation of the deletion fragments. Conditions that could be optimised were: 1) to ensure sufficient gDNA transfer, 2) fixation of the DNA onto the membrane, 3) temperature at which hybridisations were performed and 4) the stringency for washing of the membrane after hybridisation. Each of these aspects are discussed below.

Fragments larger than 20 kb cannot be transferred efficiently without nicking prior to transfer onto the membrane. In the original protocol the DNA fragments were nicked by UV irradiation at a wavelength of 312 nm as recommended. The transilluminator should be regularly calibrated as the dose emitted can vary with the age of the UV bulbs in the instrument. The management and control of the dose emitted by the transilluminator could therefore result in insufficient cleavage and incomplete gDNA transfer. The transilluminator that was available during this study was not calibrated and to exclude any variable factors, the DNA fragments were instead depurinated with 0.25 M HCl.

The original protocol utilised UV crosslinking for 4 minutes for the fixing of the DNA onto the nylon membrane. As stated above, the UV dose emitted is important to ensure optimal results. For the fixing of the DNA onto the membrane, baking in an oven at 80°C for 2 hours was performed instead.

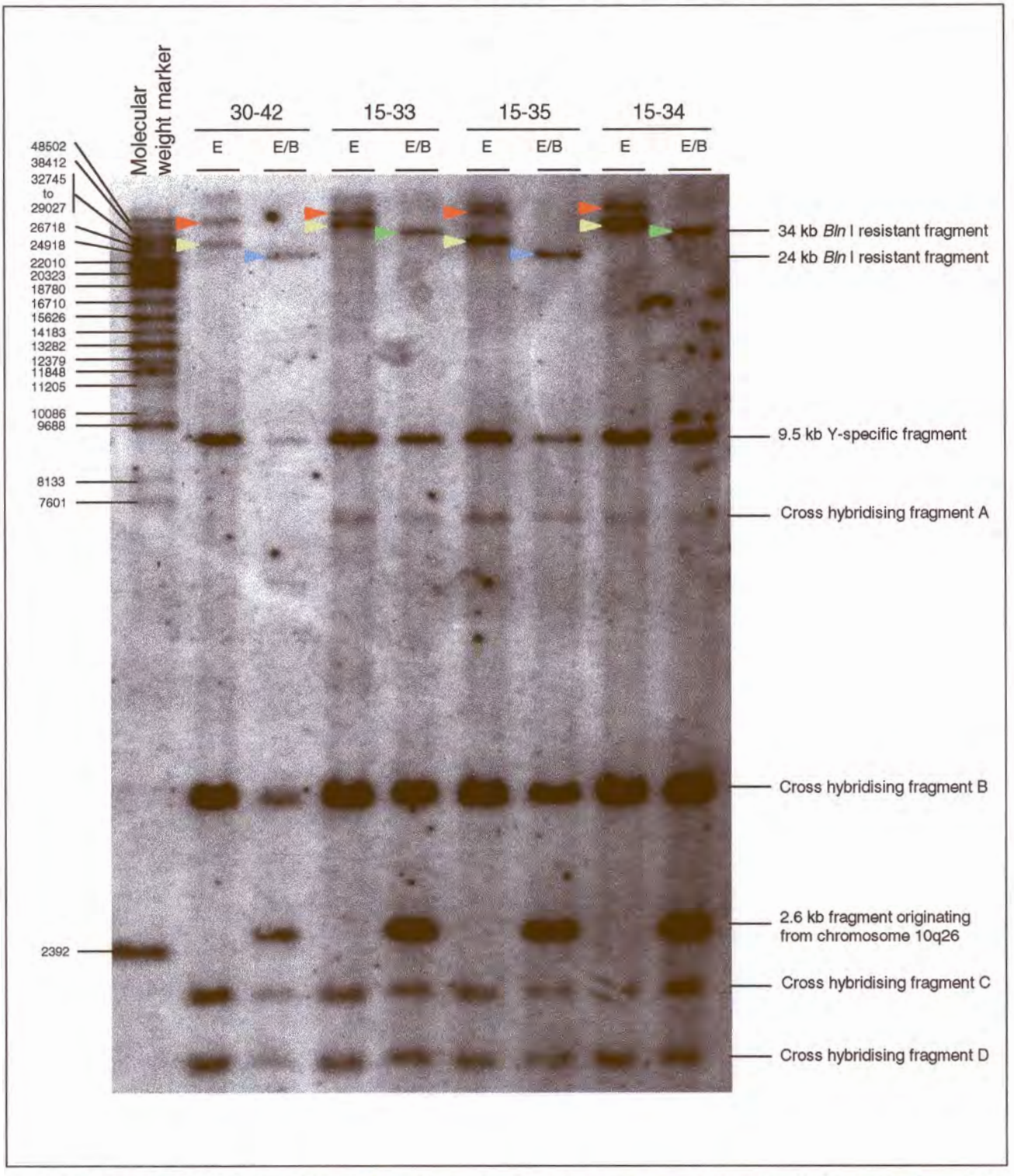
Hybridisations were performed at 65°C for 16 hours after which the membrane was washed with 2X SSC and 0.1% SDS (pre heated to 65°C) three times for 5 minutes. As previously stated, these conditions resulted in faint or no visible fragments after autoradiography. The hybridisation temperature and/or washing stringency were therefore too high and had to be lowered. The hybridisations were performed at 55°C for 16 hours and the membrane washed with 2X SSC and 1% SDS washing solution at 55°C. After autoradiography more fragments were visible, but much more background was also observed across the entire autoradiograph. This observation led to the conclusion that the stringency of the hybridisation conditions was too low. The next membrane was

hybridised at 65°C for 16 hours and washed with 2X SSC and 1% SDS washing solution at 65°C. Distinct deletion fragments as well as a Y-chromosome specific 9.5 kb fragment, a 2.6 kb *Eco* RI/*Bln* I fragment and other non-specific fragments were observed upon autoradiography. The Southern blot analysis was therefore optimised by utilising a ten times less stringent washing solution, thus at a stringency of 2X SSC and 1% SDS.

The same molecular weight markers that were loaded on the agarose gels were also random primed and hybridised with the membrane for precise sizing of the alleles. Twenty five ng of each molecular weight marker was labelled and different amounts (250 ng to 1 ng) were loaded onto the gels to determine the optimal concentration. The optimal concentration could, however, not be standardised. The signal intensity was either too intense, or no fragments were visible. The molecular weight markers were therefore end labelled instead. Fifty ng of each molecular weight marker was loaded per lane on the agarose gels and then hybridised with 500 ng of end-labelled molecular weight marker. Equal signal intensity to that of the fragments on the gels was obtained, and could be used for sizing of the fragments.

A representative autoradiograph of Southern blot analysis is displayed in Figure 4.10. Two different *Bln* I resistant deletion fragments of 24 kb and 34 kb, a 9.5 kb Y-chromosome specific fragment, a 2.6 kb chromosome 10q26 specific fragment, as well as several cross hybridising fragments are visible on this autoradiograph. The cross hybridising fragments can originate from homologous regions on the short arms of all the acrocentric chromosomes, as well as from chromosomes 1, 2, 3, 4, 10, 18, and Y, as mentioned in paragraph 2.1.2.2. The intensity of cross hybridising fragment A, just smaller than 7601 bp, is the lowest of the four cross hybridising fragments. It is evident from Figure 4.10 that all the fragments observed for individual 30-42 are of much lower intensity when compared to fragments observed in any of the other individuals. This might explain the absence of cross hybridising fragment A in individual 30-42. The dark specks visible in the lanes designated for individual 15-34 between 9.5 kb and 34 kb are due to artefact. It is evident that only samples from male individuals were included in Figure 4.10, since all of the samples displayed a 9.5 kb Y-specific fragment.

