CHAPTER THREE

Materials and Methods

This molecular investigation forms part of the ongoing MH research programme. Ethical approval for the MH project titled "Molecular analysis of malignant hyperthermia (MH) susceptibility" was obtained for this study in 2002 (approval number 02M10) and 2007 (approval number NWU-00040-07-S0) from the Ethics Committee of North-West University. Informed consent was obtained from the patients involved in this project prior to their participation.

The molecular investigation was conducted using a group of 15 probands from the South African MH population. All individuals selected had experienced an MH episode or were diagnosed as MHS with the IVCT. Only the proband was screened for mutations and family members of these individuals were not included in this study. If a positive result should be obtained in the proband for any of the mutations examined, family members of the proband would be screened. Individuals from six of these families were diagnosed through muscle contracture studies, and the results of the biopsies are discussed in Section 3.1.1 (page 74) and presented in Table 3.1. Individuals from the remaining nine families had not previously been diagnosed via the IVCT, thus their MH status is currently unknown.

As samples were continually being collected for the MH research programme, a numbering system, which included a unique family identity number followed by a unique individual number within that particular family, was used to maintain consistency. Families were numbered MH101 for example, and individuals were allocated a number following the family identification number, for example MH101-123.

3.1 PATIENT POPULATION

Blood samples for DNA extraction were collected from probands and family members of the index case. Probands included in this investigation were diagnosed as MHS based on clinical signs of MH observed during previous exposure to anaesthesia (if biopsy data were unavailable) or according to the IVCT protocol outlined by the EMHG. Individuals

included in this study were diagnosed as being susceptible to MH according to their clinical status as indicated by clinical records. In certain cases, individuals who had experienced an MH episode were subsequently diagnosed via the IVCT. Clinical records indicate that these individuals had undergone a muscle biopsy. However, these results were not available to the researcher.

In agreement with the recommendations of the EMHG (1984), a biopsy sample was characterised as MHS if the muscle strip exposed to halothane or caffeine exceeded the acceptable diagnostic contracture threshold of 0.2 g at caffeine concentrations of 2 mM or less, and halothane concentrations of 2% or less. Individuals whose muscle strips did not meet these criteria were diagnosed as MHN. Individuals were characterised as MHE if the contracture of the muscle strips at the threshold concentration occurred for either caffeine or halothane.

3.1.1 Individuals from MH families included in this study

According to the IVCT protocol, probands were typed MHS, MHN or MHE, as discussed in Section 3.1 (page 73). Equivocal results are indicated as MHEh and MHEc, depending on whether the MHEc or MHEh tests were positive. Results of the IVCT for six of the MH families included in this study are listed in Table 3.1. Diagnoses were confirmed by muscle tension studies, performed by Prof H. Isaacs from the Department of Physiology at the University of the Witwatersrand.

Table 3.1: Diagnostic *in vitro* contracture test results as determined by the European *in vitro* contracture test protocol

Family number	Caffeine (mM)	Halothane (vol %)	MH status	Family number	Caffeine (mM)	Halothane (vol %)	MH status
MH101-6	2.0	1.0	MHS	MH104-35	2.0	1.0	MHS
MH101-10	2.0	0.5	MHS	MH104-40	8.0	NR	MHN
MH101-12	4.0	4.0	MHN	MH104-41	8.0	NR	MHN
MH102-2	1.0	1.0	MHS	MH105-20	2.0	1.0	MHS
MH102-4	8.0	4.0	MHN	MH105-23	4.0	4.0	MHN
MH102-11	8.0	4.0	MHN	MH105-26	2.0	0.5	MHS
MH102-24	0.5	1.0	MHS	MH105-28	4.0	4.0	MHN
MH102-28	2.0	1.0	MHS	MH105-32	1.0	1.0	MHS
MH102-39	2.0	0.5	MHS	MH105-35	2.0	1.0	MHS
MH102-48	0.0	0.0	MHN	MH105-36	1.0	2.0	MHS
MH102-96	4.0	2.0	MHEh	MH105-37	4.0	4.0	MHN
MH102-117	2.0	0.5	MHS	MH105-38	0.5	0.5	MHS
MH102-125	1.0	2.0	MHS	MH105-39	8.0	4.0	MHN

Table 3.1: Continued...

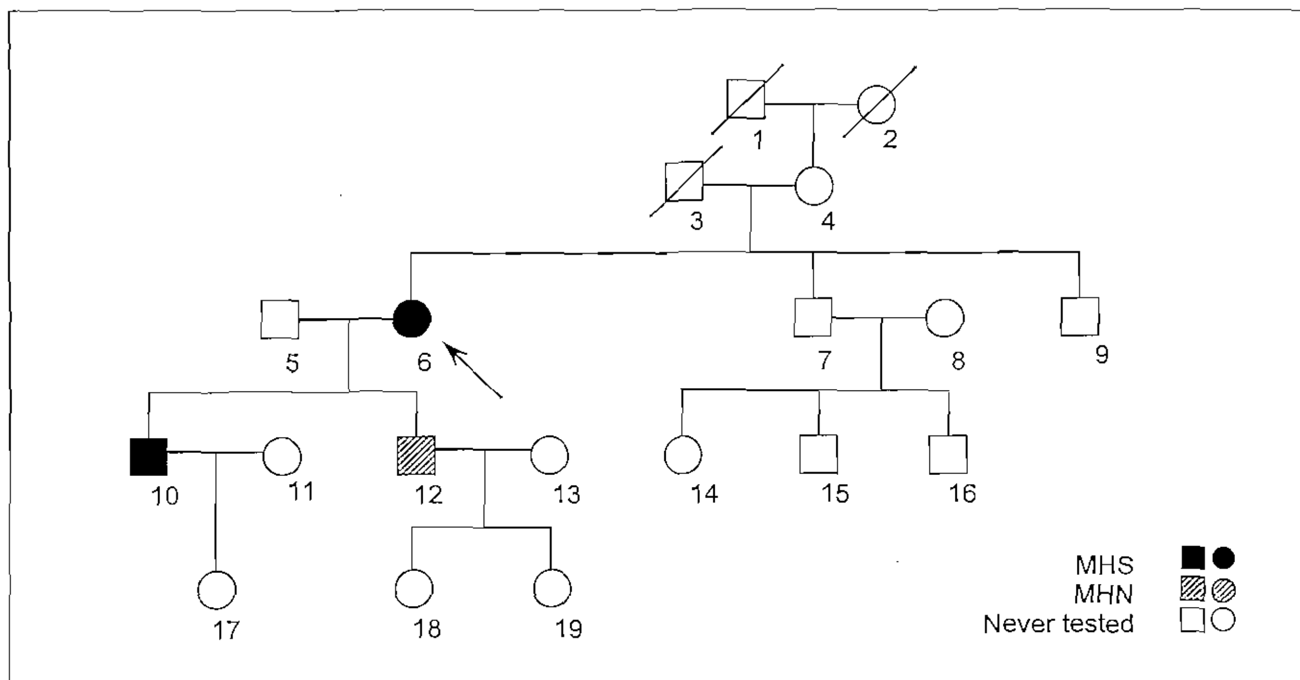
Family number	Caffeine (mM)	Halothane (vol %)	MH status	Family number	Caffeine (mM)	Halothane (vol %)	MH status
MH103-4	2.0	0.5	MHS	MH105-63	1.0	1.0	MHS
MH103-9	3.0	0.5	MHEh	MH105-64	1.5	0.5	MHS
MH104-24	8.0	4.0	False MHN	MH108-1	0.4	1.6	MHS
MH104-25	8.0	2.5	MHN	MH108-2	0.0	0.0	MHN
MH104-26	1.0	0.5	MHS	MH108-3	1.6	1.9	MHS
MH104-27	0.0	0.0	MHN	MH108-4	2.6	4.0	MHS
MH104-33	8.0	NR	MHN	---	---	---	---

MHS = malignant hyperthermia susceptible; MHN = MH normal; MHEh = MH equivocal, positive for halothane; mM = millimolar; NR = no response, vol % = percent volume per volume; (---) indicates information not available.

3.1.1.1 Malignant hyperthermia family MH101

Family MH101 consists of 19 members. The pedigree indicating all 19 individuals is presented in Figure 3.1. The proband, MH101-6, developed a high fever and experienced diaphoresis following surgery. She was subsequently diagnosed as MHS via the EMHG muscle contracture protocol.

Figure 3.1: Pedigree of family MH101



An explanation of the symbols is provided in the list of abbreviations and symbols. Adapted from Olckers (1997).

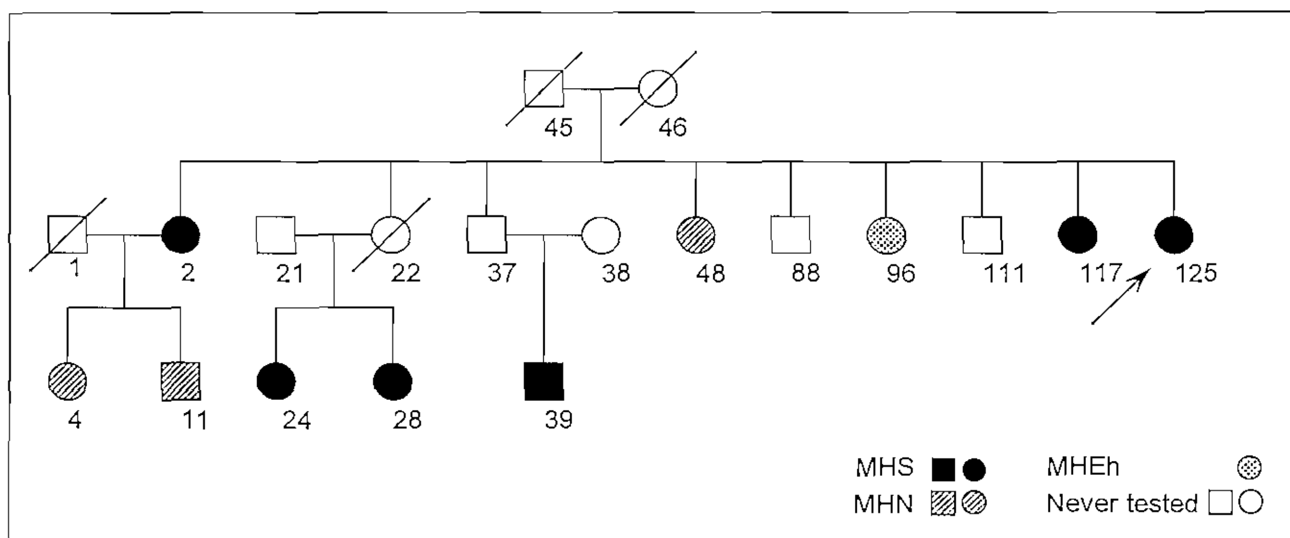
The muscle biopsy obtained from this individual indicated several small type 1 fibres that were disturbed by vacuole-like structures. In addition, electron microscopy indicated breakdown of myofilaments and many swollen mitochondria were observed in addition to extensive areas of lysosome-like structures. The two children of the proband, MH101-10

and MH101-12, were subsequently tested for MH and were diagnosed as MHS and MHN respectively. The results obtained from the IVCT for the three individuals indicated here, are listed in Table 3.1 (page 74).

