CHAPTER THREE

MATERIALS AND METHODS

The study presented in this thesis was approved by the Ethics Committee of the Faculty of Medicine of the University of Pretoria and Pretoria Academic Hospitals, and was performed after the appropriate approval was obtained (protocol number 91/96). Prior to the collection of blood samples, informed consent was obtained from all individuals included in this study.

3.1 FSHD FAMILIES

A group of five Caucasian FSHD families (families F10, F20, F30, F40 and F60) were investigated in this study. Core families, including a total of 74 individuals, were selected from the extended family pedigrees (Appendix F). The core families consisted of individuals who were relevant to the study, and from whom blood samples could be obtained. An additional 26 individuals were also included and a total of 100 individuals were thus investigated in this study. The individuals were numbered according to the family number (F10, F20, F30, F40 or F60) and the unique pedigree number, as presented in Appendix F. For example, individual 1 of family F10 would be presented in the pedigrees in Chapter three and the haplotypes in Chapter four as 10-1. The pedigree numbers of the individuals selected for this study are underlined in the pedigrees presented in this chapter.

A national referral centre for FSHD was established in South Africa as a joint collaboration between the Departments of Neurology and Human Genetics, at the University of Pretoria. The FSHD clinic, for the first time, provided the opportunity for individuals affected with FSHD, as well as their families to receive up to date clinical and molecular information. One specialised neurologist, Dr. Clara Schutte, clinically evaluated the individuals attending the clinic, therefore excluding any possibility of diagnostic bias during the clinical examination. The individuals received genetic counselling as well as information regarding the molecular project.

The majority of the individuals included in this study have, however, not been clinically diagnosed by Dr. Schutte, but by a different neurologist ca. 5-6 years ago. However, only clinical data generated by Dr. Schutte was utilised in the detailed discussion of the clinical phenotype in paragraphs 3.1.1 to 3.1.5, and for this reason detailed clinical data is only available for selected individuals included in the study

The pedigrees, listed in various figures throughout the thesis, contain clinical diagnoses, which were not made by Dr. Schutte. The main difference between the respective clinical diagnostic protocols is that our current protocol disregard clinical diagnoses of individuals below the age of 20 – as these clinical diagnoses are not reliable due to delayed onset and only 95% penetrance of the phenotype at the age of 21. However, since the clinical diagnostic data of young individuals was available it could not be excluded from this study. For this reason a high level of genotype-phenotype discordance was expected in the group of young individuals included in this study.

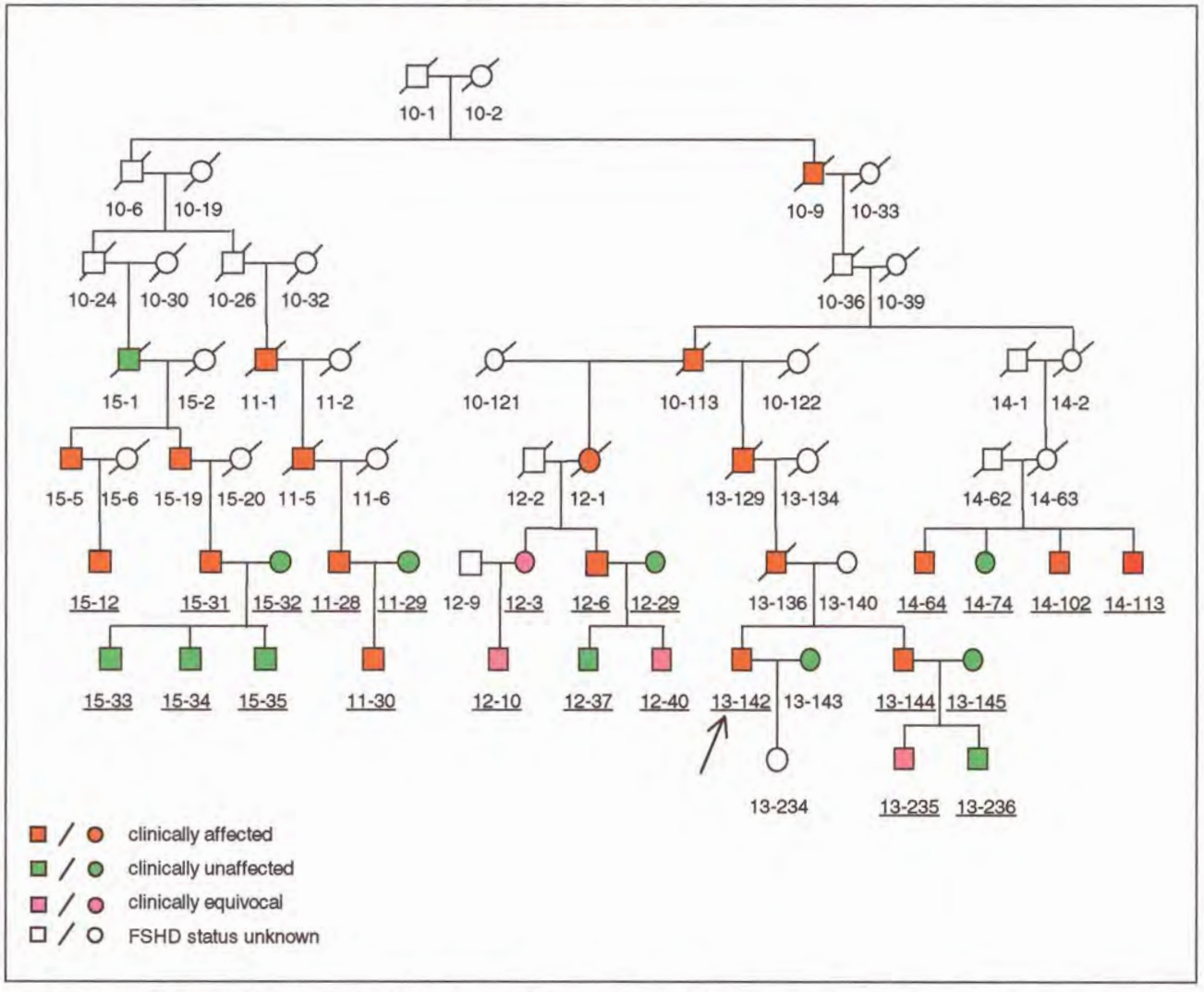
3.1.1 Family F10

Family F10 is the biggest FSHD family included in this study and was subdivided into five sub-families (F11, F12, F13, F14 and F15) for easy referral. Figure 3.1 presents an excerpt of the eight generation pedigree of family F10 which includes 480 individuals of whom 24 were genotyped. Individuals from all five of the sub-families have been included in this study.

The proband (individual 13-142 in Figure 3.1) of family F10, was clinically diagnosed with extreme facial weakness, scapula winging and high riding of the scapula. The muscles of his arms, upper legs, feet and hips were, however, only mildly affected. Two individuals not included in this phase of the FSHD project, have attended the FSHD clinic. Individual 12-31 (Figure F.3) is a 28 year old male with mild weakness of the mouth, eye and feet muscles and extreme weakness of the muscles around the shoulder area, resulting in profound high riding and winging of the scapula. His upper arm muscles are also relatively weak, no weakness of the lower arms, upper leg, hips or hand muscles are present. Individual 12-13 (Figure F.3) is 55 years old with mildly affected facial muscles but extreme weakness of the muscles around the shoulder area. Scapula winging and high riding was observed upon clinical examination. The muscles of the hips, upper legs and upper arms

were only mildly affected, while the muscles of the lower arms, hands and feet were unaffected.