Figure 4.10: Representative autoradiograph of the optimised Southern blot analysis



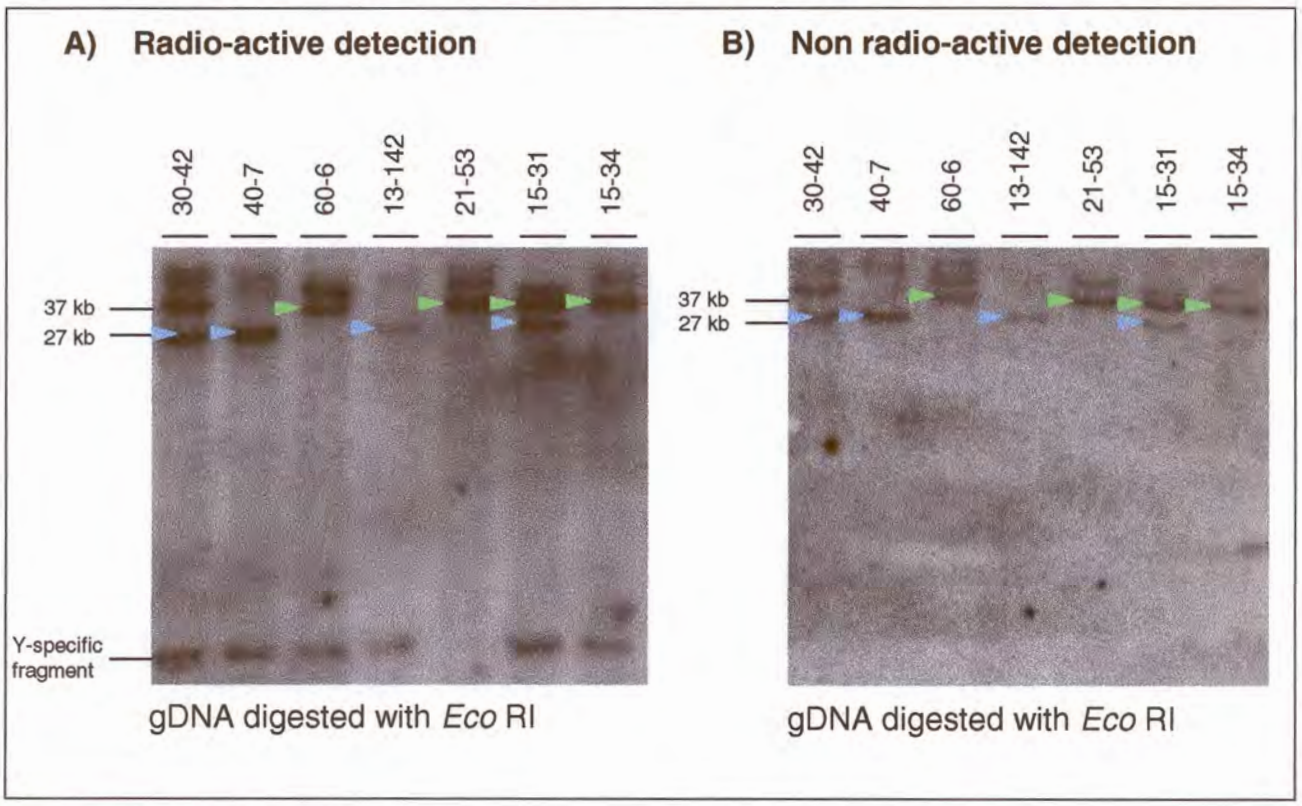
E = *Eco* RI , E/B = *Eco* RI/*Bln* I double digestion. = chromosome 10q26 fragment, = chromosome 4q35 fragment, = 24 kb *Bln* I resistant deletion fragment verifying chromosome 4q35 origin, = 34 kb *Bln* I resistant deletion fragment verifying chromosome 4q35 origin.

4.3 RADIO-ACTIVE VERSUS NON RADIO-ACTIVE DETECTION

Standard diagnosis for FSHD worldwide is Southern blot analysis utilising a radio-active labelled p13E-11 probe. The Detector™ Random Primer DNA biotinylation kit as well as

the DNADetector™ Genomic Southern Blotting kit (KPL) were utilised to standardise a non radio-active detection method for FSHD. These kits were subsequently tested on the standard optimised Southern blot protocol utilised for the radio-active detection and the results obtained are presented in Figure 4.11. In this figure two identical Southern blots are presented with different detection strategies. The non radio-active method detected the same *Eco* RI deletion fragments as the radio-active method. However, the Y-specific fragment was not detected with the non radio-active protocol. An explanation for this is that the non radio-active protocol utilised a much more stringent wash condition. The radio-active membrane was washed 3 times after hybridisation for 5 minutes in a 2X SSC/1% SDS wash solution, while the non radio-active membrane was washed twice for 15 minutes each in 2X SSPE/0.1% SDS followed by two washes for 15 minutes in 0.2X SSPE/0.1% SDS and subsequently in 2X SSPE washing solution for 5 minutes. The first wash of the non radio-active protocol (2X SSPE/0.1% SDS) is therefore a ten times more stringent solution than that utilised for the radio-active protocol. It is thus evident that with further optimisation the non radio-active detection protocol can be utilised in the detection of deletion fragments in FSHD. The radio-active protocol was utilised for the molecular diagnosis performed in this study.

Figure 4.11: Comparison between radio-active and non radio-active detection



= 37 kb *Eco* RI deletion fragments and;

= 27 kb *Eco* RI deletion fragments.

4.4 MOLECULAR ANALYSIS OF SPECIFIC FSHD FAMILIES

Markers described in paragraph 4.1 were utilised to generate genotypes for the individuals selected for this study. The genotypes generated with the nine STRP markers and the Southern blots were combined in order to elucidate the molecular basis of FSHD in the South African population. The haplotypes are indicated by coloured blocks, as indicated in Figures 4.12, 4.14, 4.15, 4.16 and 4.17, which encompass the specific allele sizes of the different markers included in the haplotype. The combined genotype analyses of the selected families are presented in the following paragraphs.

4.4.1 FSHD family F10

Family F10 was subdivided into five sub-families (F11, F12, F13, F14 and F15) for easy reference as described in paragraph 3.1.1. Twenty five individuals from family F10 were genotyped with the nine STRP markers and Southern blot marker p13E-11 as discussed in paragraphs 4.1 and 4.2 respectively. Five of the individuals are related to their respective families by marriage. Of the remaining twenty individuals, ten were clinically phenotyped to be FSHD positive, six were phenotyped to be FSHD negative and four were equivocal on a clinical level. Haplotypes for all of these individuals are presented in Figure 4.12.

The exact order of the nine STRP markers utilised in this study has not yet been mapped (Table 3.2). It is evident from Table 3.2 that marker UT1366 (locus D4S1523) is the most distal, with markers AFM238ve3 (locus D4S426) and AFMa190zf5 (locus D4S2921) being the most proximal. The exact order of the remaining six markers has not been determined. Several recombination events within this family, however, facilitated in determining the order of these markers. As indicated in Figure 4.12, recombination events in the ancestors of individuals 14-64, 14-102, 13-142, 13-144, indicated that marker UT7694 (locus D4S2688) is distal to the markers at loci D4S426 and D4S2921 and recombination events in the ancestors of individuals 12-3 and 12-6 indicated that the marker at locus D4S2390 (ATA22F02) is distal to marker UT7694 (locus D4S2688). The recombination event observed in the ancestors of individual 11-28 led to the conclusion that marker UT5785 (locus D4S2299) is distal to the marker at locus D4S2390 and that the marker at locus D4S2930 is distal to the marker at locus D4S2299. Finally the recombination event in individual 15-34 facilitated the mapping of marker UT2219 (locus D4S2283) proximal to marker UT1366 (locus D4S1523) and distal to marker AFMa224xh1 (locus D4S2930), mapping the last marker GATA5B02 (locus D4S1652) distal to marker

AFMa224xh1 and proximal to marker UT2219. The only two markers that could not be mapped through haplotype analysis in family F10 were markers AFM238ve3 (locus D4S426) and AFMa190zf5 (locus D4S2921). These two markers were, however, mapped by a recombination event in another South African family (F40). In this family, individual 40-25, as illustrated in Figure 4.16, displayed recombination allowing marker AFMa190zf5 (locus D4S2921) to be mapped proximal to marker AFM238ve3. The order in which the nine STRP markers utilised in this study, presented in the haplotypes, is thus as follows: TEL - UT1366 (D4S1523), UT2219 (D4S2283), D4S1652 (GATA5B02), AFMa224xh1 (D4S2930), UT5785 (D4S2299), ATA22F02 (D4S2390), UT7694 (D4S2688), AFM238ve3 (D4S426) and AFMa190zf5 (D4S2921) - CEN, as indicated in Figure 4.12.