3.1.1.2 Malignant hyperthermia family MH102

The pedigree for MH102 consists of 127 members. Biopsy data were available for ten individuals, and results obtained for those individuals are listed in Table 3.1 (page 74). An excerpt of the pedigree indicating the ten members for whom IVCT data are available is illustrated in Figure 3.2.

Figure 3.2: Excerpt from pedigree MH102

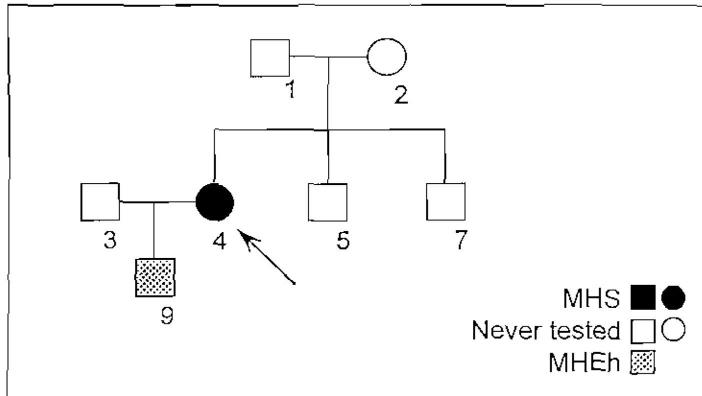


An explanation of the symbols presented is indicated in the list of abbreviations and symbols. Adapted from Olckers (1997).

The proband, MH102-125, developed pyrexia during anaesthesia, and was subsequently tested for MH. The young female yielded a positive reaction to halothane via the IVCT. However, the diagnosis was performed prior to the adoption of the protocol of the EMHG, which requires inclusion of both halothane and caffeine to diagnose MH. Consequently, this individual (MH102-125) was later re-tested using the caffeine contracture test. The positive result that was obtained further confirmed the diagnosis of MH positive. Individual MH102-96 was diagnosed as MHE following a positive reaction to halothane, but a negative reaction to caffeine. IVCT results for the remaining individuals tested, indicated an MHS diagnosis for individuals MH102-2, MH102-24, MH102-28, MH102-39 and MH102-117. The group of individuals diagnosed as MHN via the IVCT included MH102-4, MH102-11 and MH102-48.

3.1.1.3 Malignant hyperthermia family MH103

Figure 3.3: Pedigree of family MH103



An explanation of the symbols presented is indicated in the list of abbreviations and symbols. Adapted from Olckers (1997).

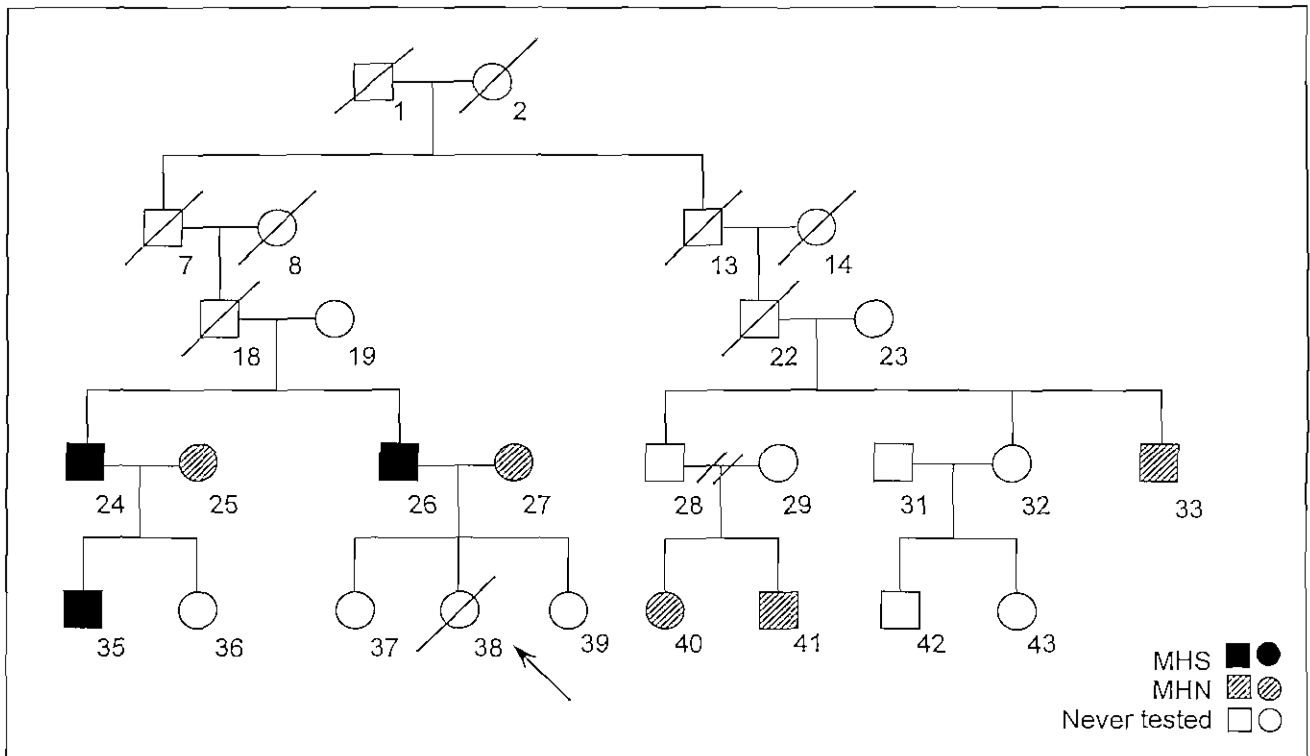
Family MH103 includes seven members. The pedigree of family MH103 indicating all seven members is illustrated in Figure 3.3. The proband, MH103-4 was diagnosed as MH positive via the IVCT. The son of the proband, MH103-9 was diagnosed as MHEh, as he showed a positive reaction to the halothane contracture but tested MH negative for the caffeine contracture. The results obtained for these two individuals are listed in Table 3.1 (page 74).

3.1.1.4 Malignant hyperthermia family MH104

The pedigree of MH104 consists of 46 individuals. An excerpt of the pedigree indicating 28 individuals is depicted in Figure 3.4. The proband, MH104-38, developed pyrexia following the administration of anaesthesia during a dental procedure when she was two years old. This was her first anaesthesia and it unfortunately resulted in her death. She was never tested for MH. The immediate family was subsequently tested for MH and available biopsy data indicated that individuals MH104-35 and MH104-26 were MH positive, while individuals MH104-25, MH104-27, MH104-33, MH104-40 and MH104-41 were MHN.

Individual MH104-24 was initially diagnosed as MHN. However, this diagnosis was actually a false negative and the individual was subsequently re-classified as MHS. False negative results have been reported previously for MH patients by Isaacs and Badenhorst (1993) with the CHCT. Individual MH104-26 was screened for mutations, as no material was available to include the proband (MH104-38) in this study. The IVCT results obtained for these individuals are listed in Table 3.1 (page 74).

Figure 3.4: Excerpt from pedigree MH104



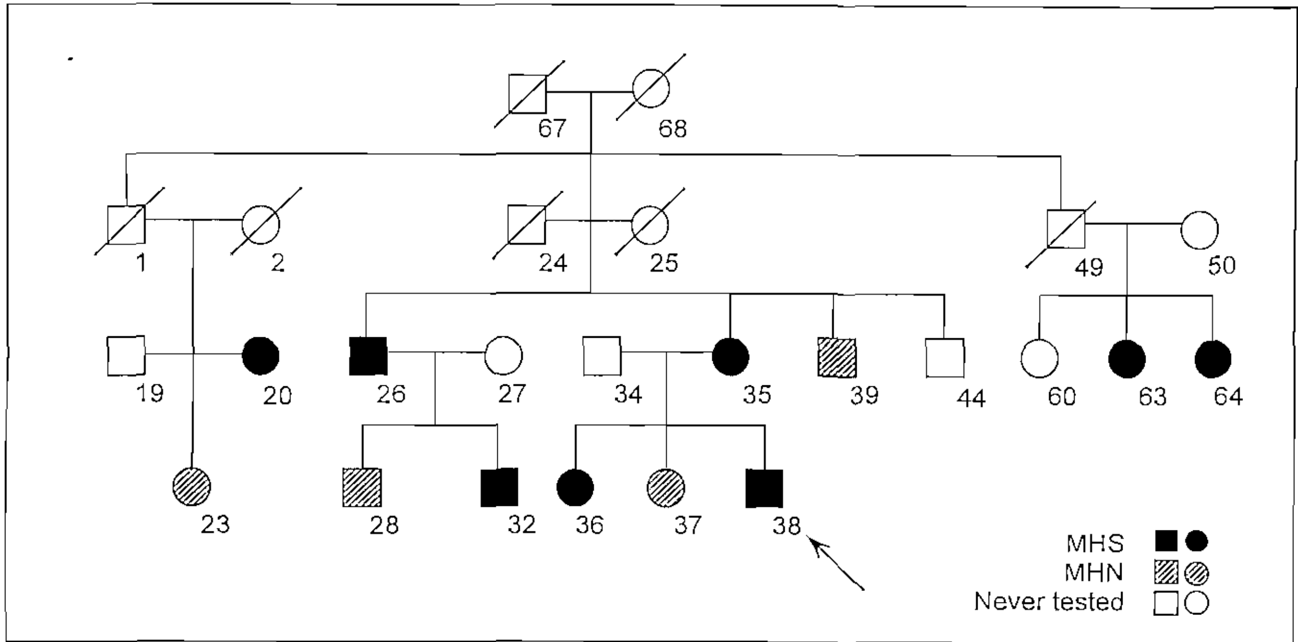
An explanation of the symbols presented is indicated in the list of abbreviations and symbols. Adapted from Olckers (1997).

3.1.1.5 *Malignant hyperthermia family MH105*

Family MH105 includes 153 members. IVCT data are available for the distant branches of this pedigree, but are not indicated. An excerpt of this pedigree, indicating 25 individuals, is displayed in Figure 3.5.

The proband, MH105-38 developed an MH-like reaction following the administration of anaesthesia. He subsequently tested MH positive via the IVCT at the age of 14. Following his positive result, some members of the extended family were also tested for MH. Biopsy data indicated that individuals MH105-20, MH105-26, MH105-32, MH105-35, MH105-36, MH105-38, MH105-63 and MH105-64 were MHS. Individuals MH105-23, MH105-28, MH105-37 and MH105-39 were all diagnosed as MHN. All biopsy results obtained for this pedigree are listed in Table 3.1 (page 74).