Figure 3.1: Excerpt from a pedigree of family F10

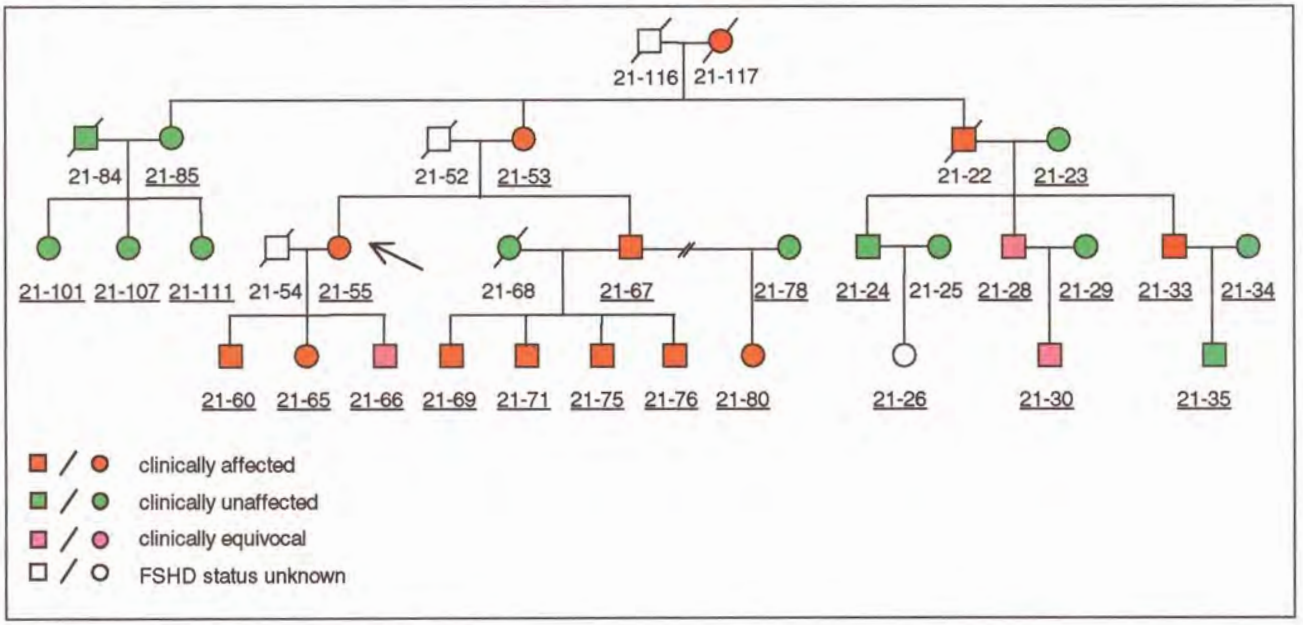


A full pedigree of family F10 is presented in Figure F.1. The full pedigrees of the sub-families are presented in Figures F.2 to F.6, Appendix F.

3.1.2 Family F20

Family F20, which include 395 individuals, was also subdivided into several sub-families for easy reference, but blood samples could only be obtained for three of the sub-families. For the purpose of this study, 25 individuals were selected from only one sub-family (F21) to represent family F20. Figure 3.2 presents an excerpt of the pedigree of family F21 and the individuals selected for this study. The proband of this family 21-55 is a 62 year old female and was classified as FSHD positive as her clinical phenotype met the clinical criteria set by the International FSHD Consortium (Appendix C).

Figure 3.2: Excerpt from a pedigree of family F21

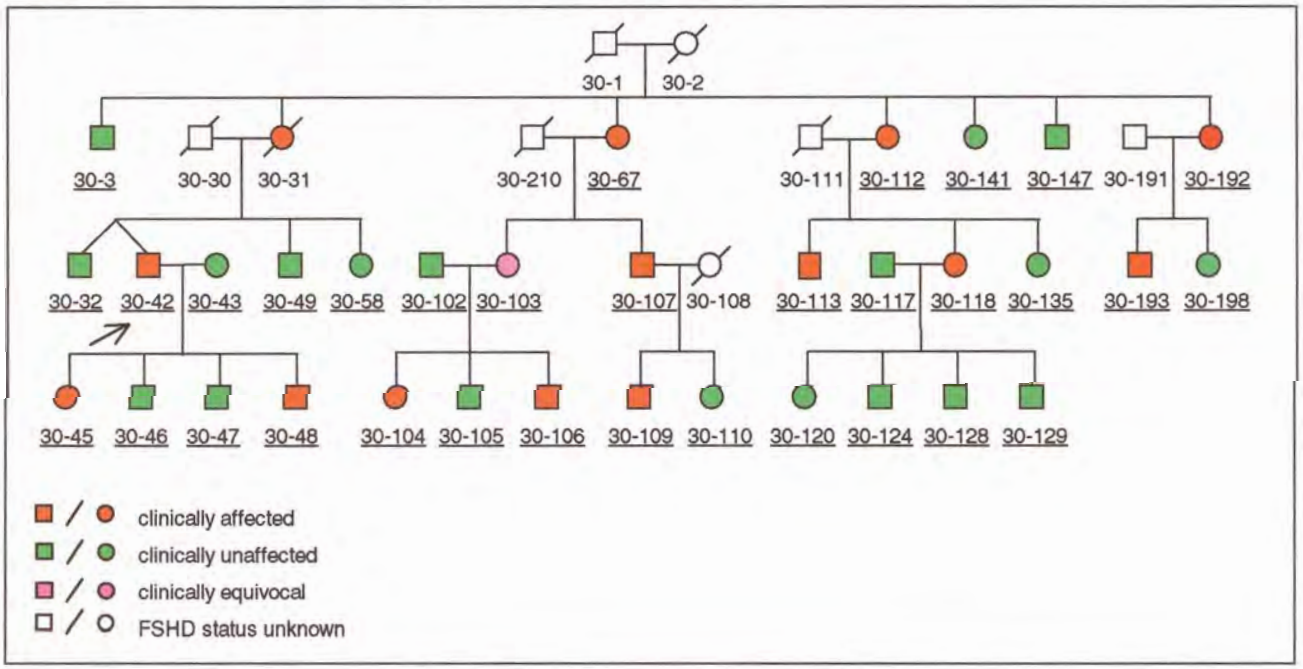


A full pedigree of this family is presented in Figure F.7.

3.1.3 Family F30

Figure 3.3 presents the excerpt from a pedigree of family F30 which includes a 65 year old male proband (individual 30-42), who was diagnosed with extreme weakness of his shoulder and surrounding muscles and pelvic girdle muscles resulting in a distinct waddling gait. This individual also has visible facial weakness. The family consists of four generations with 195 individuals of whom 33 have been genotyped in this study.

Figure 3.3: Excerpt from a pedigree of family F30

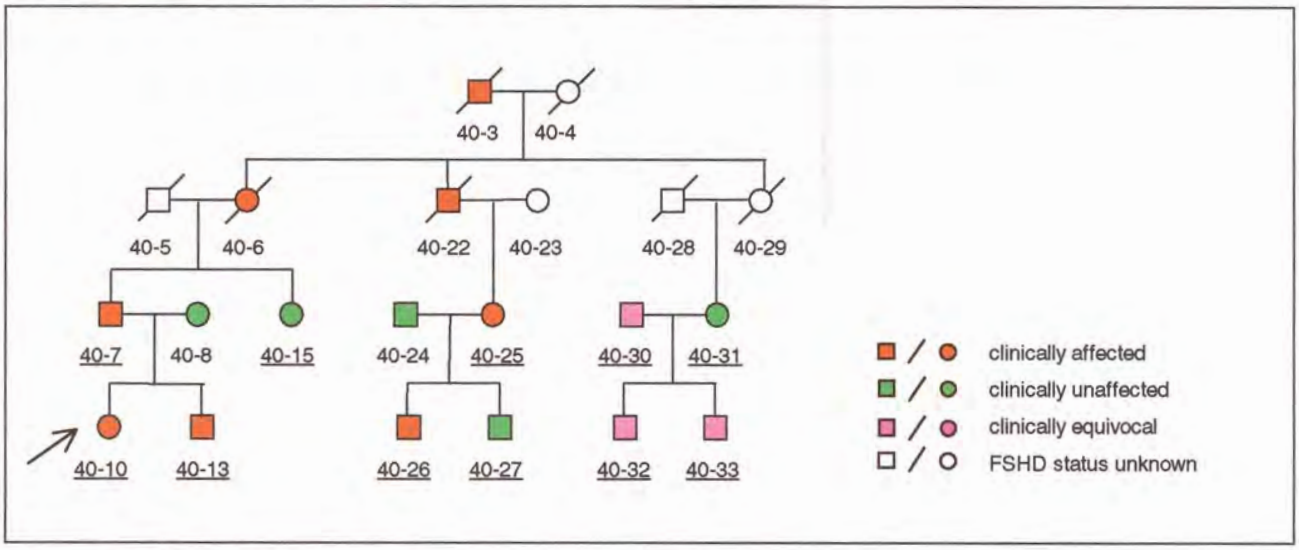


A full pedigree of this family is presented in Figure F.8.

3.1.4 Family F40

The proband of family F40 (individual 40-10 in Figure 3.4) is a middle aged female with mildly affected facial and upper arms muscles, but extreme weakness of the muscles around the scapula which resulted in extreme winging of the scapula. Her hips and upper-legs were also observed to be severely affected upon clinical examination. This individual walks with a distinct waddling gait due to the weakness of the pelvic girdle muscles. The brother of the proband (individual 40-13) has, however, only mild weakness of the eyes, mouth, upper arms and the proximal part of the lower arms. Mild weakness of the scapula resulting in only slight winging and high riding of the scapula was observed. This family consists of 40 individuals of whom 11 were genotyped.