In this family the 297-342-138-217-223-111-246-181-161 haplotype (indicated in the blue block in Figure 4.12), or a portion of the haplotype, was present in sixteen individuals from the five sub-families (F11, F12, F13, F14 and F15). This haplotype survived transmission through 32 meiotic events spanning eight generations. Upon Southern blot analysis, a *Bln* I resistant deletion fragment of 24 kb (3 kb shorter than the 27 kb *Eco* RI deletion fragment) was observed to segregate in this family. All of the individuals who inherited the haplotype marked in blue also inherited this 24 kb deletion fragment.

Nine of the ten clinically affected individuals (15-12, 15-31, 11-28, 11-30, 12-6, 13-142, 13-144, 14-64 and 14-102) inherited the FSHD associated haplotype and the 24 kb deletion fragment. Individual 14-113 did, however, not inherit the haplotype or the deletion fragment that segregated in this family. This individual was clinically diagnosed in his late fifties, an age at which the phenotype should have been fully penetrant. He was, however, not diagnosed by Dr. Schutte, a specialist in FSHD, and might therefore have been mis-diagnosed, as the clinical signs are often subtle and difficult to identify. Re-evaluation of individual 14-113, is therefore pertinent.

The four individuals diagnosed to be equivocal on a clinical level (12-3, 12-10, 12-40, and 13-235) all inherited the "blue" haplotype as well as the 24 kb deletion fragment. The FSHD status of these individuals was therefore confirmed upon molecular analysis. Two of the clinically unaffected individuals (15-35 and 13-236) also inherited the blue haplotype and the deletion fragment. These individuals are still younger than twenty-one, the age at which 95% of affected individuals generally start to show clinical symptoms. Upon clinical examination they could therefore still have been asymptomatic. Individuals 15-35 and 13-236 should also be clinically re-evaluated. It is possible that they could have been

asymptomatic at the time of analysis, but developed the symptoms associated with FSHD since that time.

Two *Bln* I resistant deletion fragments (24 kb and 34 kb) were observed for individual 15-31 upon Southern blot analysis as presented in Figure 4.13 (Van der Merwe *et al.*, 2001a). Both the deletion fragments are in the disease causing range, and being *Bln* I resistant, behave as chromosome 4-type fragments. This would imply that this individual is a compound heterozygote. It is, however, evident from the molecular data generated for family F10, that the 24 kb deletion fragment segregates with the FSHD phenotype in this family, and is therefore a true chromosome 4 type fragment. The chromosomal origin of the 34 kb deletion fragment was, however, not conclusive, since it is known that translocations occur between chromosome 4 and chromosome 10 type fragments. As depicted in Figure 4.13, individual 15-35 inherited the 24 kb deletion fragment as well as the "blue" haplotype from his father (15-31). His brothers, individuals 15-33 and 15-34, both inherited the 34 kb deletion fragment together with the distal portion of the "green" haplotype. A 9.5 kb Y-specific fragment is visible for all the male individuals, while this fragment is absent in the one female individual, 15-32, presented in Figure 4.13.

Verification of the chromosomal origin was therefore crucial for accurate molecular diagnosis of these two children, individuals 15-33 and 15-34. Haplotype analysis on chromosome 10q was performed in a different study[1] and confirmed that the 34 kb deletion fragment originated from chromosome 4 (Olckers *et al.*, 2001; Van der Merwe *et al.*, 2001b). Upon further analysis it was observed that the 34 kb deletion fragment as well as the haplotype indicated in green observed in this individual and his two children (15-33 and 15-34) also segregated in a second FSHD family, F20 (paragraph 4.3.2). The chromosomal origin of the 34 kb deletion fragment was therefore confirmed via chromosome 4 as well as chromosome 10 haplotype analyses. Individuals 15-33 and 15-34 were clinically phenotyped in their mid-teens to be unaffected but molecular analysis indicated that both individuals inherited the second disease causing deletion fragment (34 kb). These individuals might have been asymptomatic upon clinical examination, and it is thus critical that they be clinically re-evaluated at an appropriate age in the future.

[1] Chromosome 10q26 haplotype analysis performed by M. Alessandrini.