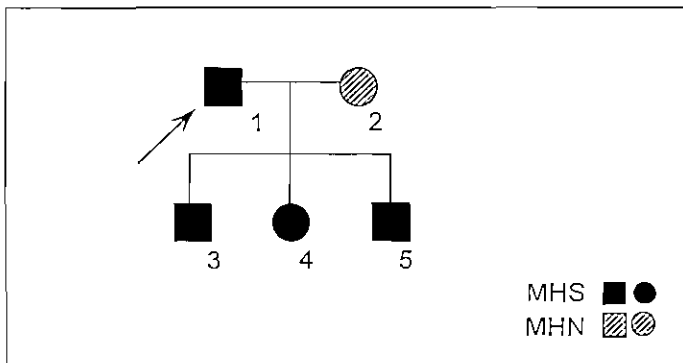
Figure 3.5: Excerpt from pedigree MH105



An explanation of the symbols presented is indicated in the list of abbreviations and symbols. Adapted from Olckers (1997).

3.1.1.6 Malignant hyperthermia family MH108

Figure 3.6: Pedigree of family MH108

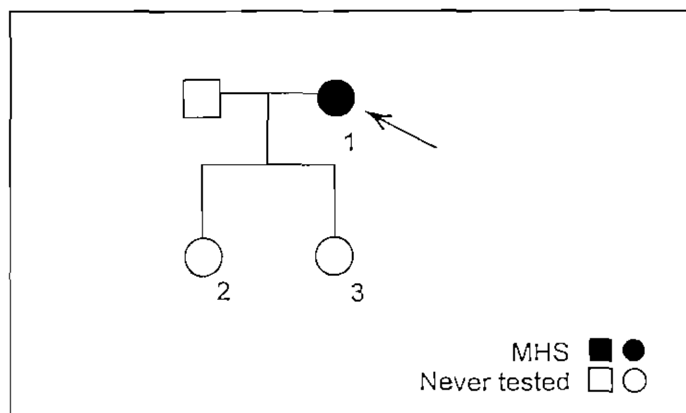


An explanation of the symbols presented is indicated in the list of abbreviations and symbols.

Family MH108 includes five members. The pedigree of family MH108 indicating all five members is illustrated in Figure 3.6. Muscle biopsies were conducted for all individuals in this family. However, IVCT data were only available for four individuals, and results obtained for those individuals are listed in Table 3.1 (page 74). The proband (MH108-1) was diagnosed as MHS. Biopsy results for this family indicated a family history of MH and designated MH108-3, MH108-4 and MH108-5 as MHS and MH108-2 as MHN.

3.1.1.7 Malignant hyperthermia family MH111

Figure 3.7: Pedigree of family MH111



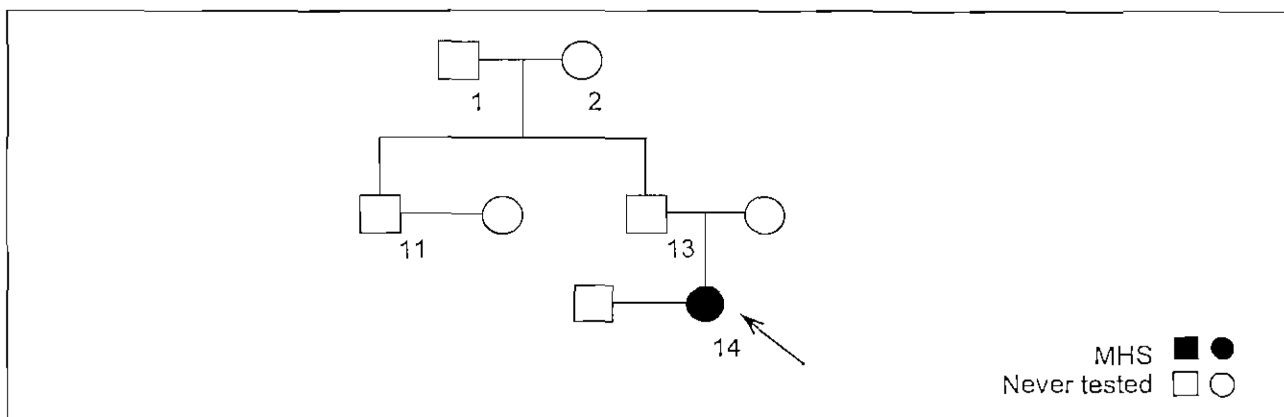
An explanation of the symbols presented is indicated in the list of abbreviations and symbols.

Family MH111 consisted of four members, as illustrated in Figure 3.7, and three individuals were available for analysis, i.e. a maternal parent and two children. The maternal parent (MH111-1) was identified as the proband and indicated scoline and halothane sensitivity during anaesthesia. She was consequently diagnosed as MHS via a muscle biopsy. Results of the biopsy were not made available to the researcher. The MH status of the children (MH111-2 and MH111-3) is unknown.

3.1.1.8 Malignant hyperthermia family MH113

The pedigree of family MH113 includes 16 members. An excerpt of this pedigree indicating eight individuals is illustrated in Figure 3.8. Individual MH113-14 (MH00381) has been identified as the proband and experienced an MH episode during a previous exposure of anaesthesia. Blood from her father, individual MH113-13, could, however, not be obtained. The MH status of all individuals from family MH113 has not been confirmed via IVCT testing and their MH status is currently unknown.

Figure 3.8: Excerpt from pedigree MH113

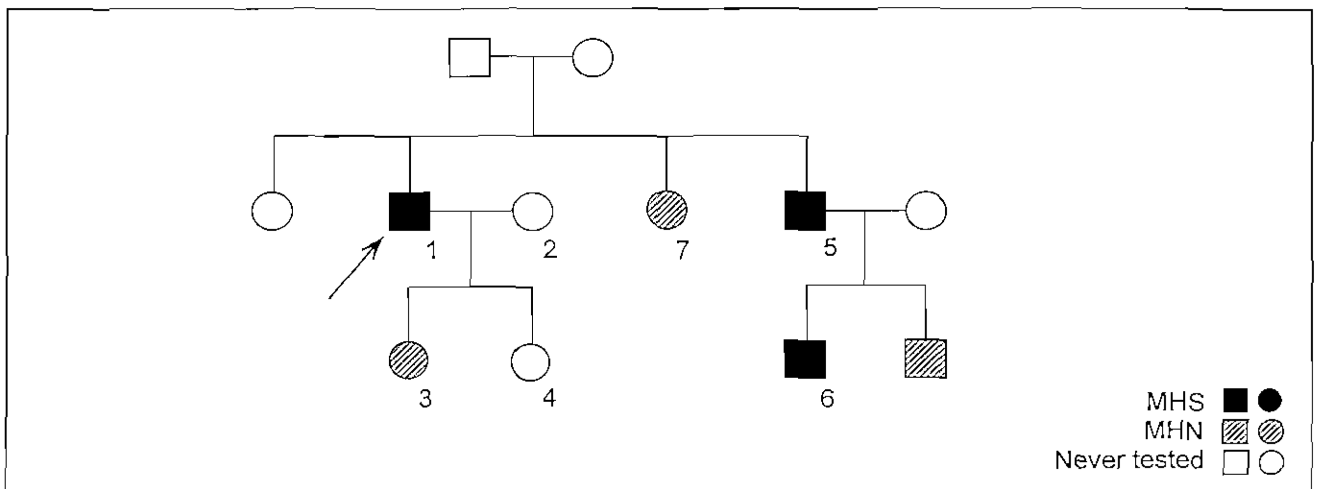


An explanation of the symbols presented is indicated in the list of abbreviations and symbols.

3.1.1.9 Malignant hyperthermia family MH114

Family MH114 consists of 12 members, as indicated in the pedigree in Figure 3.9. Only seven members were available to participate in this study. The grandparents (not numbered) were not available for testing. Muscle biopsies were conducted on all individuals in this family. However, IVCT data were not made available to the researcher. The paternal parent (MH114-1) was identified as the proband and was diagnosed as MHS via the IVCT. He has one child who was diagnosed as MHN (MH114-3) and another daughter (MH114-4) who still has to be tested. In addition, the proband's brother, MH114-5, was diagnosed as MHS. He has one son who was identified as MHS and another who was diagnosed as MHN. The MH status was provided by the individuals included in this study, and still needs to be verified.

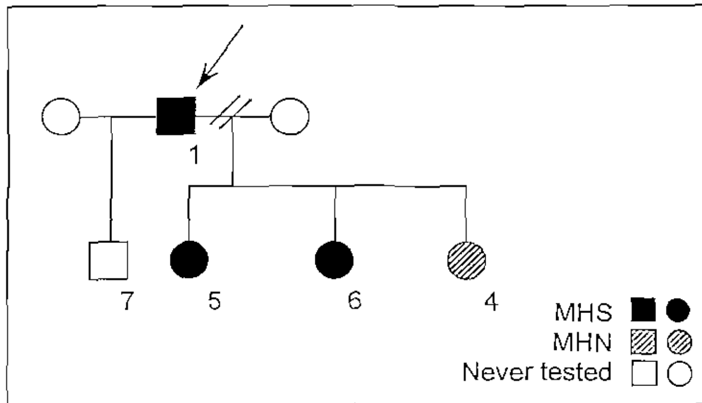
Figure 3.9: Pedigree of family MH114



An explanation of the symbols presented is indicated in the list of abbreviations and symbols.

3.1.1.10 Malignant hyperthermia family MH115

Figure 3.10: Pedigree of family MH115



An explanation of the symbols presented is indicated in the list of abbreviations and symbols. Adapted from Olckers (1997).

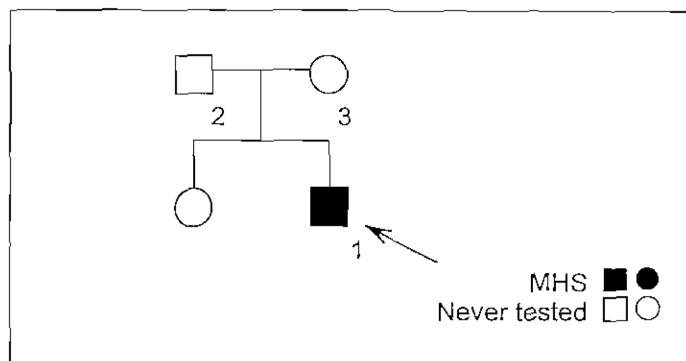
Family MH115 consists of 14 members. However, only five members were available to participate in this study, as indicated in the pedigree in Figure 3.10. The father (MH115-1) is the proband and was diagnosed as positive via the IVCT. MH115-1 had four children, of which two were diagnosed as IVCT positive (MH115-5 and MH115-6) and one was diagnosed as MHN (MH115-4). The status of one individual (MH115-7) is currently unknown.

3.1.1.11 Malignant hyperthermia family MH122

MH122-1 was identified as the proband, as he has a family history of MH and experienced an MH episode during previous anaesthesia. The MH status of his child (MH122-2) is unknown. A family tree was not constructed for this pedigree, as the family structure was not made available to the researcher.