Figure 3.4: Excerpt from a pedigree of family F40

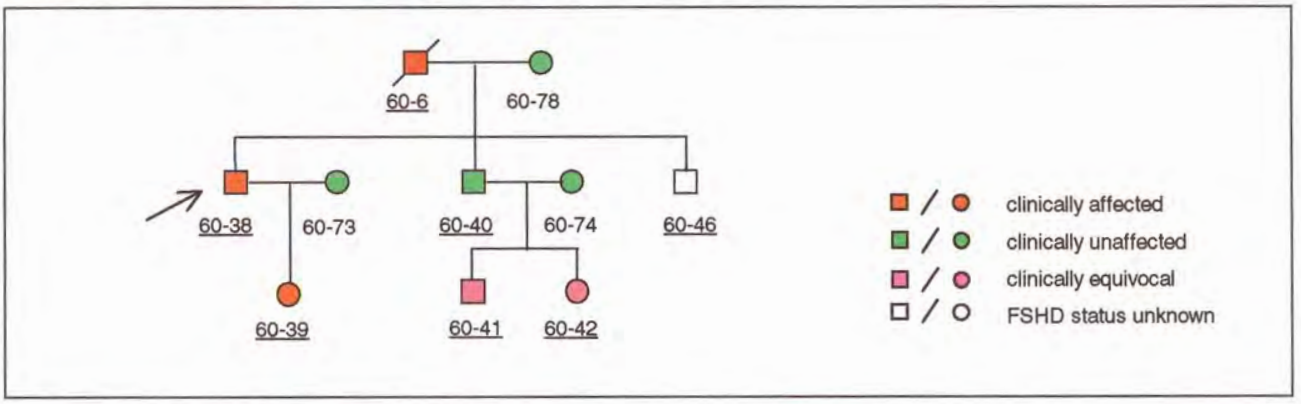


A full pedigree of this family is presented in Figure F.9.

3.1.5 Family F60

Family F60, displayed in Figure 3.5, includes a middle aged (49 years old) male proband (individual 60-38) with atrophic pectoralis major muscles and facial weakness. One deceased individual (60-6) could be genotyped as blood samples were collected from him prior to his death. Family F60 contains 75 individuals of whom seven were genotyped in this study.

Figure 3.5: Excerpt from a pedigree of family F60



A full pedigree of this family is presented in Figure F.10.

3.2 DNA ISOLATION

Whole blood was collected in tubes containing either ethylenediamine tetraacetic acid (EDTA) or acid citrate dextrose (ACD) as the preservative. The samples were divided into aliquots of 10 milliliters (ml) each. The blood was stored at -70°C until the DNA was isolated. The DNA was initially isolated with the use of sodium perchlorate and chloroform and subsequently by the Wizard Genomic DNA Purification Kit® [1] [Promega].

3.2.1 Isolation of genomic DNA using sodium perchlorate and chloroform

DNA was extracted from the 10 ml aliquots of whole blood using a modified method of the one described in 1989 by Johns and Paulus-Thomas (Olckers, 1997). If frozen, the blood was first thawed on ice and transferred to a 50 ml polypropylene centrifuge tube [Elkay or Sterilin]. Thirty five ml cell lysis buffer (0.32 M sucrose; 10 mM Tris-HCl (pH 8.0); 5 mM $MgCl_2$; 1% Triton X-100®) was added to the 10 ml whole blood and incubated on ice for 10 minutes. The solution was centrifuged at 1000 xg for 20 minutes at 4°C after incubation. The supernatant was discarded and the pellet resuspended in 9.5 ml suspension buffer (50 mM Tris-HCl (pH 8.0); 150 mM NaCl; 100 mM EDTA). Half a millilitre of a 30% SDS solution was added to the suspension, adjusting the final SDS concentration to 1.5%. Subsequently, 2.5 ml of a freshly prepared 5 M $NaClO_4$ solution and an equal volume of chloroform:IAA (24:1) were added to the suspension. The

[1] Wizard® is a registered trademark of the Promega Corporation, Madison, WI, U.S.A.

suspension was mixed on an orbital shaker for 30 minutes to extract the DNA. After the extraction, it was centrifuged at 1,000 xg for 10 minutes at 20°C.

Two volumes of ice cold absolute ethanol (stored at -20°C) were added to the aqueous phase, which was transferred to an Erlenmeyer flask, to precipitate the DNA. The DNA was spooled onto a sterile glass rod and resuspended in 5 ml of suspension buffer. The DNA was precipitated once more, where after the DNA was washed with 70% ethanol, air dried, transferred to Eppendorf®[1] tubes and dissolved in 1.0 ml of TE buffer (10 mM Tris-HCl (pH7.5), 1 mM EDTA) overnight.

The average DNA yield per 10 ml whole blood obtained with the method as described by Johns and Paulus-Thomas, (1989) was 250-450 µg and the A_{260}/A_{280} ratio for all samples was between 1.6 and 2.1. DNA preparations were treated with proteinase K (100 $\mu g.ml^{-1}$) at 60°C overnight when protein contamination was present.

3.2.2 Isolation of genomic DNA utilising the Wizard® Genomic kit

The Wizard® Genomic DNA purification kit enables the isolation of DNA from white blood cells and various other sample types. Different amounts of starting material may be used depending on the application and DNA yield required (Table 3.1). The Wizard® system was selected due to the fact that it yields DNA which is suitable for a variety of applications, including PCR, RFLP and Southern Blot.

Table 3.1: Average DNA yield from various amounts of starting material

Amount of whole blood	Typical DNA yield[a]
300 µl	5–15 µg
1.0 ml	25–50 µg
10.0 ml	250–500 µg

a = Yield depends on the quantity of white blood cells present. Adapted from Wizard® Genomic DNA purification kit, technical manual, 1998.

The kit is based on a four-step protocol. The red blood cells were lysed in the first step with the addition of 30 ml of the Cell Lysis Solution to a sterile 50 ml polypropylene tube [Elkay or Sterilin] for 10 ml whole blood samples. The tube of blood was gently rocked

[1] Eppendorf® is a registered trademark of Eppendorf, Hamburg, Germany.

until thoroughly mixed, transferred to the 50 ml tube containing the Cell Lysis Solution, inverted 5-6 times, and incubated for 10 minutes at room temperature during which the solution was mixed twice by inversion. After centrifugation at 2,000 xg for 10 minutes at room temperature as much supernatant as possible was removed without disturbing the visible white pellet.

To ensure efficient cell lysis of the white blood cells and their nuclei in the second step of the protocol, the tube was vigorously vortexed until all the cells were resuspended. Ten ml of Nuclei Lysis Solution was added to the tube containing the resuspended cells and mixed 5-6 times to lyse the white blood cells. The solution became very viscous and if clumps of cells were visible after mixing it was incubated at 37°C until the clumps were dissolved.

The cellular proteins were removed in the third step, leaving the genomic DNA in solution. 3.3 ml of the Protein Precipitation Solution was added to the lysed white blood cell solution and vortexed vigorously until small protein clumps were visible. The samples were centrifuged at 2,000 xg for 10 minutes at room temperature to collect the cellular proteins as a brown protein pellet at the bottom of the centrifuge tube.

Finally, the high molecular weight genomic DNA was precipitated and desalted by an isopropanol precipitation step. The supernatant was transferred to a 50 ml polypropylene tube containing 10 ml of isopropanol at room temperature. The tube was gently mixed until the white thread-like strands of DNA formed a visible mass. After centrifugation at 2,000 xg for one minute at room temperature the DNA was visible as a small white pellet. The supernantant was decanted and one sample volume of 70% ethanol (at room temperature) was added to the DNA. The tube was gently inverted several times to wash the DNA and the sides of the centrifuge tube. This was followed by centrifugation at 2,000 xg for one minute. The ethanol was carefully aspirated and the pellet air dried for 10–15 minutes after which it was rehydrated in 500-830 µl of DNA rehydration solution. For rapid DNA rehydration the DNA was incubated at 65°C for 1 hour, or alternatively the DNA was incubated overnight at room temperature or 4°C.

The average DNA yield per 10 ml whole blood obtained with the Wizard® Genomic kit was 450-800 µg and the A_{260}/A_{280} ratio for all samples was between 1.7 and 2.1. The concentrated stock DNA was stored at -20°C after working dilutions of 50 $ng.\mu l^{-1}$ (used for

PCR applications) and 250 ng.μl^{-1} (used for Southern blotting) were made. The working dilutions were stored at 4°C.

3.3 HAPLOTYPE ANALYSIS

Five short tandem repeat polymorphism (STRP) markers were initially identified for haplotype analysis as illustrated in Table 3.2. Four additional markers were, however, also included for more conclusive results (Table 3.2). A total of nine STRP markers, consisting of three dinucleotide, one trinucleotide and five tetranucleotide repeat markers on chromosome 4q35, were therefore included for haplotype analysis in this study. The sex average map was utilised, since both male and female individuals were included in the study.

Table 3.2: Genetic map of human chromosome 4q35

Locus[a), b)]	DNA marker	Sex average map (cM)
D4S426*	AFM238ve3	206.98
D4S2921*	AFMa190zf5	206.98
D4S2688□	UT7694	208.07
D4S2283□	UT2219	208.07
D4S2299□	UT5785	208.07
D4S2390*	ATA22F02	208.07
D4S2930*	AFMa224xh1	208.07
D4S1652*	GATA5B02	208.07
D4S1523□	UT1366	211.65

a) * Markers initially identified for this study. b) □ Additional markers utilised. Table adapted from MFD, 2001.