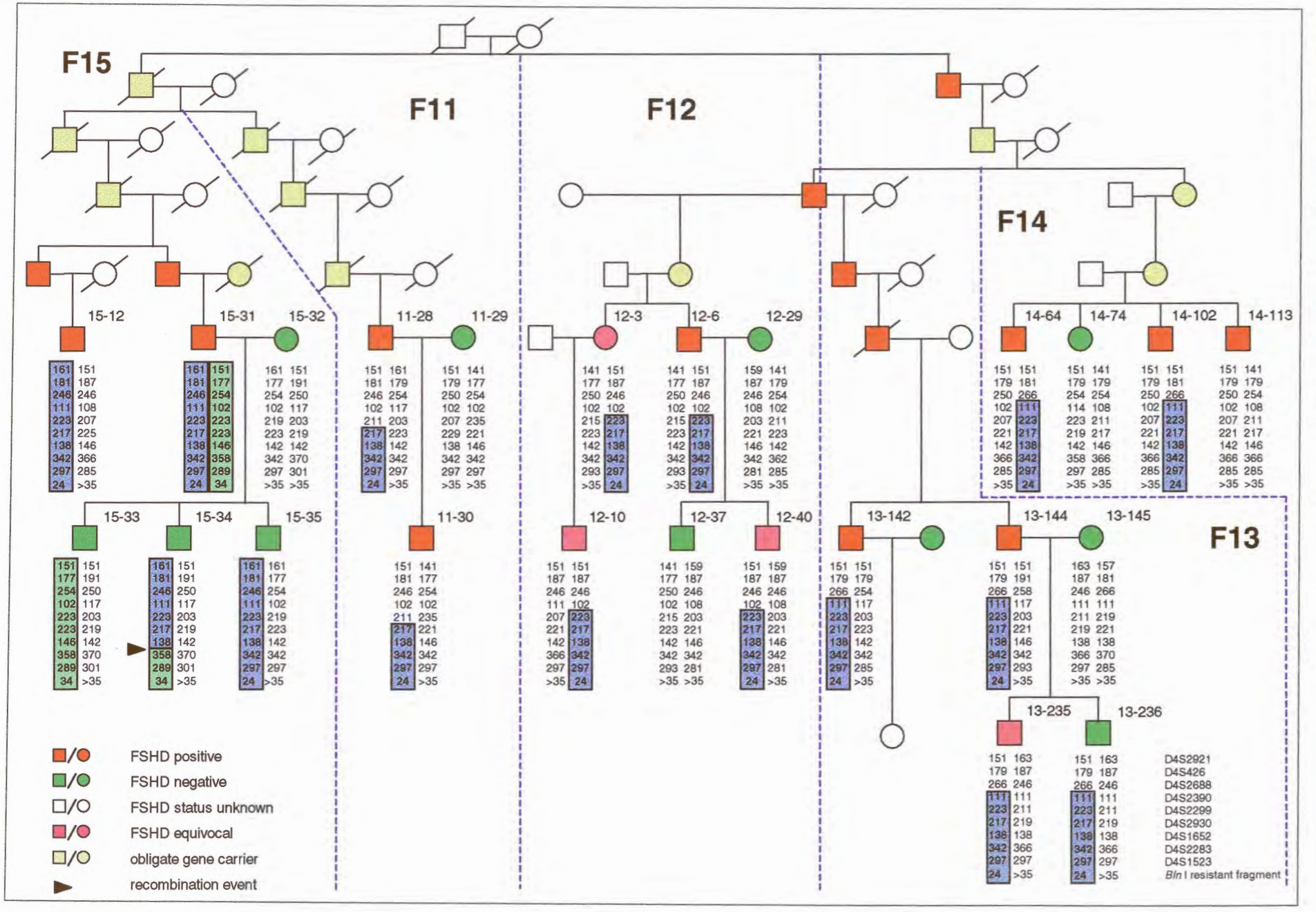
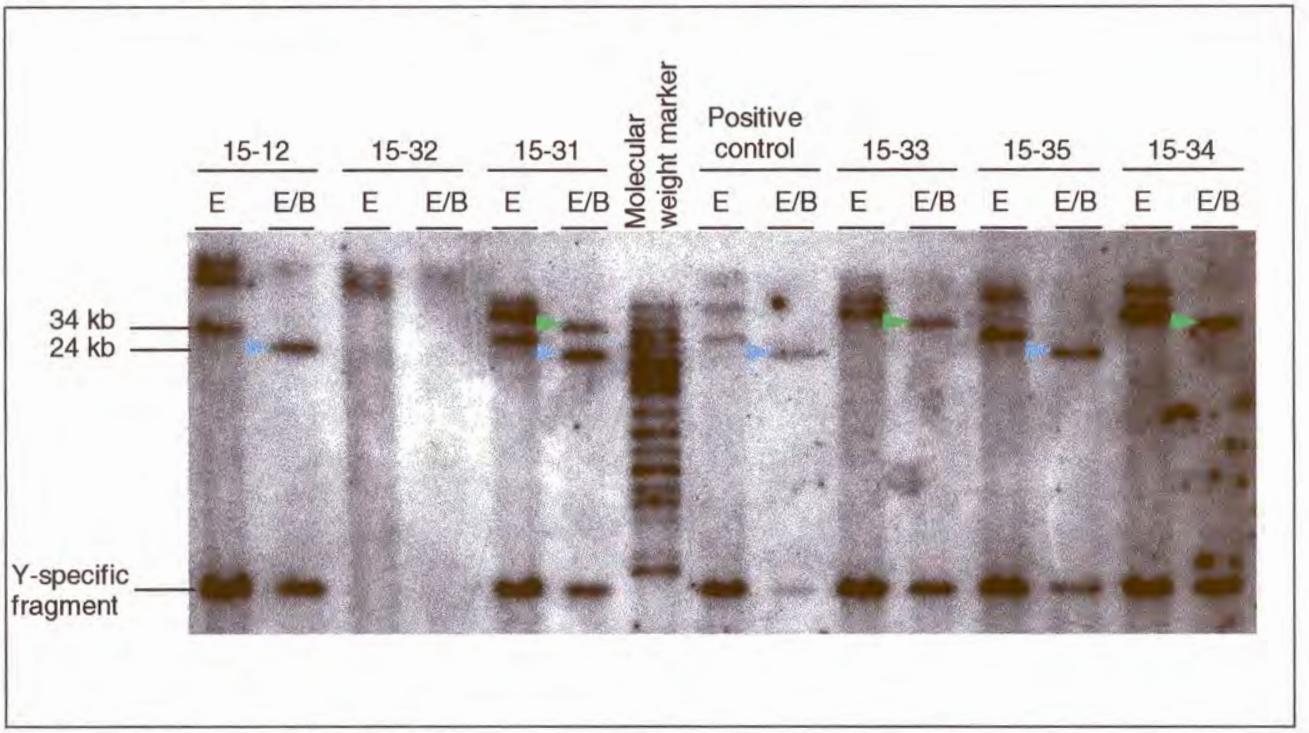


Figure 4.12: Haplotypes of selected individuals from family F10

Figure 4.13: Southern blot analysis for family F15



= 34 kb *Bln* I resistant deletion fragment;

= 24 kb *Bln* I resistant deletion fragment.

Several recombination events were observed within family F10 and facilitated in determining the order of seven of the nine STRP markers utilised in this study. FSHD could be confirmed via the presence of a *Bln* I resistant deletion fragment of 24 kb that segregated in family F10. The first South African compound heterozygote individual (15-31) for FSHD was observed in this family, as identified by the presence of two *Bln* I resistant deletion fragments. This might be useful in the elucidation of the phenotypic-genotypic correlation in FSHD due to the presence of two different sizes of *Bln* I resistant deletion fragments within one family. If the phenotype can be correlated with the genotype, the father, being a compound heterozygote, should be severely affected and the son with the 24 kb deletion fragment ought to be more severely affected than his brothers harbouring the 34 kb deletion fragments. The clinical diagnosis of these individuals is therefore essential to elucidate the correlation between the phenotype and the genotype.

4.4.2 FSHD family F20

Of the twenty five individuals genotyped from the three generations in this family, eleven were FSHD positive, ten were FSHD negative, three were phenotyped to be equivocal on a clinical level, and the FSHD status of one individual was unknown. A haplotype

indicated on a green background in Figure 4.14 was observed to segregate in family F20. As mentioned in paragraph 4.3.1 this haplotype was also observed in individuals 15-31, 15-33 and 15-34.

The proband of this family (21-55) inherited the TEL-289-358-146-223-223-102-CEN haplotype from her clinically affected mother (21-53). Four other FSHD positive individuals 21-60, 21-67, 21-80 and 21-33 also inherited this haplotype. The haplotype was also observed to segregate in two of the FSHD negative individuals (21-24 and 21-35), two of the individuals who were found to be equivocal on a clinical level (21-28 and 21-30) and in individual 21-26, whose FSHD status was unknown prior to this study. No haplotype was observed in individuals 21-65, 21-69, 21-71, 21-75 and 21-76 who were clinically phenotyped to be affected, or in individual 21-66 who was diagnosed as clinically equivocal. Haplotypes, or portions thereof, were inferred for those individuals (21-116, 21-117, 21-84, 21-52, 21-22, 21-54 and 21-68) of whom blood samples were not available. The haplotype observed to segregate in this family survived transmission through 12 meiotic events spanning four generations.

Southern blot analysis detected the presence of a 37 kb *Eco* RI deletion fragment in all of the individuals who inherited the FSHD associated haplotype. This 37 kb *Eco* RI fragment was reduced by 3 kb resulting in a 34 kb *Bln* I resistant deletion fragment. Genotype-phenotype discordance was, however, observed for individuals 21-65, 21-66, 21-69, 21-71, 21-75 and 21-76 who did not inherit this 34 kb deletion fragment. All of these individuals were younger than twenty upon clinical examination. As pointed out in family F10 the clinical diagnosis of individuals before the age of twenty is complex and may be misleading, and for this reason these individuals need to be re-examined on the clinical level.

As mentioned previously no 34 kb *Bln* I resistant deletion fragment was observed in individuals 21-69 and 21-75. However, Southern blot analysis revealed a 37 kb *Eco* RI deletion fragment in both individuals. The 37 kb *Eco* RI deletion fragment was, however, completely digested with *Bln* I, thus displaying a chromosome 10 restriction endonuclease pattern. The Southern blot analysis of individual 21-75 was repeated twice, but the 37 kb *Eco* RI fragment was *Bln* I sensitive in both analyses. It is therefore highly unlikely that

this could have been due to experimental errors. The possibility of a translocation event is also unlikely but needs to be excluded with either PFGE or the *Bgl* II/*Bln* I dosage test, as all four alleles as well as the presence of translocations will be visible via these methods of analysis.