3.1.1.12 Malignant hyperthermia family MH123

Figure 3.11: Pedigree of family MH123



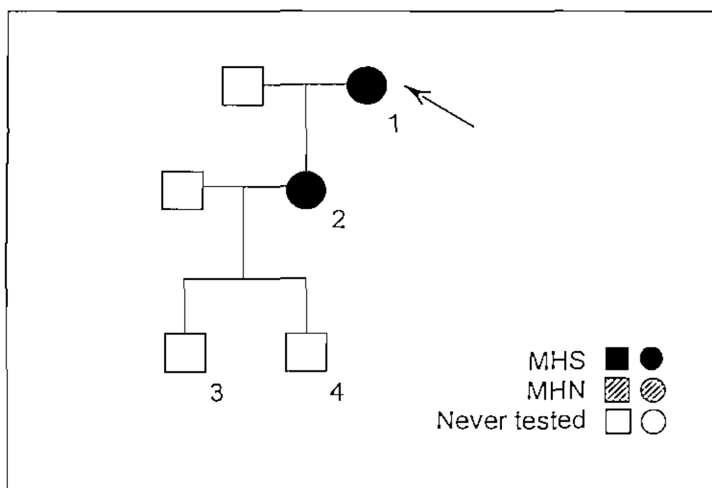
An explanation of the symbols presented is indicated in the list of abbreviations and symbols.

The pedigree of MH123 consists of four family members, of which three were available for testing, as illustrated in Figure 3.11. The child (MH123-1) is the proband and developed an MH episode during anaesthesia. Clinical symptoms developed during anaesthesia included acidosis and fever. The MH status of the parents of the proband, MH123-2 and MH123-3, is unknown. The sister of MH123-1 was not available for testing.

3.1.1.13 Malignant hyperthermia family MH125

In family MH125 only four members were available to participate in this study. The pedigree of MH125, indicating six members, is illustrated in Figure 3.12. The grandmother was identified as the proband and has a family history of MH. Her uncle died from cardiac arrest due to an MH episode during an anaesthetic procedure. The proband also experienced cardiac arrest during a back operation and took a long time in recovery. Muscle biopsies were conducted for individuals in this family. However, IVCT data were not made available to the researcher. The proband was diagnosed as MHS via an IVCT. The proband's daughter (MH125-2) was also diagnosed as MHS and displayed clinical symptoms of MH during a previous anaesthetic procedure. She has two children (MH125-3 and MH125-4). The MH status of both her sons is currently unknown, however, it is suspected in the one child, as he took a long time in recovery following an anaesthetic procedure and displayed an increase in body temperature. The second son (MH125-4) was diagnosed with liver-specific lysosomal acid phosphatase deficiency (API).

Figure 3.12: Pedigree of family MH125



An explanation of the symbols presented is indicated in the list of abbreviations and symbols.

3.1.1.14 Malignant hyperthermia family MH00630

DNA samples were obtained from a proband (MH00630) and the father of this individual. However, both individuals have not yet been assigned a family identification number and the MH status of the father is unknown. MH00630 was identified as the proband and was identified as MHS based on clinical symptoms observed during previous anaesthesia. During a previous anaesthetic procedure the individual experienced an increase in carbon dioxide partial pressure (pCO_2) and elevated body temperature was recorded during an anaesthetic procedure. In addition, the proband has a family history of MH, as his sister died from MH following an anaesthetic procedure. A family tree was not constructed for this pedigree, as the family structure was not made available to the researcher.

3.1.1.15 Malignant hyperthermia family MH00654

MH00654 was identified as the proband and was identified as MHS based on clinical symptoms observed during previous anaesthesia. During a previous anaesthetic procedure the individual experienced symptoms associated with MHS. A DNA sample was also collected from the parents of the proband, with unknown MHS status. A family tree was not constructed for this pedigree, as the family structure was not made available to the researcher.

3.2 MUTATION ANALYSIS

The complete structure of the human RYR1 gene was first described by Phillips *et al.* (1996). The length of the gene was determined by aligning 16 genomic phage clones, a cosmid clone and several long polymerase chain reaction products. The RYR1 gene encompasses 158,000 bp of gDNA and consists of 106 exons. The length of exons ranges from 15 to 813 bp, while introns range from 85 to 16,000 bp in length.

3.2.1 Primer design

Oligonucleotide primers that were used for the study presented, were specifically designed and synthesised in order to amplify all 106 exons of the RYR1 gene. In general, primers were selected that demonstrated homology to both chromosome 19 at location 43615580 - 43770612 and to the published human RYR1 gene sequence. The chromosome 19 clone was obtained from a direct submission by the DOE Joint Genome Institute and Stanford Human Genome Centre to Genbank® in 2000 (unpublished) and was retrieved in the study presented, using Ensembl, version 36 (Hubbard *et al.*, 2005). The human RYR1 sequence was obtained from Phillips *et al.* (1996).

A selection of primers for a specific region or exon was designed via the Oligo Analyzer program obtained from the Integrated DNA Technology (IDT) website, the sequence provided was analysed and the program supplied a selection of primer sets, all with a relatively narrow melting temperature (T_m) range. Each set of primers was subsequently analysed using the IDT Oligo Analyser, which allows for online calculation of oligonucleotide parameters such as T_m, self-dimerisation, hairpin loop formation, GC content and primer length. The Santa Lucia nearest-neighbour method was used by the IDT program to determine the T_m of the oligonucleotides. The method takes into account nearest-neighbour interactions (Breslauer *et al.*, 1986; Sugimoto *et al.*, 1996), salt

concentration and oligonucleotide concentration. Initially, the nearest-neighbour parameters to estimate the melting temperature of an oligonucleotide were calculated according to the method described by Breslauer *et al.* (1986). However, in recent years an improved set of parameters was published by Sugimoto *et al.* (1996) and SantaLucia (1998). Therefore, these parameters were used to calculate the T_m of the oligonucleotides. All primers were further analysed using the Basic Local Alignment Search Tool (BLAST) program version 2.2.9 (Altschul *et al.*, 1997), to determine if primers annealed to other regions of the human genome. Oligonucleotides that preferentially annealed with highest complementarities to the regions of interest in the human RYR1 gene were selected, and were synthesised by IDT.

In previous investigations, the polymerase chain reactions (PCR) protocols were optimised for specific primer sets. However, in the case where a primer set was designed for the study presented here, the reaction was optimised. Various parameters were considered and optimised, including the annealing temperature (T_a) for each primer set, the concentration of magnesium chloride ($MgCl_2$) in the reaction and the addition of formamide to the reaction. Optimisation of the T_a began at a temperature 5°C below the T_m, and was adjusted to improve specificity. All 106 exons of the RYR1 were amplified in 75 PCR reactions in order to analyse the entire coding sequence of this gene. The primers that were used for amplification and sequencing of all 106 exons are listed in Table 3.2, as well as the calculated T_m and product sizes.

Table 3.2: Oligonucleotide primers used for PCR and direct sequencing

Exon	Primer name	Sequence	Size (bp)[1]	T_m[2]
1	RYRex1F RYRex1R	F: 5'- gat ggc acg tta ctt acc gtg -3' R: 5'- cag aga gcc cca aga gat agc -3'	457	56 57
2	RYRex2F RYRex2R	F: 5'- ctg cag tat ttg tgg tat cc -3' R: 5'- ctc act ttc tct cct gtc ag -3'	300	51 52
3	RYRex3F RYRex3R	F: 5'- cat cca gac tag ggg agg gag tgt g -3' R: 5'- gtc ctc tcg ccc atc tct gcc acc -3'	246	62 65
4,5	RYRex4F RYRex4R	F: 5'- gtc cgg gga tct gtg ctt att ctg -3' R: 5'- agt ctc atg ctt gcc ttg gcg ttc -3'	378	59 62
6,7	RYRex6F RYRex6R	F: 5'- cct ggg gaa gag cat tct ggg aag -3' R: 5'- ggg caa cat taa ggg tct gtt ttg g -3'	656	61 59
8,9	RYRex8F RYRex8R	F: 5'- cat ctt ggc tcc tgg tct tcc tg -3' R: 5'- cat ctc tct ctc agg ctg ctc tg -3'	507	60 59
10,11	RYRex10F RYRex10R	F: 5'- ctc tga ctc ccc ttg gct ctc ac -3' R: 5'- gta cag tgg cat gat cac cag ctc a -3'	588	61 61
12	RYRex12F RYRex12R	F: 5'- ccc act cca gac ctc tgt ctc -3' R: 5'- gaa aga ggc caa gtg tat gga tg -3'	232	59 56
13	RYRex13F RYRex13R	F: 5'- cct ctc tgt aaa acg ggt ggg tct g -3' R: 5'- ggt ctc act ggc tgg ggt tcc tg -3'	480	61 62
14,15,16	RYRex14F RYRex14R	F: 5'- gag ggc ctg ggt ctc cta ttg -3' R: 5'- cag agt tca ggg gat gag agg -3'	605	59 57
17,18	RYRex17F RYRex17R	F: 5'- gtg cct aca cac cct tta acc tc -3' R: 5'- gga atc tag aag ctc tgg ggt tag -3'	1004	58 57
19	RYRex19F RYRex19R	F: 5'- gca ctt tcc att agg gtt tcc agg -3' R: 5'- gga agc tgt ctc agg tca gtc -3'	382	58 57
20	RYRex20F RYRex20R	F: 5'- ctc aac tcc ctg gct ctt aat tcc -3' R: 5'- ctg act cct aag aga ccc tgc -3'	489	57 57
21, 22	RYRex21F RYRex21R	F: 5'- ggt cat gat gga gga ggg tag ag -3' R: 5'- ctg ccc tgt ctc tcc atg cc -3'	565	58 61
23	RYRex23F RYRex23R	F: 5'- gtg acc tgt cgc ctc cac tc -3' R: 5'- cta tga cct tca ccc taa ccc aag -3'	256	60 57
24	RYRex24F RYRex24R	F: 5'- caa ggg tca gca gtc agg gat c -3' R: 5'- cag gtc aga gat cag gga tcg -3'	475	69 57
25	RYRex25F RYRex25R	F: 5'- cta cca act tct cga tgt ctt g -3' R: 5'- gga tga gtg gta cag tag atg -3'	402	53 52
26,27	RYRex26F RYRex26R	F: 5'- ctc tcc att tct ctg tgt gtc tcc -3' R: 5'- gag cac tgt gga agg aag gag c -3'	668	57 60

Table 3.2: Continued...