Table 3.3 lists the markers, their respective loci, primer sequence, melting temperature (T_m), product size, number of alleles, as well as the heterozygosity value for each marker. The information listed in table 3.3 was obtained from several genome mapping centers: The Co-operative Human Linkage Center (CHLC, 2001), The Centre for the Study of Human Polymorphisms (CEPH, 2001), The Genome Database (GDB, 2001) and The Center for Medical Genetics Marshfield Medical Research Foundation (MFD, 2001). The T_m was calculated for each primer as described by Thein and Wallace (1986), utilising the following equation:

$$T_m = 2(A + T) + 4(G + C)$$

Table 3.3: Primers for short tandem repeat polymorphism markers located on chromosome 4q35

Locus	DNA marker[a] (repeat type)	Primer Sequence[b]	T_m	Product size (bp)	No. of alleles[d]	HET[e]
D4S1523	UT1366 (TetNR) [1]	F: 5'-tctactcacatgcggctgg-3' R: 5'-tagtgtttggtggaatttgca-3'	60 58	285[c]	7	67%
D4S1652	GATA5B02 (TetNR) [2]	F: 5'-aatccctgggtacattatatttg-3' R: 5'-cagacattctttattctttacctcc-3'	62 64	122-158	4	69%
D4S2930	AFMa224xh1 (DNR) [3]	F: 5'-cctcatggtaggttaatcccacg-3' R: 5-tattgaatgcccgccatttg-3'	70 58	217-233	9	86%
D4S2390	ATA22F02 (TriNR) [4]	F: 5'-ctcattttccccttccact-3' R: 5-gtggttttcatcatgagatgc-3'	58 60	102-120	6	76%
D4S2299	UT5785 (TetNR) [5]	F: 5'-tgagcatgtgaaccaatgc-3' R: 5'-ctcacttcattcccaactg-3'	56 56	201-244	9	61%
D4S2283	UT2219 (TetNR) [6]	F: 5'-ccccgttatttttccatctac-3' R: 5'-ctaaagcaaaatgcagacaca-3'	60 58	383[c]	4	59%
D4S2688	UT7694 (TetNR) [7]	F: 5'-agaatgtttgtgacagatgta-3' R: 5'-cagggatgaagtaacagaag-3'	54 58	246[c]	6	52%
D4S2921	AFMa190zf5 (DNR) [8]	F: 5'-tccttcaggaactggtg-3' R: 5'-ttaaaaatctacagacaagggc-3'	52 60	141-163	8	56%
D4S426	AFM238ve3 (DNR) [9]	F: 5'-atacactgcatccatatatacaag-3' R: 5'-acattgtgaaatgaccacagtcaag-3'	68 66	177-191	6	78%

a = References for primer sets are as follows: 1) Gerken *et al.* (1993a); 2) Murray *et al.* (1995a); 3) Dib *et al.* (1996); 4) Murray *et al.* (1995b); 5) Gerken *et al.* (1993b); 6) Gerken *et al.* (1993c); 7) Gerken *et al.* (1994); 8) Dib *et al.* (1996); 9) Weissenbach *et al.* (1992). b = F and R indicate forward and reverse primers respectively. c = No allele size range was available for markers UT1366, UT2219 and UT7694. d = The number of published alleles are listed. e = The heterozygosity value for each marker is listed in this column.

3.3.1 STRP marker information

The allele frequencies and partial sequences on chromosome 4q35, encompassing each STRP marker utilised in this study are presented in the following paragraph. The allele frequencies correspond with those reported on the CEPH database (CEPH, 2001). The sequences were retrieved from the database of the National Center for Biotechnology Information with accession numbers as listed in the footnote for each marker (NCBI, 2001).

3.3.1.1 Marker UT1366 at locus D4S1523

The allele sizes of this marker have not been reported and the allele frequencies of only five of the seven alleles were reported since this marker has only been genotyped on four CEPH families (Ballard, 1999). The reported allele frequencies ranged from 0.077 to 0.346, as presented in Table 3.4.

Table 3.4: Allele frequencies for marker UT1366 at locus D4S1523

Allele (bp)	---	---	---	---	---	---	---
Frequency	0.192	0.231	0.346	0.154	0.077	0.000	0.000

No allele sizes were reported (---).

The UT1366 marker amplifies a polymorphic tetranucleotide repeat at the D4S1523 locus as presented in Table 3.5. The clone of this marker contains nine (AGAT) repeats and the amplicon is 285 bp (Gerken *et al.*, 1993a).

Table 3.5: Partial gDNA sequence at locus D4S1523 encompassing marker UT1366

Nucleotide number[a]	Genomic DNA sequence[b]
1	ccttctactc acatgcggct ggaaaaaaca acagctcaca aaggaaagaa aacctaaata
61	atagcttatt tatatagtaa ttagatgata gatgatagat agatagatag atagatagat
121	agatagatag atgagagaga gagagagagg tgaggaaaac tctttaaaat atcaccatgg
181	taaatataat atcagagatt cttcatattt gtttgttttg cttttggaag tactcatatg
241	tcaccatcta gcaggactag atggtgatgc aaattccacc aaacactaaa aagaagagtc
301	tattttcttg agactgagaa aaactcatac cctcaaagac tgaagactaa tctttgattt
361	gctgaaagct tgcctaagag ttgga

a = Nucleotide positions are as reported by Gerken *et al.* (1993a). b = The sequence was deposited in Genbank with accession number L16394. The positions of the primer set are indicated by underlined text (xxx). The STRP at this locus is indicated by double underlined text (yyy).

3.3.1.2 Marker GATA5B02 at locus D4S1652

Four alleles ranging from 138 to 150 bp have been reported for marker GATA5B02, as listed in Table 3.6. The allele frequencies of the four alleles are not equal with the 138 bp allele being the most frequent and the 150 bp allele the least frequent.

Table 3.6: Allele frequency for marker GATA5B02 at locus D4S1652

Allele (bp)	138	142	146	150
Frequency	0.357	0.286	0.268	0.089

Table 3.7 presents the gDNA sequence encompassing marker GATA5B02. The marker amplifies a $(CTAT)_n$ repeat at locus D4S1652. An amplicon of 140 bp is obtained for a clone consisting of nine $(CTAT)_n$ repeats (Murray *et al.*, 1995a).

Table 3.7: Partial gDNA sequence at locus D4S1652 encompassing marker GATA5B02

Nucleotide number[a]	Genomic DNA sequence[b]
1	ggaatccctg ggtacattat atttgttaaa ttttagaatc tatcatctat ctatttctct
61	atcatctatc tatctatcta tctatctatc tatctatcta tctacttcta gtgagtggga
121	ggtaaagaat aaagaatgtc tggctaatgg aaaattctcg tataatatct attcatatca
181	caagngtaag aagattttga tagaaaaaca aacacatttg atcattcagt gtgaactccc
241	tttgctcagt tggaatttgn tttcaacata tctgggtttt ctcatcagcc tctagttaaa
301	acagtganat tttgtatagg aa

a = Nucleotide positions are as reported by Murray *et al.* (1995a). b = The sequence was deposited in Genbank with accession number G08375. The positions of the primer set are indicated by underlined text (xxx). The STRP at this locus is indicated by double underlined text (yyy).

3.3.1.3 Marker AFMa224xh1 at locus D4S2930

Marker AFMa224xh1 generates nine alleles ranging from 217 to 233 bp at locus D4S2930 as presented in Table 3.8. Equal frequencies were reported for the 225, 231, 229 and 233 bp alleles. The 225 and 231 bp alleles are the least frequent with the 221 bp allele being the most frequent.

Table 3.8: Allele frequency for marker AFMa224xh1 at locus D4S2930

Allele (bp)	217	219	221	223	225	227	229	231	233
Frequency	0.125	0.143	0.339	0.179	0.018	0.036	0.071	0.018	0.071

This marker amplifies a polymorphic dinucleotide repeat $(CA)_n$ within the D4S2930 locus. The clone of this marker contains fourteen dinucleotide repeats corresponding to a 219 bp amplicon as illustrated in Table 3.9 (Dib *et al.*, 1996). The nine reported alleles therefore contain from twelve to twenty eight dinucleotide repeats.

Table 3.9: Partial gDNA sequence at locus D4S2930 encompassing marker AFMa224xh1

Nucleotide number[a]	Genomic DNA Sequence[b]
1	cctcatggta ggttaatccc acgctttgcc aaagattacc gtatctttac tcacatcccc
61	ttcctcatgt gtcctttcca cttattatca ttcnttgggt ttttctgtca cctcacccct
121	gtcatacatt ctcacacaca cacacacaca cacacacaca cgtgtatata caataagttt
181	ctattagggt agcattgtac aaatggcggg cattcaataa atgnttaata tgcattttaa
241	aacagtgtat gcaagngtaa tcatcnaaag tcatatgact cttctctttt catgtagcat
301	ccttcagaac tgcattatag aagggagata gct

a = Nucleotide positions are as reported by Dib *et al.* (1996). b = The sequence was deposited in Genbank with accession number Z52598. The positions of the primer set are indicated by underlined text (xxx). The STRP at this locus is indicated by double underlined text (yyy).