The 146 bp allele for marker GATA5B02 at locus (D4S1652) was observed to segregate in the haplotype indicated in green. However, individual 21-28, who displays the haplotype, inherited a 150 bp allele. The son of individual 21-28 (21-30) inherited the haplotype with this 150 bp allele from his father. The genotypes of these individuals (21-28 and 21-30) were repeated multiple times, with identical results. The father of individual 21-28 could not be genotyped as he was deceased before a sample could be obtained. If the father had a 150 bp allele at locus D4S1652, individual 21-28 could have inherited this allele due to a recombination event between loci D4S2930 and D4S1652, and loci D4S1652 and D4S2283. This is, however, very unlikely due to the short genetic distances between these markers. The mutation rate of tetranucleotides was reported to be as high as $1.8x10^{-3}$ (Xu *et al.*, 2000). It is therefore most likely that the 146 bp allele mutated to a 150 bp allele in individual 21-28 as both his brothers (21-24 and 21-33) inherited a 146 bp allele from their father and the mother of individual 21-28 (21-23) does not harbour a 150 bp allele at this locus.

A different *Bln* I resistant deletion fragment and FSHD associated haplotype were observed to segregate in family F20 than in family F10. This indicated the presence of at least two *Bln* I resistant deletion fragments and FSHD associated haplotypes in the South African FSHD population. Although FSHD could be confirmed on a molecular level for family F20, genotype-phenotype discordance was, however, also observed. The individuals displaying discordance were all clinically diagnosed before the age of 20 and should therefore be re-examined as they could have developed symptoms since their last examination.

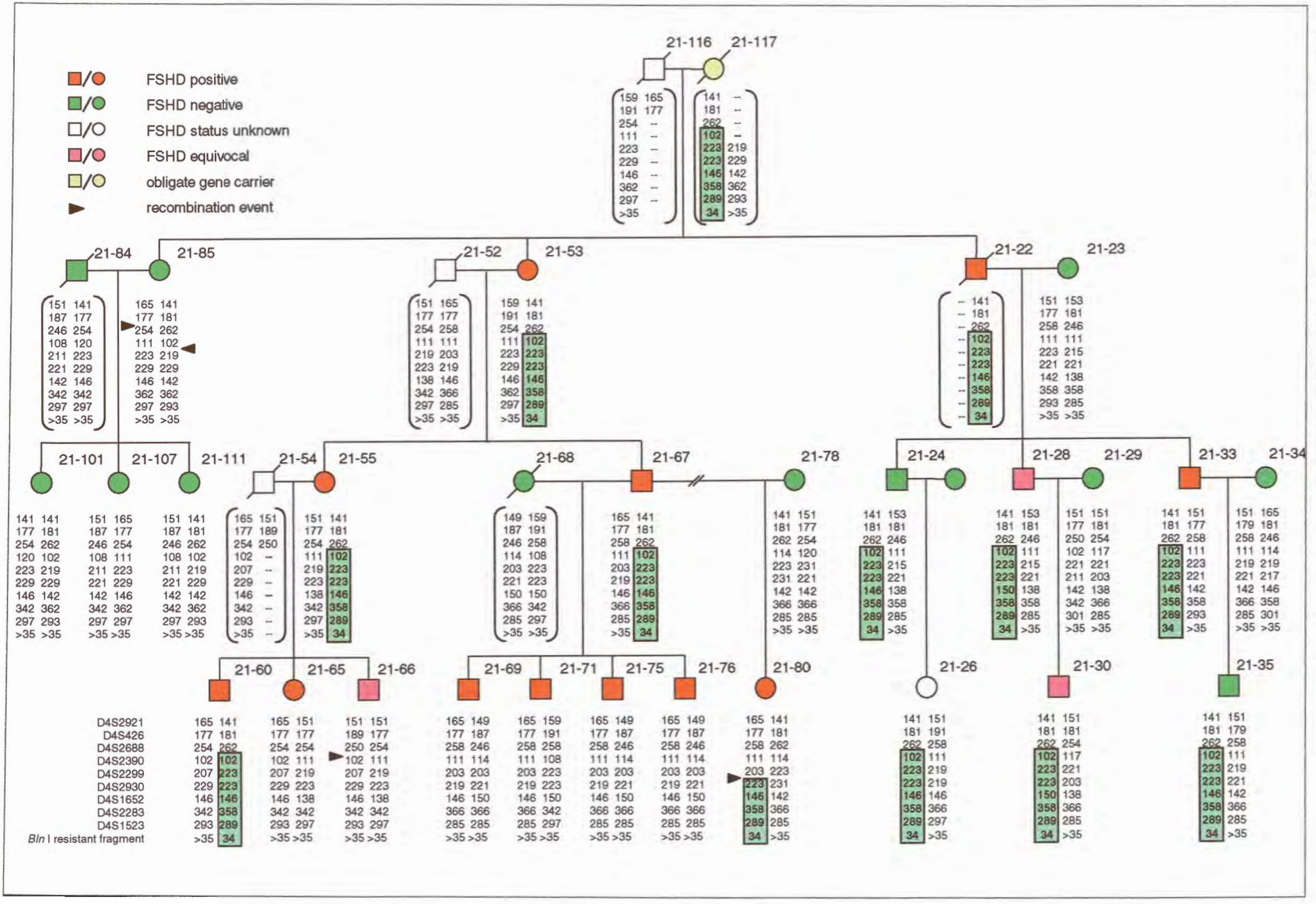


Figure 4.14: Haplotypes of selected individuals from family F20

4.4.3 FSHD family F30

Thirty three individuals were genotyped in family F30. Three of the individuals are only related to their respective families by marriage and of the remaining thirty individuals, twelve were clinically diagnosed to be FSHD positive, sixteen were diagnosed to be negative on a clinical level and two were equivocal upon clinical examination. Haplotypes for the thirty three individuals are presented in Figure 4.15.

The FSHD associated haplotype, indicated in blue in Figure 4.12, which segregated in family F10 was also observed to segregate in family F30. The entire haplotype, consisting of all nine STRP markers, that was observed in sub-family F15 also segregated in family F30. The haplotypes were deduced for those individuals (30-1, 30-2, 30-111 and 30-108), of whom blood samples were not available. Only a portion of the haplotypes of individuals 30-30 and 30-210 could be deduced as their children inherited the sample haplotype in this region. This haplotype survived transmission through 14 meiotic events spanning four generations. Nine of the twelve clinically affected individuals inherited this FSHD associated haplotype and the remaining three individuals, 30-48, 30-104 and 30-106 as well as two individuals (30-103 and 30-193) who were equivocal on the clinical level did not inherit this haplotype.

Four clinically unaffected individuals (30-3, 30-147, 30-135 and 30-46) also inherited the FSHD associated haplotype. Individual 30-3 was in his nineties upon clinical examination, individual 30-147 in his late seventies and individual 30-135 in her mid forties. These individuals were therefore clinically diagnosed at an age at which the FSHD phenotype was expected to have been fully penetrant, yet they displayed no clinical FSHD phenotype and were in fact diagnosed as asymptomatic. Individual 30-46 was clinically diagnosed to be unaffected in his mid twenties, the age at which the phenotype is reported to be 95% penetrant. This individual might, however, still have been asymptomatic by that age, and should therefore be clinically re-evaluated in the future, as symptoms might have developed since his last clinical evaluation. Molecular data therefore indicated that the four clinically unaffected individuals, 30-3, 30-147, 30-135 and 30-46, are FSHD positive on a genetic level. Genetic counselling is therefore essential for these clinically asymptomatic individuals who are treated as affected individuals, since their molecular diagnosis was confirmed to be positive.

An *Eco* RI deletion fragment of 27 kb was observed to segregate in this family. The chromosome 4 origin of this deletion fragment was verified with a *Bln* I digestion. The

Eco RI deletion fragment was reduced by 3 kb, resulting in the presence of a 24 kb *Bln* I resistant deletion fragment. All the individuals who inherited the FSHD associated haplotype, indicated in blue in Figure 4.15, inherited the 24 kb *Bln* I resistant deletion fragment. Individual 30-48 who was phenotyped to be FSHD positive, but who did not inherit the FSHD associated haplotype did, however, inherit the 24 kb *Bln* I resistant deletion fragment from his clinically affected father. This is the result of a recombination event between the D4S1523 and D4Z4 loci and therefore argues against the use of haplotype analysis alone for the diagnosis of FSHD and confirms the importance of Southern blot analysis.