Exon	Primer name	Sequence	Size (bp)[1]	T_m[2]
28	RYRex28F RYRex28R	F: 5'- gtg tga cca ggt gta gga cca ac -3' R: 5'- gtt tct cag gtt act gtg gtt gcc -3'	551	60 58
29	RYRex29F RYRex29R	F: 5'- cat gaa tat tgc ggt ggg agg -3' R: 5'- cag gag tgc cta tgc tat gcg -3'	258	56 58
30	RYRex30F RYRex30R	F: 5'- ggg act cag atc caa caa ctt cct g -3' R: 5'- gag ctc tga ctg cct cct gcc -3'	356	59 62
31	RYRex31F RYRex31R	F: 5'- gtg tcc agg gtc cag agc tac -3' R: 5'- ctg gcc tca ggg gac atc tat aag -3'	309	59 58
32,33	RYRex32F RYRex32R	F: 5'- gtc ctc ttc tcc tct gcc agg tg -3' R: 5'- gtt gtg ttg agg agg ggc act gag -3'	599	61 62
34	RYRex34F RYRex34R	F: 5'- atg ggt gga tag tga tga agg aaa t -3' R: 5'- gat gca tgt atc tct gga gtt ttg g -3'	842	56 56
35	RYRex35F RYRex35R	F: 5'- ggc agg tct gga gaa tga gg -3' R: 5'- cat ccc acc tac cct gtg tct c -3'	416	58 59
36,37	RYRex36F RYRex36R	F: 5'- ggg aga gga agc aag aga agt ttc -3' R: 5'- gca tgg gga gga ctc tct gat c -3'	773	57 59
38	RYRex38F RYRex38R	F: 5'- gag tgt gta agc agg tga ata agc -3' R: 5'- ccc tct cac tcc tgc cta tc -3'	342	56 57
39	RYRex39bF *Val2168R	F: 5'-gag ggc gca ggt ggt agt aac tg -3' R: 5'-gac tga gat cac cca gag gat ggg cc -3'	519	62 65
40	*RYRE40F *RYRE40R	F: 5'-ccc ctg gtg acc ccg cac act ctg -3' R: 5'- ctg gga cag gca ggg tgg tca ggg -3'	229	69 69
41,42	RYRex41F RYRex41R	F: 5'- agg gga ggc agc cac aga g -3' R: 5'- cag ccc tgc cct cca cac -3'	613	62 62
43	RYRex43F RYRex43R	F: 5'- gtg gca tgg gtc tgg tct ctg act g -3' R: 5'- gga ggt gtg tga cca gtg act c -3'	238	63 59
44,45	*RYRex43F *Glu2434R	F: 5'-atg ctt gtg gcc aaa ggg tac –3' R: 5'-ctg cat gag gcg ttc aaa g -3'	936	53 56
46	*RYRE46F *RYRE46R	F: 5'-ggg agg gag cag agc agt cac tg -3' R: 5'-ctc cct ccc cag cat cac tcc ttc -3'	242	56 56
47	RYRex47F RYRex47R	F: 5'- gaa ctt ggc gaa gga gtg atg ctg -3' R: 5'- cat ctc ctg tcc ctc tgt gga ag -3'	308	60 59
48,49	RYRex48F RYRex48R	F: 5'- cag tcg ctc aag aca ggt gcc ag -3' R: 5'- gac aca aat gag ccc cgc agt agg -3'	624	62 62
50,51,52	RYRex50F RYRex50R	F: 5'-cct cat ttg tgt gtc ccc ctc ttg -3' R: 5'-gct ggg gtc ttg agg gtt tct tgg -3'	943	60 62

Table 3.2: Continued...

Exon	Primer name	Sequence	Size (bp)[1]	T_m[2]
53, 54	RYRex53F RYRex53R	F: 5'- gga ttc tct gtc ctc ggc tcc tc -3' R: 5'- ctc tcc atc cct tcc ctg tct g -3'	814	61 59
55,56,57	RYRex55F RYRex55R	F: 5'- ctt cct gct agc cca tca gcc c -3' R: 5'- ctt agc tcc tcc cct ctg gtt cc -3'	830	63 61
58	RYRex58F RYRex58R	F: 5'- ctg aga agg gtg gga aac tgt agg -3' R: 5'- gca taa gcg ggg ggt att tct c -3'	209	59 58
59,60	RYRex59F RYRex59R	F: 5'- cca gcc ttg aac cca ctg tga acc -3' R: 5'- cgg tca gta ccc aac acc cag cac -3'	429	62 64
61	RYRex61F RYRex61R	F: 5'- ctg tcc ctg tct cct cta att gg -3' R: 5'- ggc aga acc tgg gag cta ttt c -3'	226	57 58
62,63	RYRex62F RYRex62R	F: 5'- ggc act gtc ctc tgt cct ctt ag -3' R: 5'- cac att caa aca ccc agg gac tct c -3'	566	59 60
64	RYRex64F RYRex64R	F: 5'- gct gag aga gag ttg gta act tg -3' R: 5'- ctt aac atc tac cct gct ttt cac c -3'	355	55 56
65	RYRex65F RYRex65R	F: 5'- cac atg gat gaa tgg cag ctc tg -3' R: 5'- cca gcc aca cta ccc cca aat tag -3'	349	58 60
66	RYRex66F RYRex66R	F: 5'- ggt ggc aat tca atg gtg tct gat g -3' R: 5'- gac cat ctg cca agg gag cc -3'	500	59 61
67	RYRex67F RYRex67R	F: 5'- ctg ttt ggg agt cgg gct ggg aac g -3' R: 5'- gct gga gga cgt ggg agg tc -3'	382	66 62
68,69	RYRex68F RYRex68R	F: 5'- cat ctc ctc ctc caa gat ctc tct c -3' R: 5'- cag gta gga agt cct aga ggg tgc t -3'	437	58 61
70	RYRex70F RYRex70R	F: 5'- tgt ctc ctt cct cct cct gta tct t -3' R: 5'- gga aca gaa gca ggg gtt ttc t -3'	486	58 58
71	RYRex71F RYRex71R	F: 5'- gaa att gag gtg tcg tcg gca gtt g -3' R: 5'- cac agt gag tcc tca gca tcc -3'	478	60 58
72	RYRex72F RYRex72R	F: 5'- gtt gtg ggt cag gaa gga gga tg -3' R: 5'- caa tgc ctg gtc ttt ggt aaa tgc -3'	215	60 57
73	RYRex73F RYRex73R	F: 5'- ccc aaa aac gga aag ggg aca tc -3' R: 5'- cac ctg ccg ccc agt aga aag ac -3'	259	59 64
74,75,76	RYRex74F RYRex74R	F: 5'- cct tct gcc gtg tga gtc tta acc -3' R: 5'- cag gac ctt ggg ggc att tct gg -3'	918	60 64
77,78	RYRex77F RYRex77R	F: 5'- gac cac tcc cct gct tac ttc –3' R: 5'- cac atg ctg aat gaa tgg gag atg -3'	351	57 56

Table 3.2: Continued...

Exon	Primer name	Sequence	Size (bp)[1]	T_m[2]
79,80,81	RYRex79F RYRex79R	F: 5'- gga ggg cag aag tga gaa tgt gag g -3' R: 5'- gca gtg gca cca aac aca gct taa c -3'	805	61 61
82	RYRex82F RYRex82R	F: 5'- gcc caa cca tat gtc cta gct tc -3' R: 5'- ggg aac cag tgt ctt gga gga ag -3'	365	58 60
83	RYRex83F RYRex83R	F: 5'- ggg ttg ttc ctc tct ctc tgt gtg -3' R: 5'- cct cca cgt ccc aga tcc tca g -3'	252	59 61
84	RYRex84F RYRex84R	F: 5'- ctt ggg tct ccg tct gct gat gtc -3' R: 5'- gga ttg aca ctg gct gga gag tg -3'	431	61 60
85,86,87	RYRex85F RYRex85R	F: 5'- gtt cat ctc ccc tag cac atg g -3' R: 5'- caa agg ggc aag act tgg aaa tg -3'	662	57 57
88	RYRex88F RYRex88R	F: 5'- caa cag agg tgg ggg agg tgt atg -3' R: 5'- ggc ttc ttc atc aac cca tgg atc c -3'	278	61 60
89	RYRex89F RYRex89R	F: 5'- gtg gtg gct cct ggg ctg gaa ag -3' R: 5'- gag gca gcc agc cag aag ggt atg -3'	359	62 63
90	RYRex90F RYRex90R	F: 5'- gaa ttg agg ctc tcc agg tca c -3' R: 5'- cag ata tgc gag gca cgc aca g -3'	470	59 61
91	RYRex91cF RYRex91cR	F: 5'- gtg acc cct tgt agc tgc cac -3' R: 5'- ggg ctc tct tcc tcc ctc caa tc -3'	966	61 61
92	RYRex92F RYRex92R	F: 5'- gag gac tca gcc ctg atg ctt g -3' R: 5'- ctc tag gag gga ggc agt gat ag -3'	166	60 58
93	RYRex93F RYRex93R	F: 5'- ctc atc atc cca tgt acc cag tac -3' R: 5'- gaa cag atg aac tca aga aca agg -3'	381	57 54
94	RYRex94F RYRex94R	F: 5'- ctg tct gtg gcg ctt tct c -3' R: 5'- ctt cag tgg agg aac cct g -3'	224	56 55
95	*RYRex95F RYRex95bR	F: 5'- cca aga ctg tat ctg gta tgg tcc c -3' R: 5'- ctc tgt ccc aac cac ttt gag g -3'	473	58 58
96	RYRex96F RYRex96R	F: 5'- aag gtg cct gac gcc cac -3' R: 5'- agg tcc cct cct gct gcc -3'	218	61 63
97	RYRex97F RYRex97R	F: 5'- gag ttt cag cca acc ctg tcg tg -3' R: 5'- caa ggt cac aca cca agc aag tgc -3'	228	60 61
98,99	RYRex98F RYRex98R	F: 5'- gtc tac aca gcc tga tgc tct ctt g -3' R: 5'- gag tcc ctc ccc agt ctg tgg -3'	347	59 62
100	RYRex100aF RYRex100bR	F: 5'- gtg ctc ctc gtg tgt ccc tgc ctt c -3' R: 5'- ctt atc cct tca cca ccc act gcc -3'	274	65 62
101	*RYRex100F *RYRex100R	F: 5'- ggc tgg tat atg gtg atg tcc ct -3' R: 5'- aca gat gcg aga agg aag ggt cc -3'	554	65 67

Table 3.2: Continued...

Exon	Primer name	Sequence	Size (bp)[1]	T_m[2]
102	RYRex102F RYRex102R	F: 5'- ctg atg ccg tat ctg tga gcc -3' R: 5'- gcg aga ggt aga gat ggg gta tg -3'	415	58 58
103	*RYRex103F *RYRex103R	F: 5'- gtc ggg cac tga ctt gtg tc -3' R: 5'- gac ccc ctg aat ccc gta atc -3'	147	63 63
104,105	RYRex104F RYRex104R	F: 5'- gga gga tat gga ggt agg tca tgt c -3' R: 5'- ctt atg tta aag ggc tcc acg tcc -3'	643	58 58
106	RYRex106F RYRex106R	F: 5'- ggg ttt gaa gat gtg acc aat g -3' R: 5'- ctt tag cct ctg ctg tca tc -3'	375	54 53

[1] = size indicated in base pair (bp); [2] = calculated annealing temperature for the primer pair; * = oligonucleotides used in previous studies conducted at the Centre for Genome Research; F = forward primer and R = reverse primer.