3.3.1.4 Marker ATA22F02 at locus D4S2390

This marker generates six alleles as presented in Table 3.10. The reported alleles range from 102 to 120 bp with the allele frequency of the 120 bp allele being twenty times less than that of the most frequent allele (111 bp).

Table 3.10: Allele frequency for marker ATA22F02 at locus D4S2390

Allele (bp)	102	108	111	114	117	120
Frequency	0.135	0.212	0.385	0.096	0.154	0.019

Marker ATA22F02 is the only trinucleotide marker included in this study. This marker amplifies a $(ATA)_n$ repeat at the D4S2390 locus. The deposited sequence in Genbank, presented in Table 3.11, contains twelve trinucleotide repeats, corresponding to an amplicon size of 111 bp (Murray *et al.*, 1995b).

Table 3.11: Partial gDNA sequence at locus D4S2390 encompassing marker ATA22F02

Nucleotide number[a]	Genomic DNA Sequence[b]
1	aactcctaag tttggctaga tacttaatgg cccaaactct ttgcaaataa gtgtggttat
61	attttagaca atagaatgca aacggatacc aacaccagtt tgtgattgtn tttttataag
121	gaggctgtgt gtctttctct ctcattttcc cctttccact aattgcaaaa tgatacataa
181	taataataat aataataata ataataataa tannncnggag gagttgcctt gcatctcatg
241	atgaaaacca caatcaaagg atgaaagagt caccctacta gcttgaattc cttgattatg
301	ttgtngggna

a = Nucleotide positions are as reported by Murray *et al.* (1995b). b = The sequence was deposited in Genbank with accession number G08314. The positions of the primer set are indicated by underlined text (xxx). The STRP at this locus is indicated by double underlined text (yyy).

3.3.1.5 Marker UT5785 at locus D4S2299

The allele frequencies of five of the eight alleles have been reported on the CEPH-database as presented in Table 3.12. The allele sizes of the eight alleles were, however, not reported for this marker. The presence of 10 alleles in an extended family of 150 individuals, ranging from 201-244 bp have, however, been reported by the Eccles Institute of Human Genetics (Ballard, 1999). No allele frequency information was available for these 10 alleles.

Table 3.12: Allele frequency for marker UT5785 at locus D4S2299

Allele (bp)	---	---	---	---	---	---	---	---
Frequency	0.200	0.233	0.200	0.233	0.133	0.00	0.00	0.00

No allele sizes were reported (---).

Marker UT5785 at locus D4S2299 amplifies a polymorphic tetranucleotide repeat $(AGAT)_n$. The sequence presented in Table 3.13 includes twelve tetranucleotide repeat units generating an amplicon of 209 bp after PCR amplification (Gerken *et al.*, 1993b).

Table 3.13: Partial gDNA sequence at locus D4S2299 encompassing marker UT5785

Nucleotide number[a]	Genomic DNA Sequence[b]
1	gatcagccct cctacttttg gactcaggca gaattacacc acaggctttc ttgggtctcc
61	agctcacaga gggcagatgg tgggtcttct cagcttccat gagcatgtga accaatgccc
121	atgttaatta gtgtgtgcat atgtatacac acataccgat agattagata gacagatgat
181	agatgataga cagatgatag atgatagaca gatagatgat agatagatag atagatagat
241	agatagatag atagatagat agatagattc tggagaacct tgactaatac agttgggaat
301	gaagtgagtt gtcaaatctg acagaggtag tgtatgtaga ctgagaaaaa ttagaaaatg
361	aagcttcacc agtagaacgg cacaaaatta taagtgacca ttaaggctgg gagagtttga
421	tc

a = Nucleotide positions are as reported by Gerken et al. (1993b). b = The sequence was deposited in Genbank with accession number L18154. The positions of the primer set are indicated by underlined text (xxx). The STRP at this locus is indicated by double underlined text (vvv).

3.3.1.6 Marker UT2219 at locus D4S2283

As previously mentioned for markers UT1366 (locus D4S51523) and UT5785 (locus D4S2299), no allele sizes were available for the four alleles reported for marker UT2219

(Table 3.14). The frequencies of the four alleles range from 0.062 to 0.40. The allele with the lowest frequency is therefore six and a half (6.5) times less frequent than the most frequent allele.

Table 3.14: Allele frequency for marker UT2219 at locus D4S2283

Allele (bp)	---	---	---	---
Frequency	0.406	0.375	0.156	0.062

No allele sizes were reported (---).

A tetranucleotide polymorphic repeat $(CTAT)_n$ at the D4S2283 locus is amplified with marker UT2219. The sequence presented in Table 3.15 contains eleven tetranucleotide repeats which correspond with a 383 bp amplicon after PCR analysis (Gerken *et al.*, 1993c).

Table 3.15: Partial gDNA sequence at locus D4S2283 encompassing marker UT2219

Nucleotide number[a]	Genomic DNA Sequence[b]
1	cagccccgt tatttttcca tctactaatt tttgggacat agcatttcta cagcagaaat
61	gcatttacat ggcctctacc tgtgtggttg catctatcta tctatctatc tatctatcta
121	tctatctatc tatctatcca tccatccatc cattctccca tccatccatc cacccactca
181	cccactcatc tatctgtctt ctgaaataaa agtttaataa aatgacattt tctttttttg
241	taatatattt gtataatttc aattatatcc tggtcgattt ctttttaaaa aaaatgctgg
301	taattagtaa ctaaatttat tttacaactt atttttgtaa atcagtggca taaatagtag
361	cagttatgtg tctgcatttt gctttagatt

a = Nucleotide positions are as reported by Gerken *et al.* (1993c). b = The sequence was deposited in Genbank with accession number L17998. The positions of the primer set are indicated by underlined text (xxx). The STRP at this locus is indicated by double underlined text (yyy).

3.3.1.7 Marker UT7694 at locus D4S2688

Six alleles, with frequencies as presented in Table 3.16, have been reported for marker UT7694 at locus D4S2688. The allele sizes of the six alleles were, however, not reported. The frequencies of only five of the six alleles have been reported with frequencies range from 0.062 to 0.375.

Table 3.16: Allele frequency for marker UT7694 at locus D4S2688

Allele (bp)	---	---	---	---	---	---
Frequency	0.375	0.281	0.219	0.062	0.062	0.00

No allele sizes were reported (---).

Marker UT7694 amplifies a tetranucleotide repeat $(AGAT)_n$ at locus D4S2688 as illustrated in Table 3.17. An amplicon of 246 bp, consisting of six tetranucleotide repeats, is generated after PCR analysis (Gerken *et al.*, 1994). A second repeat sequence (AGAC) can be observed in the sequence presented in Table 3.17. Upon STRP analysis it was, however, observed that this repeat is not polymorphic.

Table 3.17: Partial gDNA sequence at locus D4S2688 encompassing marker UT7694

Nucleotide number[a]	Genomic DNA Sequence[b]
1	tcgaactcct gacctcaggt gatccacccg cttcagcctc ccaaagtgct gggaatatag
161	gcgtgagcan ctgcacctgg cccacacatt aagtttacaa aacaagacac agaaggggaa
121	actatggaat aacaggacta gaaagagtag gagattttgt aaatgttttt attatgaaaa
181	tatttgatac acataagaga atgtttgtga cagatgtata tttatataca cacaaacata
241	tacatctgta gataggatgg atagataaga ttagataga gatagataga tagagagata
301	gatagataga cagacagaca gacagatagc agntggggta ggttttcatg aaacaaacac
361	cattgacccc ttcacccaca ctaagacatg gaacgtcact gttatctctg aagctcctgt
421	tcgcttctgt tacttcatcc ctggccctgg gttttctcca agagttccat tcacatacgt
481	gttgcacagc tgttaataca ctgcctagtg tgcttggctt tgaatttata atacagtatc
541	atgctgcata tctttccaca acttgcttcc acaatgtatt aactttctga gtttcga

a = Nucleotide positions are as reported by Gerken *et al.* (1994). b = The sequence was deposited in Genbank with accession number L30309. The positions of the primer set are indicated by underlined text (xxx). The STRP at this locus is indicated by double underlined text (yyy). A non polymorphic repeat is indicated by dashed underlined text (zzz).

3.3.1.8 Marker AFMa190zf5 at locus D4S2921

Table 3.18 displays the allele sizes and frequencies of the eight alleles reported for marker AFMa190zf5 at locus D4S2921. The allele sizes range from 141-163 bp, with the 151 bp allele being 36 time more frequent than the 153 bp and 163 bp alleles. The smallest allele (141 bp) is the second most frequent and is six time less frequent than the most frequent allele (151 bp).

Table 3.18: Allele frequency for marker AFMa190zf5 at locus D4S2921

Allele (bp)	141	151	153	155	157	159	161	163
Frequency	0.107	0.643	0.018	0.036	0.036	0.071	0.071	0.018

This STRP marker amplifies a $(CA)_n$ repeat at the D4S2921 locus. Tale 3.19 displays the sequence of the clone for this marker containing fourteen dinucleotide repeat units concurring with a 151 bp fragment after PCR analysis (Dib *et al.*, 1996).