Individuals 30-104 and 30-106 were clinically diagnosed with FSHD but did not inherit the haplotype or the 24 kb *Bln* I resistant deletion fragment. Both of them were clinically examined before the age of 20 and will have to be re-examined. Phenotype-genotype discordance was observed in families F10, F20 and F30. It was, however, evident that the discordant individuals were generally present in the youngest generation. The discordance might therefore be due to clinical bias as the clinical phenotype is extremely heterogeneous and complex, especially in young children. The clinical diagnosis by a clinician specialised in FSHD is therefore essential and it will be optimal if all the individuals could be diagnosed by one clinician.

Genomic DNA from individual 30-3 was amplified with the nine STRP markers described in paragraph 4.1. Individual 30-3 inherited the 297-342-138-217-223-111-246-181-161 haplotype, indicated in a blue block in Figure 4.15, from his mother. Three of the nine markers utilised in this study (D4S2930, D4S2390 and D4S2921), generated alleles that were incompatible with Mendelian segregation of alleles from his father (individual 30-1). These markers were scored again to confirm that the discrepancies observed were not due to scoring errors. It is also possible that incorrect labelling of the original tubes of whole blood might have resulted in the observed discrepancies. To eliminate this possibility, a fresh blood sample should be obtained from individual 30-3 to re-type these markers. If the alleles indicated in Figure 4.15 for individual 30-3 is, however, correct it would seem that non-paternity is indicated between individuals 30-1 and 30-3.

Via molecular analysis the presence of the same size *Bln* I resistant deletion fragment, of 24 kb, and FSHD associated haplotype was confirmed in families F10 and F30. The presence of a recombination event between the most distal STRP marker and locus D4Z4, observed in individual 30-48, highlighted the fact that Southern blot analysis is essential in the molecular diagnosis of FSHD.