3.3 DNA EXTRACTION

Approximately 3 mL of venous blood was collected in ethylenediamine tetra-acetic acid (EDTA) tubes and was stored at -70°C until required. DNA had previously been extracted as part of the ongoing MH research programme[1]. Isolations were performed using the Promega Wizard®[2] Genomic DNA purification kit. The extraction protocol as outlined in the manufacturer's guideline was followed. To lyse the red blood cells, 9 mL of cell lysis solution was added to 3 mL blood in a sterile 15 mL centrifuge tube. The mixture was inverted, incubated for 10 min at room temperature and centrifuged at 2,000 gravitational acceleration (x g) for 10 min. The pellet containing the white blood cells was collected and vortexed in the remaining supernatant to resuspend the cells. Nuclei lysis solution (3 mL) was added to lyse white blood cells, followed by the addition of 1 mL protein precipitation solution for deproteinisation. The sample was vortexed and centrifuged at 2,000 x g for 10 min at room temperature. DNA present in the aqueous top phase was transferred to a 15 mL centrifuge tube and precipitated with 3 mL isopropanol, equilibrated at room temperature. Following mixing, the sample was centrifuged at 2,000 x g for 1 min at room temperature to precipitate the DNA. Subsequent to discarding the supernatant, 3 mL of 70% ethanol (EtOH) was added to the DNA pellet, subsequent to discarding the supernatant.

[1] DNA isolations were performed by Y. Havenga and D. Prosser.
[2] Wizard® is a registered trademark of the Promega Corporation, Madison, WI, USA.

The DNA was air-dried and rehydrated in 250 microlitre (μL) DNA rehydration solution via incubation for 24 hours (h) at room temperature. The typical DNA yield for 3 mL of blood ranged from 75 -150 microgram.mL^{-1} (μg.mL^{-1}). DNA was stored at 4°C until required.

Samples collected as part of the continuous MH programme were isolated during the current phase of the study. Extraction was performed using the QIAgen FlexiGene®[1] DNA kit. The extraction procedure as outlined in the manufacturer's protocol was followed. To lyse the red blood cells, 7.5 mL FG1 buffer was added to 3 mL blood in a sterile 15 mL centrifuge tube. The mixture was inverted and centrifuged at 2,000 x *g* for 5 min in a swing-out rotor. Once the supernatant was removed, 1.5 mL of a mixture consisting of 1.5 mL of buffer FG2 and 15 μL of QIAgen Protease solution was added to the pellet. The tube was vortexed, inverted and placed in a waterbath for 10 min at 65°C. In order to precipitate the DNA 1.5 mL isopropanol (100%) was added and the sample was centrifuged at 2,000 x *g* for 3 min. Subsequent to discarding the supernatant, 1.5 mL of 70% EtOH was added to the DNA pellet and the sample was centrifuged at 2,000 x g for 3 min. The supernatant was discarded and the DNA was air-dried for 5 min. DNA was rehydrated in 300 μL DNA rehydration solution (buffer FG3) via incubation for 1 h at 65°C in a water bath. The typical DNA yield for 3 mL of blood is between 75 and 90 μg.mL^{-1}. DNA was stored at -20°C until required. Following DNA isolation, working dilutions of DNA were prepared by dilution with sterile distilled water to a final concentration of 50 nanogram (ng).μL^{-1} and were stored at 4°C.

3.4 DETERMINATION OF DNA CONCENTRATION

DNA concentration can be measured by determining the absorbance at 260 nm (A_{260}) in a spectrophotometer. The calculation indicated in Equation 3.1A takes into account that an absorbance of one unit at 260 nm corresponds to 50 μg gDNA per mL. As there is a linear relationship between absorbance and DNA concentration, the concentration of DNA can be determined by the equation depicted in Equation 3.1B (Sambrook and Russell, 2001). DNA concentrations were determined spectrophotometrically using the formula indicated in Equation 3.1.

[1] FlexiGene™ is a trademark of QIAGEN Pty. Ltd., Victoria, Australia.

Equation 3.1: Spectrophotometric conversion for calculating the concentration of nucleic acids from the absorbance at 260 nm

3.1A	Unknown (μg.mL^{-1}) / $A_{260\ nm}$ = 50 (μg.mL^{-1}) / 1.0 $A_{260\ nm}$
3.1B	Unknown μg.mL^{-1} = 50 μg.mL^{-1} x $A_{260\ nm}$ x dilution factor

A_{260} = absorbance at 260 nm.

3.5 POLYMERASE CHAIN REACTION

PCR, which offers a fast and convenient method of amplifying specific DNA segments, was first described by Mullis *et al.* (1986). This technique involves denaturation of the DNA sample, annealing at a temperature depending on the T_m of the expected amplified product, and template elongation synthesis. The primers flank the DNA segment of interest and direct the DNA polymerase to synthesise new complementary strands. Multiple cycles of this process, each doubling the amount of DNA present, exponentially amplify the DNA (Voet and Voet, 1999).

DNA concentration, primer T_a and $MgCl_2$ concentration were optimised to ensure high specificity during amplification of specific regions in the RYR1 gene. The T_a was optimised for each reaction with a range of temperatures spanning the melting temperature of the primer pairs and was experimentally analysed for each primer set to ensure that non-specific amplification did not occur. Higher temperatures were preferentially chosen in order to increase the specificity of the reaction. PCR was conducted with Promega Go *Taq*®[1] Flexi DNA polymerase, which has a 1 x buffer containing 10 mM Tris®-HCl (pH 9.0), 50 mM potassium chloride (KCl) and 0.1% Triton® X-100 or with Super-therm®[2] polymerase, which has a 1 x buffer containing 20 mM Tris®-HCl (pH 8.0), 100 mM sodium chloride (NaCl), 0.1 mM EDTA, 1 mM dithiothreitol (DTT), stabilisers and 50% glycerol, as discussed in Section 4.2 (page 159). PCR reactions were performed in a total volume of 12.5 μL.

[1] Promega Go *Taq*® Flexi DNA polymerase is a registered trademark of the Promega Corporation, Madison, WI, USA.
[2] Super-therm® polymerase, is a registered trademark of JMR Holdings, Sevenoaks, Kent, UK.

The PCR reaction consisted of the following components:

- 1 X PCR buffer
- 0.5 to 2.0 mM $MgCl_2$
- 200 µM of each 2'-deoxynucleotide triphosphate (dNTP)
- 5 picomol (pmol) of each of the forward and reverse primer
- 0.25 units (U) *Taq* DNA polymerase
- 100 ng gDNA template

Each 12.5 µL reaction was overlaid with 12.5 µL of mineral oil to prevent evaporation. Thermal cycling was carried out via a Thermo Hybaid®[1] Multiblock System using temperature cycles listed in Table 3.3.

Table 3.3: Temperature cycles of the standard PCR reaction protocol

PCR step	Temperature	Time	Number of cycles
Denature	94°C	10 min	1
Denature Anneal Extend	94°C T_a 72°C	30 s 30 s 60 s	30
Elongate	72°C	7 min	1
Hold	4°C	Hold	Indefinite

°C = degree Celsius; s = seconds; min = minutes; T_a = optimised annealing temperature for each reaction.

In certain instances, amplification of an RYR1 region or exon was achieved via a two-step fast PCR protocol using Promega Go *Taq*® Flexi DNA polymerase, which has a 1 x buffer containing 50 mM Tris-HCl (pH 9.0), 50 mM NaCl and 0.1% Triton® X-100. Primer sets which had an average T_m higher than 60°C and amplified a target region of more than 250 bp were selected for this modified PCR protocol. The PCR reaction consisted of the following components:

- 1 X PCR buffer
- 0.5 to 2.0 mM $MgCl_2$
- 200 µM of each dNTP
- 5 pmol of each of the forward and reverse primer
- 0.25 U *Taq* DNA polymerase
- 100 ng gDNA template

[1] Thermo Hybaid® is the registered trademark of Hybaid Limited, Ashford, Middlesex, UK.

Each 12.5 μL reaction was overlaid with 12.5 μL of mineral oil to prevent evaporation. Thermal cycling was carried out via a Thermo Hybaid® Multiblock System using temperature cycles listed in Table 3.4.

Table 3.4: Temperature cycles of the two-step fast PCR reaction protocol

PCR step	Temperature	Time	Number of cycles
Denature	98°C	30 s	1
Denature Anneal and Extend	92°C 70°C	1 s 10 s	35
Elongate	72°C	15 s	1
Hold	4°C	Hold	Indefinite

°C = degree Celsius; s = seconds.

3.6 AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis is a simple and effective method that can be used for separating and identifying 0.5 to 25 kilo base pair (kb) DNA fragments. Detection was carried out using 2% weight per volume (w/v) mini agarose gel. The 2% mini agarose gel was made up to a final volume of 30 mL and contains 0.6 g low electroendosmosis (LE) analytical grade agarose, 3.0 mL 10 x Tris® borate-EDTA (TBE) buffer [89.15 mM Tris® (pH 8.1), 88.95 mM boric acid, 2.498 disodium EDTA (Na_2EDTA)], and 0.5 $\mu g.mL^{-1}$ ethidium bromide (EtBr). PCR product (2.5 μL) was added to 2 μL of a 2 X loading buffer [0.04% orange G (Sigma®[1]) and 50% glycerol] and loaded. Electrophoresis was carried out for 30 min at 10 volts per centimetre (V/cm) in 1 X TBE buffer. DNA was visualised by illumination with ultraviolet (UV) light and the images were captured on a video documentation system.

3.7 CHAIN TERMINATION SEQUENCING

Sanger *et al.* (1977) developed a method that allows for the determination of DNA nucleotide sequence. The method employs 2',3'-dideoxynucleotide triphosphates (ddNTP) that lack a 3'-OH group, necessary for the formation of phosphodiester bonds. Consequently the DNA chain is specifically terminated at the position where a ddNTP is incorporated (Alphey, 1997). The method of sequencing used in this molecular investigation was based on dye-labelled terminators, i.e. fluorophores attached to dideoxynucleotides. All four reaction products are assembled into one tube and the output

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

generated from the electrophoresis run is in the form of intensity profiles for each differently coloured fluorophore.