Table 3.19: Partial gDNA sequence at locus D4S2921 encompassing marker AFMa190zf5

Nucleotide number[a]	Genomic DNA Sequence[b]
1	cctcttcctt caggaactgg tgcantttca tgtattctga cacaanaacc tgtttacaag
61	tctttgtttc ntcctgccaa aacacacaca cacacacaca cacacacaca cgagataaag
121	ttagtaagag aaatgccctt gtctgtagat ttttaancct tacaagtcct agagtttctt
181	tctttcnttt ttttttttt ttgagatgga gtctcactct gtcgccagtc tgaagtgcag
241	tggcgcaatc tcggctcact gcaacctctg cctcctgagt tcaaatgatt ctcctgcctc
301	agcctncctg agtagct

a = Nucleotide positions are as reported by Dib *et al.* (1996). b = The sequence was deposited in Genbank with accession number Z52419. The positions of the primer set are indicated by underlined text (xxx). The STRP at this locus is indicated by double underlined text (yyy).

3.3.1.9 Marker AFM238ve3 at locus D4S426

The allele frequencies of the six reported alleles are listed in Table 3.20. The alleles range from 177 to 191 bp, with the 189 bp allele being the least frequent and the 179 bp allele the most frequent of the six alleles. The 181 bp and 191 bp have equal reported frequencies.

Table 3.20: Allele frequency for marker AFM238ve3 at locus 426

Allele (bp)	177	179	181	187	189	191
Frequency	0.232	0.375	0.107	0.125	0.054	0.107

This dinucleotide marker amplifies a $(CA)_n$ repeat at locus D4S426. The sequence presented in Table 3.21 includes sixteen dinucleotide repeats generating an amplicon of 189 bp upon PCR analysis (Weissenbach *et al.*, 1992).

Table 3.21: Partial gDNA sequence at locus D4S426 encompassing marker AFM238ve3

Nucleotide number[a]	Genomic DNA Sequence[b]
1	agcttctact cnnttagaca atttnnagta tacactgcat ccatatatac aaggnggcag
61	tgaatacttg aaattgtctg agacagtaga ncttnggtgt cctaaccaca tacacataat
121	acacanacac acacacacac acacacacac acacacacag aggtaactat gtgtgatgat
181	gattgttaaa taacttgact gtggtcattt cacaatgtaa acatatctca aattaccata
241	ttgtattact tgaatatata taattttaat ttatcaatta tacctaaata aagct

a = Nucleotide positions are as reported by Weissenbach *et al.* (1992). b = The sequence was deposited in Genbank with accession number Z17062. The positions of the primer set are indicated by underlined text (xxx). The STRP at this locus is indicated by double underlined text (yyy).

3.3.2 5' - End labelling of PCR primers

Sixty pico moles (pmol) of the selected primer were 5'-end labelled with γ^{32}P-dATP and polynucleotide kinase [Promega]. Reaction mixtures contained 70 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM Dithiothreitol (DTT), 5 units polynucleotide kinase and 60 µCi γ^{32}P-dATP [Amersham Life Science Inc.][1] in a total volume of 14 µl. After incubation at 37°C for one hour, 26.0 µl ddH_2O was added. The end labelled primers were stored at -20°C, until required. All of the markers were end labelled, except markers D4S2390 and D4S2930 for which multiplex PCR and internal incorporation were utilised.

3.3.3 The polymerase chain reaction (PCR)

PCR conditions were optimised for each primer set with regard to the annealing temperature and the magnesium chloride ($MgCl_2$) concentrations. The optimisation reactions were performed under non-radioactive conditions.

A modified method described by Mullis and Faloona (1986) was utilised to perform PCR. The PCR reactions were prepared in a total volume of 12.5 µl in 0.5 ml thin wall PCR tubes [Hybaid] or 96 well plates [Sterilab]. The PCR reagents were thawed on ice and thoroughly mixed by vortexing prior to use. PCR were performed in reactions containing 10 mM Tris-HCl (pH 8.3), 1.0-1.5 mM $MgCl_2$, 50 mM KCl, 200 µM of each nucleotide [2'-deoxyadenosine-5'-triphosphate (dATP), 2'-deoxycytidine-5'-triphosphate (dCTP), 2'-deoxyguanosine-5'-triphosphate (dGTP) and 2'-deoxythymidine-5'-triphosphate (dTTP)], 60 nM (0.75 pmol) ^{32}P end labelled primer, 40 nM (5.0 pmol) unlabelled primer, 0.5 units (U) Taq polymerase [Promega]. 100 ng gDNA was added and the reactions were overlaid with a drop of mineral oil to prevent evaporation.

A standard PCR program consisted of: denaturation at 94°C for ten minutes, and 30 cycles consisting of denaturation at 94°C for 30 seconds (sec), annealing at the optimised temperature for 30 sec and extension at 72°C for 30 sec. A final extension of 7 minutes at 72°C ensured that all PCR products were full length products. The PCR reactions were performed in a Hybaid Touchdown™ [2] thermocycler.

---

[1] Amersham™ is a trademark of Amersham International plc., Buckinghamshire, UK.
[2] Touchdown™ is a trademark of Hybaid Limited, Ashford, Middlesex, UK.

After PCR amplification was completed an equal volume of 2X stop buffer (95% formamide; 0.05% xylene cyanol FF (XC); 0.05% bromophenol blue (BPB); 20 mM EDTA) was added to each sample. The samples were denatured at 85°C for 5 minutes and placed on ice before loading on a denaturing polyacrylamide gel. Whenever the PCR products could not be electrophoresed immediately, the products were stored at 4°C.

3.3.4 Multiplex PCR

Multiplex PCR was performed for three of the nine STRP markers (D4S2921, D4S2390 and D4S2930). The primers of these markers were not end-labelled, but internal incorporation was performed instead. The PCR reaction mixture was prepared as described in paragraph 3.3.2, without adjusting any of the reagents except for using α^{32}P-dCTP instead of the end-labelled primer. The PCR program was also the same as described in paragraph 3.3.2, except for increasing the annealing time to 60 sec. Stop buffer was added to each sample as described in paragraph 3.3.2.

3.3.5 Single stranded DNA sequencing

DNA sequencing was performed according to the chain termination protocol described by Sanger *et al.* (1977). Sequencing was performed with the Sequenase® Version 2.0 (Sequenase) kit [USB]. The control DNA included with the kit was sequenced as size reference for accurate allele sizing. The included DNA is a single-stranded phage DNA from a clone of a *Sau* 3AI fragment of bacteriophage λ DNA inserted at the *Bam* HI site of M13mp18.

Annealing of the template and primer was accomplished by adding 1 µg of the template, 3.33 pmol of the –40 forward 23-mer primer and ddH_2O, up to a final volume of 10 µl. The mixture was incubated at 99.9°C for 3 minutes and placed on ice for 5 minutes. The rapid cooling promotes primer annealing over template re-annealing. The labelling solution contained 2 µl of a 5X T7 Sequenase reaction buffer (200 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 250 mM NaCl), 0.1 µmol DTT suspension, 3 µM dATP, 3 µM dGTP, 3 µM dTTP, 5 µCi α^{32}P-dCTP and 3.2 U Sequenase enzyme.

The labelling solution was added to the 10 µl annealing mixture and incubated at room temperature for 3 minutes. Two and a half microlitres of the four termination mixtures (80 µM of each dNTP, 8.0 µM of one ddNTP, 50 mM NaCl) were aliquoted into four separate 0.5 ml eppendorf tubes. The labelling reactions were terminated by adding 3.5 µl of each sample to the four termination tubes. The sequencing reactions were incubated at 37°C for 7 minutes after which 4 µl of a 2X stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) were added to each tube. The reactions were stored at 4°C until loaded onto a denaturing polyacrylamide gel.

3.3.6 Denaturing gel electrophoresis and autoradiography

A gel stock solution containing an acrylamide monomer concentration of 6% was prepared, containing 75 ml of a 40% acrylamide mixture (acrylamide [Stratagene]/ bis-acrylamide [Promega] (19:1) and 210 g (7 M) urea [USB] dissolved in TBE buffer (89.15 mM Tris base [USB]; 88.95 mM Boric acid [USB]; 2.498 mM Na_2EDTA [ACE], at a pH of 8.0-8.3). A catalyst (30 µl TEMED) and an oxidising agent (500 µl of a 10% ammonium persulphate solution) were added to 75 ml gel stock solution prior to casting of the 0.4 mm thick gel to achieve polymerisation.

The gel was left for at least an hour to allow complete polymerisation, after which it was pre-run at 60 watts until the temperature of the gel reached 45-50°C (30 min-45 min). Samples were loaded and electrophoresed in 1X TBE buffer at 45 watts for the required length of time. After termination of electrophoresis, the gels were transferred to Whatman®[1] 3MM Chromatography blotting paper, covered with Cling Wrap and vacuum-dried at 80°C for approximately 45 min. The dried gels were exposed to X-Ray film at room temperature for the required length of time.