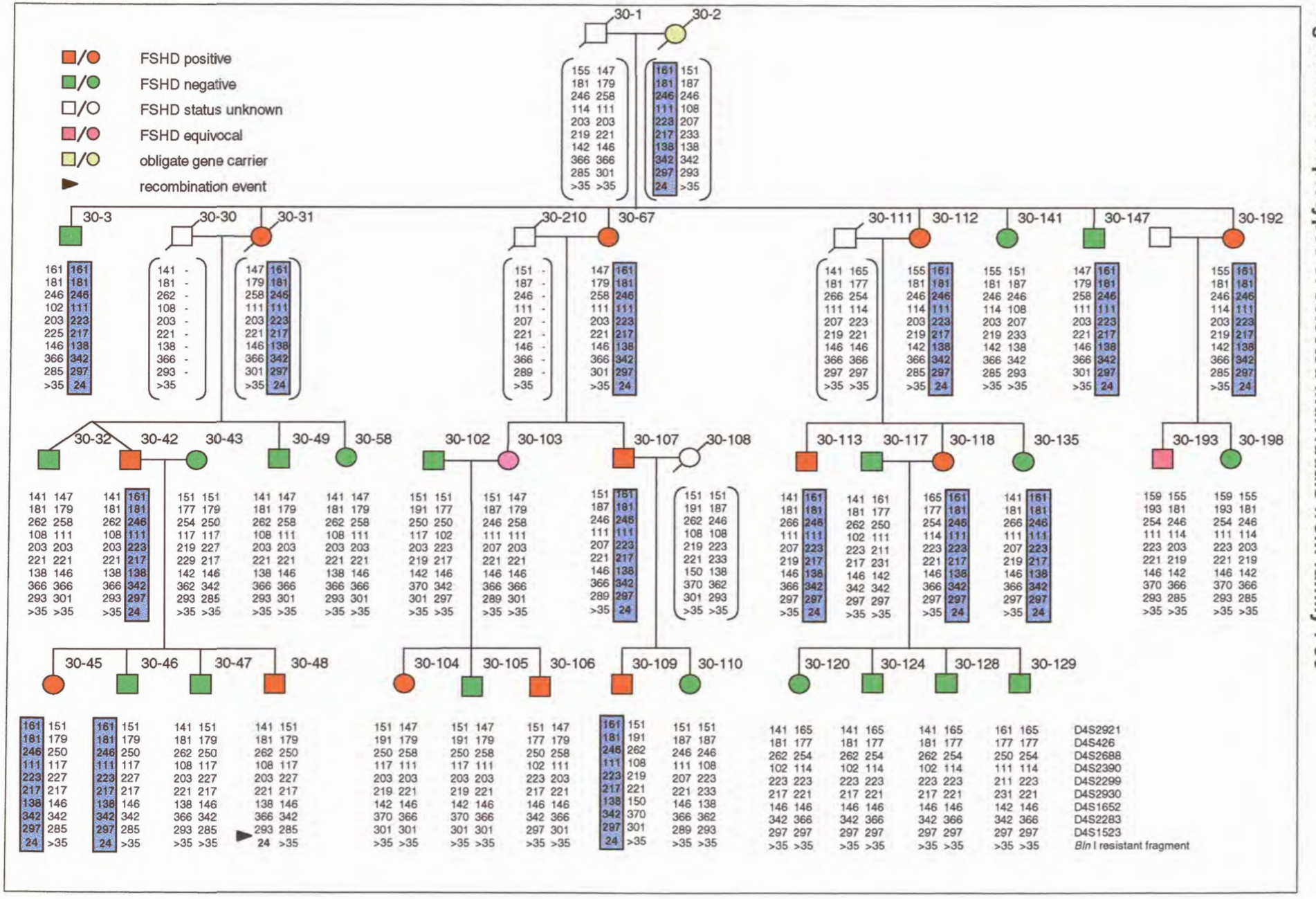


Figure 4.15: Haplotypes of selected individuals from family F30

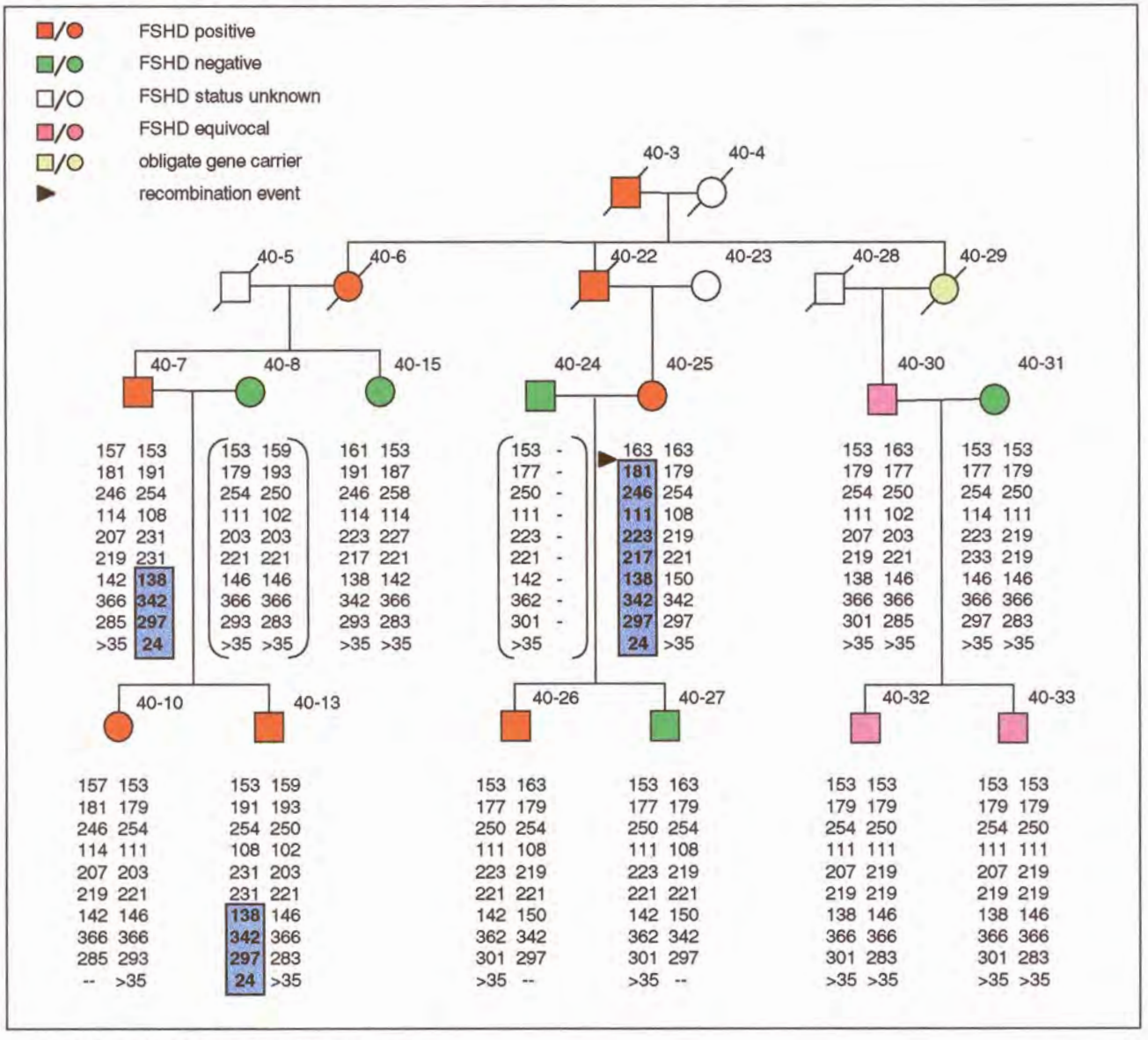
4.4.4 FSHD family F40

This family consists of 40 individuals of whom 11 were genotyped. Five of these individuals were FSHD positive, three were FSHD negative, and three were diagnosed to be equivocal on a clinical level.

Haplotypes of these 11 individuals are presented in Figure 4.16. The haplotypes were inferred for those individuals (40-8, and 40-24) of whom blood samples were not available. Only a portion of the haplotype of individual 40-24 could be inferred, as both his children inherited the same haplotype in this region. The FSHD associated haplotype segregating in family F40 is identical to the haplotype observed to segregate in families F10 and F30. As mentioned in paragraph 4.4.1 the recombination event observed in individual 40-25 facilitated elucidation of the exact order of the STRP markers. Individuals 40-7 and 40-13 inherited only a portion of the FSHD associated haplotype, including three markers, and confirmed the STRP marker order in that region. Individuals 40-10 and 40-26 who were phenotyped to be FSHD positive and the three equivocal individuals (40-30, 40-32 and 40-33) did, however, not inherit the FSHD associated haplotype.

Identical to families F10 and F30, a 24 kb *Bln* I resistant deletion fragment was observed to segregate in this family. The three individuals who inherited the FSHD associated haplotype all inherited the 24 kb *Bln* I resistant deletion fragment. The proband of family F40, individual 40-10, was clinically diagnosed in 1999 to be severely affected. Upon Southern blot analysis no *Bln* I resistant deletion fragment was observed in this individual. However, she did inherit a 27 kb *Eco* RI deletion fragment, the same as in her affected father and brother, albeit a *Bln* I sensitive fragment. The Southern blot analysis of individual 40-10 was repeated four times, but no *Bln* I resistant deletion fragment could be detected. The same scenario was observed for individuals 40-26 and 40-27. The 27 kb *Eco* RI deletion fragment observed in these three individuals, 40-10, 40-26 and 40-27, therefore displayed a chromosome 10 restriction endonuclease pattern. To exclude chromosome 4 or 10, a *Xap* I digestion needs to be performed in the future. If the fragment originates from chromosome 4 it should be *Xap* I sensitive, and *Xap* I resistance will designate it as a fragment from chromosome 10. The possibility of a translocation event is not very likely, but it can be excluded with either the *Bgl* II/*Bln* I dosage test or PFGE analysis.

Figure 4.16: Haplotypes of selected individuals from family F40



-- = no *Bln* I resistant deletion fragment was detected.

The same *Bln* I resistant deletion fragment of 24 kb and FSHD associated haplotype, indicated in blue in Figure 4.16, was observed to segregate in families F10, F30 and F40, indicating the presence of a common ancestor for these families. The presence of *Bln* I sensitive fragments in clinically affected individuals (40-10, 40-26 and 40-27) confirmed the importance of PFGE in the diagnosis of FSHD on a molecular level. This will enable the visualisation of all four alleles, two from chromosome 4 and two from chromosome 10, at the *D4Z4* locus.

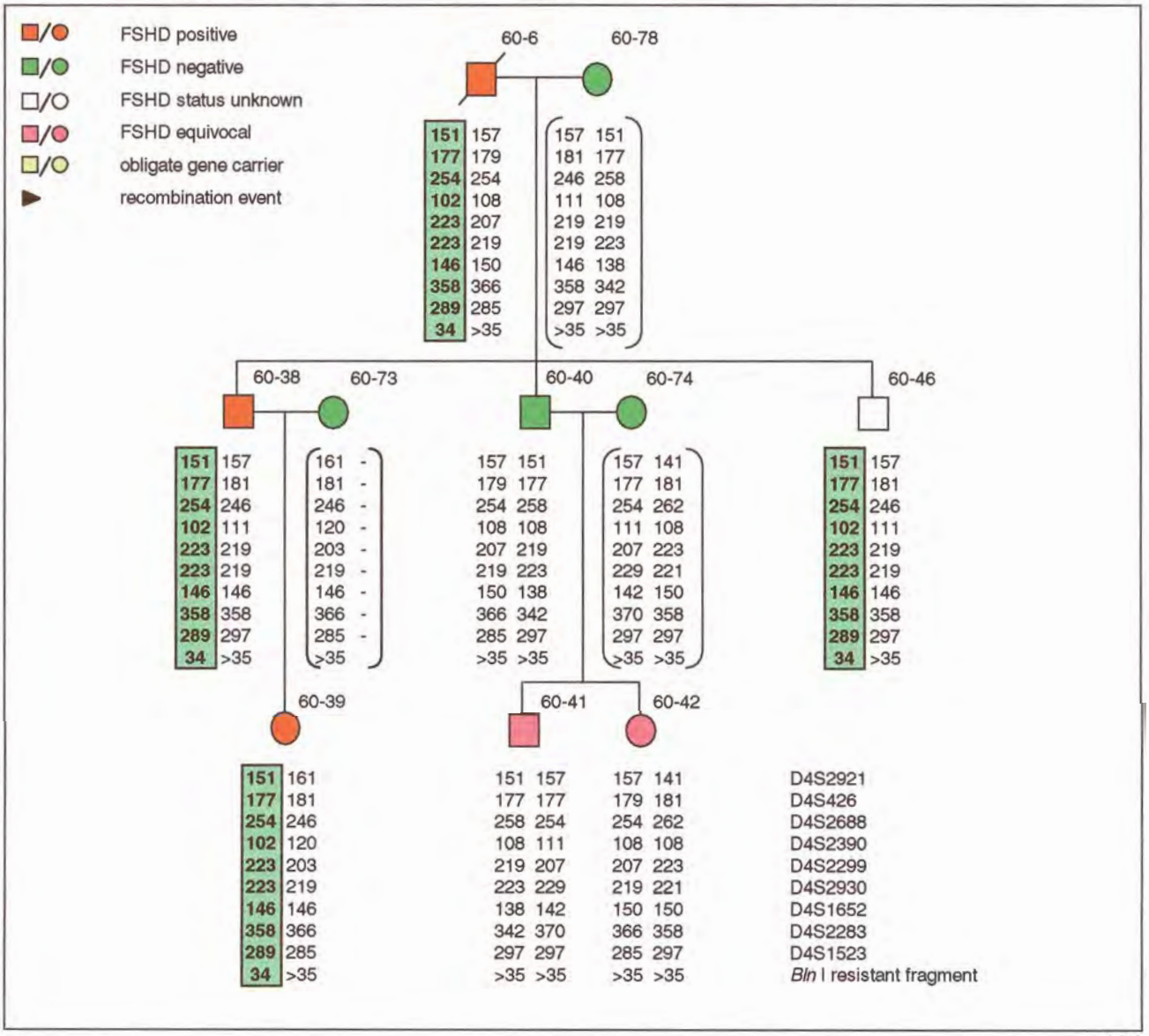
4.4.5 FSHD family F60

The core family selected for this study from family F60 included three FSHD positive, one FSHD negative, and three individuals of whom the clinical diagnosis was unknown. Figure 4.17 presents haplotypes for ten individuals from this family who were genotyped at

nine loci with the STRP markers discussed in paragraph 4.1. Deduced haplotypes are indicated for individuals 60-78, 60-73 and 60-74, of whom blood samples were not available. Only a portion of the haplotype of individual 60-73 could, however, be deduced, as only one child was genotyped.