PCR purification of samples was performed using either the QIAquick®[1] PCR purification kit or the Zymo Research DNA Clean and Concentrator-5™[2] (DCC-5™) kit, for direct purification of PCR product. The purification procedure as outlined in the manufacturer's protocol was followed. For the QIAquick® PCR purification kit, five volumes of Buffer PB were added to one volume of PCR sample and mixed. The mixture was incubated at room temperature for 1 min and transferred to the QIAamp spin column. The column was centrifuged at 17,900 x g for 1 min. The filtrate was discarded and 750 µL buffer PE was added to wash the sample via centrifugation at 17,900 x g for 1 min. The filtrate was discarded and the sample was again centrifuged at 17,900 x g for 1 min. The spin column was placed in a clean 1.5 mL microcentrifuge tube. To elute the bound DNA, 50 µL elution buffer was added to the centre of the membrane and the sample was centrifuged at 17,900 x g for 1 min. For the DCC-5™ kit two volumes of DNA binding buffer was added to one volume of PCR sample and mixed. The mixture was transferred to the Zymo-Spin™ column and was centrifuged at 13,000 revolutions per minute (rpm) for 1 min. The filtrate was discarded and 200 µL wash buffer was added to wash the sample by centrifugation at 13,000 rpm for 1 min. The filtrate was discarded and the wash step was repeated. The spin column was placed in a clean 1.5 mL microcentrifuge tube. To elute the bound DNA, 30 µL double distilled water (ddH_2O) was added to the centre of the membrane and the sample was centrifuged at 13,000 rpm for 1 min. Prior to sequencing the DNA quantity of the PCR product was determined. The quantity of PCR product used for sequencing was determined by the appropriate amount of template required to provide optimum results as determined by the spectrophotometer. The amount of template that should be used for a sequencing reaction is listed in Table 3.5.

Following DNA purification, samples were sequenced using the ABI PRISM®[3] Big Dye™ Terminator version 3.1 Ready Reaction Cycle Sequencing Kit. The kit contains a premixed terminator Ready Reaction Mix which consists of dye terminators, dNTPs, AmpliTaq®[4] DNA polymerase, $MgCl_2$ and buffer (Tris-HCl, pH 9.0). The sequencing reaction was performed in a 0.2 mL microcentrifuge tube and included 2 µL Ready Reaction Premix,

[1] QIAquick™ is a trademark of QIAGEN Pty. Ltd., Victoria, Australia.
[2] DNA Clean and Concentrator -5™ is a registered trademark of Zymo Research Corporation, Orange, CA, USA.
[3] ABI PRISM® Big Dye™ is a registered trademark of Applera Corporation, Foster City, CA, USA.
[4] AmpliTaq® DNA polymerase, FS, is a registered trademark of Roche Molecular Systems Inc., Alameda, CA, USA.

2 µL 5 X sequencing buffer (Tris-HCl, pH 9.0 and $MgCl_2$), 3.2 pmol primer and 10 - 20 ng purified PCR product.

Table 3.5: Template quantity used in sequencing

Template	Quantity	Template	Quantity
100 – 200 bp 200 – 500 bp	1 – 3 bp 3 – 10 bp	500 – 1000 bp 1000 – 2000 bp >2000 bp	5 - 20 ng 10 - 40 ng 40 - 100 ng

bp = base pair; ng = nanogram. Adapted from ABI PRISM® BigDye™ Terminator version 3.0 Ready Reaction Cycle Sequencing kit protocol.

Forward or reverse primers used for PCR were also used as sequencing primers. Deionised water was added to a final volume of 10 µL. Following mixing, sequencing was conducted on the Thermo Hybaid® Multiblock System using temperature cycles listed in Table 3.6. Detection of a mutation resulted in both strands being sequenced to permit identification of ambiguities. Following sequencing, sodium dodecyl sulfate (SDS) was used in order to remove unincorporated dye terminators that occur due to an imbalance in the primer:BigDye:template. SDS treatment disrupts non-covalent binding involving unincorporated dye terminators. A 2.2% SDS solution was added to the sequencing reaction and the samples were then heated for 5 min at 98°C and cooled for 10 min at 25°C.

Table 3.6: Temperature cycles of the sequencing reaction

PCR step	Temperature	Time	Number of cycles
Denature	96°C	10 s	
Anneal	50°C	10 s	25
Extend	60°C	4 min	
Hold	4°C	Hold	Indefinite

°C = degree Celsius; s = seconds; min = minutes.

Purification to remove unincorporated dye terminators from the sequencing reaction was conducted prior to electrophoresis of the sample. Two different methods were used in order to precipitate the sequencing reaction. Initially, the ethanol/sodium acetate method of precipitation was employed to purify the samples. In this regard, a solution was made, composed of 3 µL of 3 M sodium acetate (NaOAc), pH 4.6, 62.5 µL non-denatured 99% EtOH and 14.5 µL deionised water. The solution was added to 10 µL of the PCR sequenced product and vortexed. The tubes were centrifuged at 10,621 x g for 20 min and 250 µL 70% EtOH was added to the pellet and vortexed briefly. The samples were centrifuged at 10,621 x g for 10 min and the supernatants were discarded. Finally, the

samples were air-dried for 30 min. However, in order to obtain a higher uniform signal intensity, sequences were also precipitated using the Centri-sep™[1] 96 well clean-up kit. The Centri-sep™ 96 well filter plate removes excess dye terminators via a cross-linked preservative-free gel. Following precipitation the sequences were submitted for electrophoresis. Precipitation of sequences via the Centri-sep™ 96 well filter plate and electrophoresis of the sequenced product were not performed by the author, but were analysed on contract. Sequences were either run on a SpectruMedix™[2] SCE2410 genetic analysis system sequencer or on an Applied Biosystems®[3] 3130xl genetic analyser. DNA sequences were analysed and compared with the reference sequence of RYR1 provided by the National Centre for Biotechnology Information (NCBI) database (NM_000540) using the BioEdit Sequence Alignment Editor version 5.0.9 software (Hall, 1999).

3.7.1 Detection of alterations in exon 1 of the RYR1 gene

Analysis of a 457 bp region was conducted in order to detect novel and reported alterations that may occur in exon 1 of the RYR1 gene. The partial gDNA sequence of exon 1 from the RYR1 gene is presented in Table 3.7.

Table 3.7: Partial gDNA sequence of exon 1 of the RYR1 gene

Nucleotide number	DNA sequence: exon 1
43615940	tgggagaatg atggcacgtt acttaccgtg gtggggagaa agcgcaggta cctcctagat
43616000	actctctctc ccaccccacc tccggcggcc aacggccaag caaacctcca gccaagattt
43616060	ggggtatgtg ggcagggctc cggcgaaggg gagtggccgg ggagtcctgg tccaatgggg
43616120	cccgggggcg gggacttcct cccatctctg tccagcatgc gtgtactcct cgcagttcca
	↓ **exon 1**
43616180	TCTACCTCGC GGGTGCCTCT GGTGTCTCCA GAGGTCTCCG ACCCCAGCCC GCCCCCAGCC
43616240	CTCCCGCCCA GCCCGCAGCC CCCTCCCTCT GTTCCCCGAC CTCAGACCCT GGGCTTCCGA
43616300	CCTCGACATC ATGGGTGACG CAGAAGGCGA AGACGAGGTC CAGTTCCTGC GGACGgtgcg
43616360	tatctctggg ttaggggcct gtggggctat ctcttggggc tctctgaggg tctctctgtc

The partial gDNA sequence amplified for exon 1 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Two single nucleotide polymorphisms are observed in this region, the A433T nucleotide transition is indicated in blue and the T623C nucleotide transition is indicated in orange. The codon that correlates to Leu13 is indicated in a solid box (–) and the nucleotide position of the mutation is indicated in **bold**. The forward primer (RYRex1F) is the single underlined sequence, while the reverse primer (RYRex1R) is the double underlined sequence; the beginning of exon 1 is indicated with an arrow.

[1] Centri-sep™ 96 is a registered trademark of Princeton Separations, Adelphia, NJ, USA.
[2] SpectruMedix™ is a trademark of the SpectruMedix LLC, State College, PA, USA.
[3] Applied Biosystems® 3130xl genetic analyser is a registered trademark of Applera Corporation, Foster City, CA, USA.

The sequence was also analysed for novel and reported polymorphisms that may be observed in this region. Currently, exon 1 has been reported to harbour one alteration, Leu13Arg that occurs due to a T38G nucleotide transition (Ibarra *et al.*, 2006), even though this exon resides outside the first mutational hotspot.

3.7.2 Detection of alterations in exon 2 of the RYR1 gene

Analysis of a 300 bp region was conducted in order to detect novel alterations that may occur in exon 2 as well as reported mutations associated with MHS. The partial gDNA sequence of amplified exon 2 from the RYR1 gene is depicted in Table 3.8.

Table 3.8: Partial gDNA sequence of exon 2 of the RYR1 gene

Nucleotide number	DNA sequence: exon 2
43623140	agggtgggag gaggggcctg tggtctgcag tatttgtggt atccgggcca ggccccctg
	↓ **exon 2**
43623200	gagacgctgc ccctcggttc cgcagGACGA TGAGGTGGTC CTGCAGTGCA GCGCTACCGT
43623260	GCTCAAGGAG CAGCTCAAGC TCTGCCTGGC CGCCGAGGGC TTCGGCAACC GCCTGTGCTT
43623320	CCTGGAGCCC ACTAGCAACG CGCAGgtctg tgcaggaggg agaggggcct ggggacaggg
43623380	gcgtctgaag gggcagagaa tcttgggtcc aaagaagagg gttctgggag tctgaaagga
43623440	ggtgctgaca ggagagaaag tgaggagggg ggctaaggct aagaggggct acctgaggtg

The partial gDNA sequence amplified for exon 2 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. A single nucleotide polymorphism is observed in this region, the G7839C nucleotide transition is indicated in blue. The codon that correlates to Asp17del is indicated in a solid red box (–), the codon that correlates to Cys35 is indicated in a dashed box (---), the codon that correlates to Arg44 is indicated in a solid box (–) and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex2F) is the single underlined sequence, while the reverse primer (RYRex2R) is the double underlined sequence; the beginning of exon 2 is indicated with an arrow.

Exon 2 harbours two reported mutations. The first is the Cys35Arg alteration, which occurs due to a T103C nucleotide transition and was first described by Lynch *et al.* (1997). Halsall and Robinson (2004) reported an Arg44His substitution in the RYR1 gene segregating in one family, which occurs due to a G131A transition. In addition, a deletion, Asp17del, has been reported to occur in one MH patient of Japanese origin (Ibarra *et al.*, 2006). Exon 2 is observed in the first mutational hotspot of the RYR1 gene and harbours one reported polymorphism.

3.7.3 Detection of alterations in exon 3 of the RYR1 gene

Exon 3 of the RYR1 gene is located in hotspot one. Analysis of a 246 bp region was conducted in order to detect novel alterations associated with MH that may occur in this exon, as well as to identify any reported or novel polymorphisms that may occur in this

region. Currently, exon 3 harbours two alterations, the Asp60Asn was reported in a patient diagnosed with CCD and the Ser71Tyr alteration was reported in a family with both CCD and MmD (Zhou *et al.*, 2005; Wu *et al.*, 2006). The two alterations occur due to G178A and C212A nucleotide transitions, respectively. The partial gDNA sequence of amplified exon 3 from the RYR1 gene is depicted in Table 3.9.