[1] Whatman® is a registered trademark of Whatman Scientific Ltd., Kent, UK.

3.4 SOUTHERN BLOT ANALYSIS

Southern blot analysis was utilised to detect the FSHD associated DNA rearrangements at the D4Z4 locus in the South African FSHD families. Probe p13E-11 was utilised for the detection of the deletion fragments and was kindly donated to the South African FSHD research project by Dr. Silvère van der Maarel, Department of Human Genetics, Leiden University, Medical Center.

3.4.1 Restriction fragment length polymorphism (RFLP) analysis

Restriction fragment length polymorphism (RFLP) analysis was performed on high molecular weight gDNA. Two restriction enzyme digestion reactions were performed for each patient. Five micrograms of gDNA were utilised in each reaction. Reactions were incubated at 37°C for at least six hours, in the presence of 25 U *Eco* RI restriction endonuclease [Amersham], 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreithol and 3.3 mM spermidine [Sigma[1]] in a final volume of 30 µl. The double restriction enzyme reaction contained 25 U *Eco* RI restriction endonuclease [Amersham] and 25 U *Bln* I restriction endonuclease [Amersham], 2X one-phor-all buffer (20 mM Tris-acetate (pH7.5), 20 mM magnesium acetate, 100 mM potassium acetate), to prevent star activity [Amersham], and 3.3 mM spermidine in a final volume of 40 µl.

3.4.2 Agarose gel electrophoresis

Two and a half microlitres of the restriction endonuclease digestion reaction was mixed with 1 µl of a 2X loading buffer (0.04 % Orange G [Sigma®] and 50 % glycerol) prior to loading on a gel. The 0.5% agarose gel contained 1.25 g molecular grade agarose [Promega] and 1.268 µM EtBr [Sigma®]. The agarose was dissolved in 1X TBE buffer in a total volume of 25 ml. The mini submarine agarose gel was electrophoresed in 1X TBE buffer at 100 V for 30 minutes and then visualised via UV transillumination. After confirmation of complete digestion the remaining product was loaded onto a maxi or midi (both 23 cm in length) 0.5% submarine agarose gel along with a molecular weight marker to determine the fragment sizes. The agarose gel was electrophoresed in 1X TBE buffer at 35V ($1.5V.cm^{-1}$) for 30-48 hours. After 24 hours, the 1X TBE buffer was replaced.

[1] Sigma® is a registered trademark of Sigma Chemical Company, St. Louis, MO, U.S.A.

3.4.3 Genomic DNA Transfer

The agarose gel was removed from the gel apparatus and stained for 20 minutes in 2 $\mu g.ml^{-1}$ EtBr dissolved in 1X TBE buffer (if not added directly to gel mixture) with gentle shaking after which it was visualised under an UV light before blotting. The gDNA was subsequently nicked with 0.25 M HCl for 15 minutes and then denatured with fresh alkaline blotting solution (0.4 M NaOH, 1.6 M NaCl) twice for 20 minutes with gentle shaking. Hybond™[1] N^+ membrane [Amersham] was simultaneously prewet for 10 minutes in distilled water and subsequently 15 minutes in alkaline blotting solution. The DNA was transferred to the Hybond N^+ membrane overnight utilising the alkaline blotting solution and capillary action. After Southern transfer, the blot was neutralised for 5 minutes in neutralization solution (0.2 M Tris-HCl (pH 8.0), 2X SSC - 1.75% NaCl [ACE], 0.88% Na-citrate [Merck]). Fixation of the DNA was accomplished by baking the membrane for 2 hours at 80°C. The membrane was subsequently heat sealed in a plastic bag and stored at 4°C until hybridisation. The transfer efficiency was determined by staining the gel after the overnight transfer with 2 $\mu g.ml^{-1}$ EtBr dissolved in 1X TBE buffer for 30 minutes followed by visualisation via UV light.

3.4.4 Isolation of p13E-11

Probe p13E-11 was cloned as an 800 bp insert into the pBluescript plasmid. Five millilitres of LB-broth containing ampicillin was inoculated with the p13E-11 stab culture and incubated with shaking overnight at 37°C. Five millilitres of the overnight culture was added to 250 ml of LB broth containing ampicillin. The larger culture was incubated overnight at 37°C with shaking. The Wizard® Purefection Plasmid DNA Purification system was utilised for the isolation of the plasmid. Two reactions of 40 ml each were performed and the remaining culture was frozen at 70°C in the presence of 15% glycerol for future use.

The bacterial cells were pelleted by centrifugation at 10,000 xg for 10 minutes at 22°C. The supernatant was discarded and the excess liquid was blotted on a paper towel. Six millilitres of Cell Resuspension Solution was added to each tube and the cell pellet

[1] Hybond™ is a trademark of Amersham International plc., Buchinghamshire England.

resuspended by vortexing. The cells were lysed by adding 6 ml of Cell Lysis Solution and mixed thorougly by inverting the tube 6-8 times. The mixture was incubated at 22°C for 5 minutes after which 8 ml of Neutralization Solution was added and the tube again thoroughly mixed by inverting it 6-8 times. The bacterial lysate was centrifuged twice at 10,000 xg for 20 minutes at 22°C and the supernatant was then transferred to a clean 50 ml centrifuge tube.

After thorough resuspension of the Endotoxin Removal Resin 1 ml was added to the supernatant. The reaction was incubated for 10 minutes at 22°C, with vigorous shaking for 5 seconds at several intervals during the incubation. The tube was placed onto the MagneSil™[1] Magnetic Separation Unit for 30 seconds until the solution turned clear. While keeping the tube on the magnet, the supernatant was transferred to a new tube. Four and a half millilitres of 5 M guanidine thiocyanate was added to the supernatant followed by an addition of 3.5 ml of MagneSil™ Paramagnetic Particles. The reaction was mixed and incubated at 22°C for 3 minutes. The tube was placed onto the magnetic unit and the solution allowed to clear for 30 seconds before the supernatant was discarded. The tube was placed on the magnetic unit for another 3 minutes and the residual liquid discarded.

The particles, to which the plasmid DNA was bound, were then washed with 4.5 ml of 4.2 M guanidine-HCl/40% isopropanol solution. The particles were completely resuspended by vortexing for 10 seconds. The tube was placed on the magnetic unit and allowed to clear for 30 seconds after which the supernatant was discarded and the tube placed onto the magnetic unit for another 3 minutes and the residual liquid discarded.

Ten millilitres of 80% ethanol wash solution was added and the particles completely resuspended by vortexing for 10 seconds. The tube was placed on the magnetic unit and the solution allowed to clear for 30 seconds after which the supernatant was discarded. The washing step was repeated for a total of 3 washes. After the final wash, the 80% ethanol wash solution was carefully removed and the tube was left open for 10 minutes on the magnet to allow evaporation of any remaining ethanol. Any residual liquid was removed from the bottom of the tube. The tube was removed from the magnet and 6.0 ml

[1] MagneSil™ is a trademark of Promega Corporation, Madisin, WI, U.S.A.

high quality water (freshly distilled and free from enzymes) was added to the particles and vortexed for 10 minutes. The reaction was incubated at 22°C for 1 minute and then placed onto the magnet where the solution was allowed to clear. The supernantant was transferred to a sterile 15 ml centrifuge tube.

The DNA was precipitated by the addition of 0.5 volume of 7.5 M ammonium acetate and 2.5 volume of 95 % ethanol. The DNA was collected by centrifugation at 14,000 xg for 15 minutes at 22°C and the supernatant discarded. The pellet was then rinsed in 250 µl 70% ethanol followed by centrifugation at 14,000 xg for 5 minutes. The ethanol was carefully aspirated and the pellet air-dried for 5 minutes. The DNA was resuspended in 1.5 ml high quality water and the concentration determined.

The insert was excised from the plasmid through a double digestion with *Sac* I [Amersham] and *Eco* RI [Amersham]. The restriction enzyme digestions were performed in reactions containing 1.7 µg plasmid DNA, 20 U *Sac* I, 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$ and 1 mM dithiothreitol. The reactions were incubated at 37°C for 2 hours followed by inactivation of the enzyme by incubation at 65°C for 15 minutes. Subsequently 30 U *Eco* RI, 50 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 1 mM dithiothreitol and 100 mM NaCl was added. The reactions were incubated at 37°C for 2 hours. A portion of the reaction was loaded on a 1% agarose gel to determine the efficiency of the digestions. Upon verifying that the plasmid was completely digested the remaining product was loaded on a 0.8 % low melt agarose gel [FMC]. Agarose gel electrophoresis was performed as described in paragraph 3.4.2. The 0.8 kb insert was excised from the gel with a razor blade and was placed in an eppendorf tube. Three millilitres water per gram of gel was added and the mixture placed in a boiling water bath for 7 minutes to melt the gel and denature the DNA. The DNA was divided into aliquots and stored at -20°C.