The same FSHD associated haplotype (indicated in green in Figures 4.13 and 4.14) observed in family F20 and individuals 15-31, 15-33 and 15-34, from family F10, was observed to segregate in family F60. It is, however, evident from Figure 4.14 that only the six distal markers of the haplotype observed in family F20 are identical to the haplotype observed in family F60, whereas families F10 and F60 have the entire haplotype of nine markers in common. The three affected individuals (60-6, 60-38 and 60-39) as well as individual 60-46, whose clinical FSHD status was unknown, inherited this FSHD associated haplotype. The two clinically equivocal individuals (60-41 and 60-42) did not inherit the haplotype.

Figure 4.17: Haplotypes of selected individuals from family F60



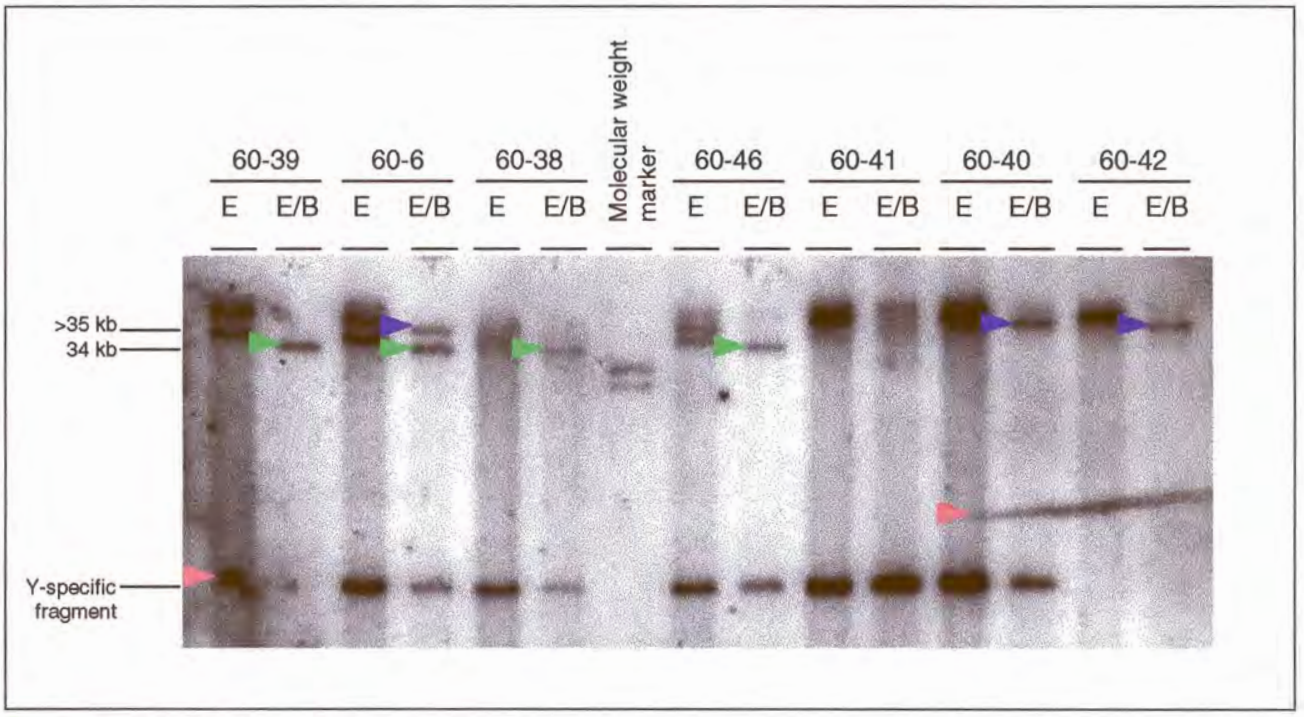
A *Bln* I resistant deletion fragment of 34 kb was observed to segregate in family F60 as presented in Figure 4.18. Complete concordance between the FSHD associated haplotype and the deletion fragment was observed. This deletion fragment was also detected to segregate with the FSHD associated haplotype observed in family F20.

In Figure 4.18, the horizontal line stretching across the last three lanes, indicated by a pink arrow is due to artefact. No 9.5 kb Y-specific fragment was observed for the female individuals 60-39 and 60-42. An artifact is, however, visible in the first lane, also indicated by a pink arrow, but it is evident that this is not a fragment, as individual 60-39 is female, and the artefact is not present in the second lane.

Two *Bln* I resistant deletion fragments are present in individual 60-6, one of 34 kb and the other of greater than 35 kb, as presented in Figure 4.18. The exact size of the larger deletion fragment could not be determined due to the limitation of the conventional gel utilised. It is however evident from Figure 4.18 that this deletion fragment is larger than the 37 kb *Eco* RI deletion fragment that was reduced to the 34 kb *Bln* I resistant fragment, and is therefore outside the reported pathogenic range. Caution should, however, be taken as the pathogenic allele size threshold for the South African population might be different than that reported for other populations (10 to 35 kb). Individual 60-40 (clinically unaffected) and his son, individual 60-42 (clinically equivocal), inherited the >35 kb *Bln* I resistant deletion fragment. Individual 60-41, the oldest son of individual 60-40, did not inherit the >35 kb *Bln* I resistant deletion fragment from his father. This was also confirmed with the haplotype analysis (Figure 4.17), as the two sons, individuals 60-41 and 60-42, inherited different chromosomes from their father, individual 60-40. It is, therefore essential that individuals 60-40 and 60-42 be clinically re-examined. The exact size of the deletion fragment should be determined with PFGE.

Family F60 harbours the same FSHD associated haplotype and *Bln* I resistant deletion fragment of 34 kb as family F20 and individuals 15-31, 15-33 and 15-34. Upon molecular analysis, it is evident that individual 15-31 forms the link between families F20 and F60.

Figure 4.18: Southern blot analysis for family F60



= 34 kb *Bln* I resistant deletion fragment;

= >35 kb *Bln* I resistant deletion fragment;

= artefact.

FSHD was confirmed for the five families investigated in this study via molecular analysis. It will therefore be possible to provide a diagnostic service for these families as well as other families in the South African population. Genotype-phenotype discordance, where individuals were clinically diagnosed to be affected, but did not inherit the FSHD associated haplotype or *Bln* I resistant deletion fragment was also observed. These individuals were, however, all diagnosed at a young age and further clinical examination is required.

To date, two different FSHD associated haplotypes and *Bln* I resistant deletion fragments (24 kb and 34 kb) have been observed to segregate in the South African population. This revealed the fact that two distinct ancestral origins are responsible for the current FSHD phenotype segregating in the South African population. It is not known how each of the FSHD genetic backgrounds evolved or how and when these mutations entered South Africa. These issues remain to be resolved in one of the next phases of this ongoing research programme.