3.4.5 Radio-active detection

The fragments on the membrane were detected after hybridisation with a radio-actively labelled probe. The membrane was washed to remove any non-specific binding and exposed to X-ray film for visualisation.

3.4.5.1 Labelling of probe p13E-11 and molecular weight markers

Twenty five nanograms of the p13E-11 probe DNA was labelled by the random nonamer priming method (Multiprime Kit, Amersham) with α-^{32}PdCTP for each hybridisation reaction. The DNA was denatured by heating to 95°C for one minute then chilled on ice. Ten µl reaction buffer containing dATP, dGTP, and dTTP in a concentrated buffer solution with Tris-HCl (pH 7.8), magnesium chloride and 2-mercaptoethanol (the exact concentrations were not provided in the kit), 5 µl primer (the primer concentration was not provided in the kit), 2 U enzyme, 50 µCi α-^{32}PdCTP and the appropriate amount of water to give a final reaction volume of 50 µl were added to the denatured DNA on ice. The reactions were incubated at room temperature overnight.

DNA molecular weight marker XV [Roche] and DNA molecular weight marker III [Roche] were utilised for allele sizing in this study. Molecular weight marker XV consists of fragments ranging from 2,392 to 48,502 bp, while molecular weight marker III consists of fragments ranging from 125 to 21,226 bp. The molecular weight markers were initially also labelled by random priming, but the signal intensity was too strong, and end-labelling was therefore utilised instead.

3.4.5.2 Hybridisation conditions

The membrane was prehybridised for at least 30 minutes at 65°C in 40 ml hybridisation solution (0.125 M Na_2PO_4 (pH 7.2) [USB], 0.25 M NaCl [ACE], 1 mM EDTA [ACE], 7% SDS [USB], 10% PEG-6000 [ACE] and supplemented with 10 µg.ml^{-1} denatured salmon sperm DNA [Sigma]. The labelled probe and molecular weight markers were denatured for 10 minutes at 95°C and placed on ice prior to hybridisation. After addition of the denatured probe and molecular weight markers to the prehybridisation solution, the blot was incubated overnight (16 hours) at 65°C in a Techne hybridisation oven.

3.4.5.3 Wash conditions

After overnight incubation with the denatured probe and molecular weight markers, the blot was washed three times for 5 minutes with washing solution (2X SSC (1.75% NaCl, 0.88% Na-citrate), 1% SDS) at 65°C. The membrane was heat sealed in a plastic bag, after removal of excess air bubbles.

3.4.5.4 Autoradiography

The hybridised membrane was exposed to X-ray film (Fuji RX-U) utilising an intensifying screen at -70°C for the appropriate time. The X-ray film was developed and fixed to allow the visualisation of the fragments.

3.4.6 Non radio-active detection

The Detector™[1] Random Primer DNA biotinylation kit and the DNADetector™ Genomic Southern Blotting kit [Kirkegaard & Perry Laboratories] were utilised for the non radio-active detection of the DNA rearrangements at the D4Z4 locus. The random primer kit utilises six base random sequence oligonucleotides as primers for the replication of the template DNA. The probes are labelled through the incorporation of biotin-N_4-dCTP during the random-primer extension by Klenow DNA polymerase. The labelled probe is then detected with the DNADetector™ kit.

3.4.6.1 Labelling of probe p13E-11

One hundred nanograms of probe DNA was dissolved to a final volume of 24 µl in DEPC treated water and mixed with 15 µg of 2.5X random primer solution. The DNA was denatured by heating the sample to 95°C for 5 minutes and immediately transferred to ice. 5 µl of the 10X dNTP mixture (0.1 mM biotin–N_4-dCTP, 0.1 mM dCTP, 0.2 mM dATP, 0.2 mM dGTP and 0.2 mM dTTP in 1 mM Tris-HCl (pH 7.5) and 0.1 mM Na_2EDTA) and 10 U Klenow polymerase was added to obtain a final volume of 50 µl. The reaction was gently mixed and incubated at 37°C for 1-4 hours. One molar Na_2EDTA (pH 7.5) was subsequently added to terminate the reaction.

3.4.6.2 Quantification of labelled probe

The amount of labelled probe was quantified utilising the DNADetector™ Genomic Southern Blotting Kit [KPL]. This kit was designed for the hybridisation and

[1] Detector™ is a trademark of Kirkegaard & Perry Laboratories, Inc, Gaithersburg, MD, U.S.A.

chemiluminescent detection of biotinylated DNA probes. An initial 1:200 dilution, with 2X SSC/0.1% SDS as the diluent, of the labelled probe was made after which a series of two-fold dilutions from both the 1/200 probe dilution and the undiluted biotinylated DNA standard were prepared. One microlitre of each dilution was spotted on a membrane which was pre-soaked in water and 5X SSC for 10 minutes where after it was dried for 10 minutes. The spotted dots were subsequently dried for 10 minutes and fixed onto the membrane by baking for 30 minutes at 80°C.

A blocking/diluent solution was prepared by diluting the 5X Detector Block solution 1:5 in ddH_2O and adding 0.2% (w/v) Detector Block Powder (DBP). The DBP was completely dissolved in a 50°C waterbath and cooled to room temperature before use. The dot blot was incubated for 30 minutes with 0.1 ml diluted blocking solution per cm^2 membrane after which the dot blot was incubated for 30 minutes in 0.1 ml per cm^2 membrane phosphatase-labelled streptavidin (AP-SA) diluted 1:10,000 in blocking/diluent solution. A 1X Phosphatase Wash Solution was prepared and the membrane was washed 3 times, for 5 minutes each, using at least 1 ml of wash solution per cm^2 membrane. The membrane was subsequently rinsed twice for 2 minutes each in 1X Phosphatase Assay Buffer (PAB) using at least 0.5 ml per cm^2 membrane. The excess PAB was drained off and the membrane was incubated in CDP-Star®[1] Chemiluminescent Substrate using at least 0.05 ml per cm^2 membrane for 5 minutes. Excess CDP-Star was removed by blotting the membrane on filter paper after which the membrane was heat sealed and exposed to X-ray film for ca. 2 minutes.

The total yield of biotinylated DNA probe was then determined by the comparison of the signal intensities of the biotinylated DNA standard and the biotinylated DNA probe. The ratio of probe to standard (P/S) was determined by the following equation:
P/S = probe endpoint dilution / standard endpoint dilution, after which the relative specific activity of the labelled probe was calculated by: Probe RSA = standard RSA x P/S. The relative specific activity (RSA) of the labelled probe is defined by the relative amount of biotin attached to the DNA. The total yield of biotinylated DNA probe could then be determined by: Total yield = probe RSA x reaction volume. The labelling reaction generally generates a 5 to 10 fold amplification of the template after one hour, and 10 to 50 fold amplification after 4 hours.

[1] CPD-Star® is a registered trademark of Tropix Inc., Bedford, MA, U.S.A.

3.4.6.3 Hybridisation conditions

The target DNA was immobilised on a Hybond N^+ membrane and prehybridised in 0.10 ml of Formamide Hybridisation Buffer (supplemented with 200 $\mu g.ml^{-1}$ Salmon Sperm DNA) per cm^2 membrane for 1 hour at 42°C. Fifty $ng.ml^{-1}$ of the biotinylated probe was denatured at 95°C for 10 minutes and immediately placed on ice. The denatured probe was added directly into the prehybridisation solution and incubated for 16 hours at 42°C.

3.4.6.4 Wash conditions

Following hybridisation the membrane was washed to remove any excess probe. The membrane was washed twice for 15 minutes in at least 1 ml per cm^2 of membrane 2X SSPE [0.3 M NaCl, 20 mM NaH_2PO_4, 2 mM EDTA (pH 7.4)]/0.1% SDS at room temperature. A second wash with 0.2X SSPE [0.03 NaCl, 2 mM NaH_2PO_4, 0.2 mM EDTA (pH 7.4)]/0.1% SDS at 55°C was performed twice for 15 minutes. Residual SDS was removed with a final wash with 2X SSPE at room temperature for 5 minutes.

3.4.6.5 Detection

The membrane was subsequently incubated in 1X blocking/diluent solution (1X detector block solution supplemented with 1% Detector Block Powder) for 45 minutes (0.25 ml 1X blocking/diluent solution per cm^2 membrane) and subsequently incubated for 30 minutes in 0.25 ml per cm^2 membrane AP-SA solution (AP-SA conjugate diluted 1:10,000 in 1X blocking/diluent solution). A 1X Phosphatase Wash Solution was utilised to wash the membrane three times for 5 minutes each using 0.75 ml of wash solution per cm^2 membrane. The membrane was rinsed for 5 minutes in 1X Phosphatase Assay Buffer using 0.4 ml per cm^2 membrane after which the CDP-Star was added (0.04 ml per cm^2 membrane) and incubated for 5 minutes. The excess CDP-Star was removed by blotting the membrane on filter paper after which it was heat sealed in a hybridisation bag. The membrane was exposed for 10 minutes to X-ray film at room temperature where after the X-ray film was developed and fixed.