Table 3.9: Partial gDNA sequence of exon 3 of the RYR1 gene

Nucleotide number	DNA sequence: exon 3
43624700	gtggggtggg ggtggggtct ggcgtctcaa gagtgtgggc atccagacta ggggagggag
43624760	tgtggcaggg aatgttgctg gggtgggggg gtcttctgac ccctcactta catcccccctc
	↓ exon 3
43624820	ccaccccagA ATGTGCCCCC CGATCTGGCC ATCTGTTGCT TCGTCCTGGA GCAGTCCCTG
43624880	TCTGTGCGAG CCCTGCAGGA GATGCTGGCT AACACGGTGG AGGCTGGCGT GGAGgtgagg
43624940	accccacctg ggggtgggcg gggtggcaga gatgggcgag aggacccagg ggtcgtttag

The partial gDNA sequence amplified for exon 3 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. A single nucleotide polymorphism is observed in this region, the G9381A nucleotide transition is indicated in a circle. The codon that correlates to Asp60 is indicated in a dashed box (---), the codon that correlates to Ser71 is indicated in a solid box (–) and the nucleotide positions of the mutations are indicated in bold. The forward primer (RYRex3F) is the single underlined sequence, while the reverse primer (RYRex3R) is the double underlined sequence; the beginning of exon 3 is indicated with an arrow.

3.7.4 Detection of alterations in exons 4 and 5 of the RYR1 gene

Exons 4 and 5 are located in hotspot one of the RYR1 gene. The partial gDNA sequence of amplified exons 4 and 5 of the RYR1 gene is represented in Table 3.10.

Table 3.10: Partial gDNA sequence of exons 4 and 5 of the RYR1 gene

Nucleotide number	DNA sequence: exons 4 and 5
43625960	tgtgactagg ccagacctct tggggatctg gagagtccgg ggatctgtgc ttattctgtt
	↓ exon 4
43626020	ccctccctcc ccctgcagTC ATCCCAGGGC GGGGGACACA GGACGCTCCT GTATGGCCAT
43626080	GCCATCCTGC TCCGGCATGC ACACAGCCGC ATGgtgagtg caacctcggt gggcgtgggc
	exon 5 ↓
43626140	aggggccagg gcatgtgggg cctgctagaa ggaggctgac ctccctctac aaccctagTA
43626200	TCTGAGCTGC CTCACCACCT CCCGCTCCAT GACTGACAAG CTGGCCTTCG ATGTGGGACT
43626260	GCAGGAGGAC GCAACAGgtg cagcagctgg aggggatggg ggtgtgaagg ggccccgcag
43626320	cagggattca gggggtagaa ggtctgcaga acgccaaggc aagcatgaga ctaccctggg

The partial gDNA sequence amplified for exons 4 and 5 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The codon that correlates to Arg109 is indicated in a solid box (–) and the nucleotide position of the mutation is indicated in bold. The forward primer (RYRex4F) is the single underlined sequence, while the reverse primer (RYRex4R) is the double underlined sequence; the beginnings of exons 4 and 5 are indicated with an arrow.

Alterations associated with the MH phenotype have thus far not been reported to occur in these exons. However, a single alteration, Arg109Trp, which occurs due to a C325T substitution, has been reported in a patient diagnosed with CCD (Zhou *et al.*, 2005) in exon 4.

3.7.5 Detection of alterations in exons 6 and 7 of the RYR1 gene

Exons 6 and 7 are located in hotspot one of the RYR1 gene. Alterations associated with the MH phenotype have thus far not been reported for exon 7. The partial gDNA sequence of amplified exons 6 and 7 from the RYR1 gene is represented in Table 3.11.

Table 3.11: Partial gDNA sequence of exons 6 and 7 of the RYR1 gene

Nucleotide number	DNA sequence: exons 6 and 7
43626560	gagccctggg gaagagcatt ctgggaagcc atcatctgac agccaccccc attccatccc
	↓ exon 6
43626620	cacccatagG AGAGGCTTGC TGGTGGACCA TGCACCCAGC CTCCAAGCAG AGGTCTGAAG
43626680	GAGAAAAGGT CCGCGTTGGG GATGACATCA TCCTTGTCAG TGTCTCCTCC GAGCGCTACC
43626740	TGgtgagcca ttgcggttcc tcctgctccc aggtctgggg gcgcatggga tggtccccat
43626800	cttctcacca tgggtttgcc tggctgatct cccaccccca aggtcctgac tcccaatttc
43626860	ccatttcctg acccctgaca tccaattttc tgatttctga cctcccattg cccgacttga
43626920	tcatttcctg atctgtgatc tctgatgact ctgtctccca tctgccggtt tccgggtatc
43626980	cacccttgat ttctggcctc tgacgctggg actctcgccc accctgcaa tcgtctctga
	↓ exon 7
43627040	ctgccgcatc ctggtggccc ccagCACCTG TCGACCGCCA GTGGGGAGCT CCAGGTTGAC
43627100	GCTTCCTTCA TGCAGACACT **A**TGGAACATG AACCCCATCT GCTCCCGCTG CGAAGAGGgt
43627160	gagggcccca gacctccccc taaatggaga tcccccaaa acagaccctt aatgttgccc

The partial gDNA sequence amplified for exons 6 and 7 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Two single nucleotide polymorphisms are observed in this region, the C11520T nucleotide transition is indicated in **blue** and the A11541G nucleotide transition is indicated in **orange**. The codon that correlates to Gln155 is indicated in a green solid box (–), the codon that correlates to Arg156 is indicated in a **pink** dashed box (---), the codon that correlates to Glu160 is indicated in a solid box (–), the codon that correlates to Arg163 is indicated in a dashed box (---), the codon that correlates to Gly165 is indicated in a **red** dashed box (---), the codon that correlates to Asp166 is indicated in a **blue** dashed box (---), the codon that correlates to Arg177 is indicated in a solid **red** box (–), the codon that correlates to Tyr178 is indicated in a solid **blue** box (–) and the respective nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex6F) is the single underlined sequence, while the reverse primer (RYRex6R) is the double underlined sequence; the beginnings of exons 6 and 7 are indicated with an arrow.

Exon 6 however, harbours ten reported causative alterations. A Gln155Lys alteration was reported in one MH individual and occurs due to a C463A substitution (Ibarra *et al.*, 2006). Galli *et al.* (2006) reported an Arg156Lys alteration in one MH individual from Italy. The alteration occurs due to an A467G nucleotide transition. A Glu160Gly alteration that occurs due to an A479G transition and an Arg163Leu mutation which results from a G488T substitution were both reported in one UK family with MH (Halsall and Robinson,

2004). An Arg163Cys which occurs due to a C487T single base substitution was first reported by Quane *et al.* (1993) in a single CCD pedigree. A Gly165Arg alteration was detected in one French MHS family and occurs due to a G493A nucleotide transition (Monnier *et al.*, 2005). An Asp166Asn alteration that is due to a G496A substitution was detected in one MH proband from Germany (Rueffert *et al.*, 2002) and an Asp166Gly which is due to the nucleotide substitution A497G was reported in one MH proband from Japan (Ibarra *et al.*, 2006). In addition, Monnier *et al.* (2005) reported alterations Arg177Cys and Tyr178Cys that are due to nucleotide transitions C529T and A533G respectively, each in one French MHS family. Analysis of a 656 bp region was therefore conducted in order to detect novel and reported alterations that may occur in these exons, as well as identify any novel or reported polymorphisms.

3.7.6 Detection of alterations in exons 8 and 9 of the RYR1 gene

Exons 8 and 9 of hotspot one of the RYR1 gene were analysed in order to detect reported and novel alterations that may occur in these exons, as well as to identify novel polymorphisms located in this region. The partial gDNA sequence of amplified exons 8 and 9 from the RYR1 gene is represented in Table 3.12.

Table 3.12: Partial gDNA sequence of exons 8 and 9 of the RYR1 gene

Nucleotide number	DNA sequence: exons 8 and 9
43628840	ctcagccctc aggttccccc aggggaggag cagggcccct gacttcatct tggctcctgg
	↓ exon 8
43628900	tcttcctggg gctccagcct cccattgacc aacttcccttt gctcctctcc agGCTTCGTG
43628960	ACGGGAGGTC ACGTCCTCCG CCTCTTTCAT GGACATATGG ATGAGTGTCT GACCATTTCC
43629020	CCTGCTGACA GTGATGACCA GCGCAGgtct gggctgtgga cgagagggcc tggggtctag
43629080	gggtggacgt ggagggctgg gaccctatga gtaggattag ggaccagatt ccggggagct
	↓ exon 9
43629140	gaacccttga cttcactctc ttctgtgtcc ccagACTTGT CTACTATGAG GGGGGAGCTG
43629200	TGTGCACTCA TGCCCGCTCC CTCTGGAGGC TGGAGCCACT GAGAATCAGg tagggcgggg
43629260	aagatgggga gagaccaggg agaggctggg gtcacctggc aggctgggag gacagaaaag
43629320	gtcttgaggg aagatctgat aaagagactg aagggtctcg agggaaaatc agagcagcct
43629380	gagagagaga tgaaaatctc ggccaggcgt ggtgacttca tgcctgtaat cccagcactt

The partial gDNA sequence amplified for exons 8 and 9 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The codon that correlates to Gly215 is indicated in a solid box (–), the codon that correlates to Val218 is indicated in a dashed **blue** box (—), the codon that correlates to Asp227 is indicated in a dashed box (—), the codon that correlates to Gly248 is indicated in a solid **blue** box (–) and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex8F) is the single underlined sequence, while the reverse primer (RYRex8R) is the double underlined sequence; the beginnings of exons 8 and 9 are indicated with an arrow.

Exon 8 harbours three reported alterations, the Gly215Glu alteration, which is due to a G644A transition, has been associated with a family that was affected by CCD and presented with foetal akinesia (Romero *et al.*, 2003) and an Asp227Val alteration that is due to a A680T nucleotide substitution, which has been reported in one French MH family (Monnier *et al.*, 2005). In addition, the Val218Ile alteration that is due to a G652A nucleotide transition has been reported in one MH proband from Japan (Ibarra *et al.*, 2006). Exon 9 only harbours one reported alteration, termed Gly248Arg, which is due to a G742A transition (Gillard *et al.*, 1992).

3.7.7 Detection of alterations in exons 10 and 11 of the RYR1 gene

Analysis of a 588 bp region was conducted in order to detect novel and reported alterations or polymorphisms that may occur in exons 10 and 11 of the RYR1 gene. The partial gDNA sequence of amplified exons 10 and 11 from the RYR1 gene is depicted in Table 3.13.

Table 3.13: Partial gDNA sequence of exons 10 and 11 of the RYR1 gene

Nucleotide number	DNA sequence: exons 10 and 11
43630760	aaagaagaaa agactgtaat gtccatggga gaactggggg gtcctctgac tcccttggc
	↓ **exon 10**
43630820	tctcaccctc cacagCTGGA GTGGGAGCCA CCTGCGCTGG GGCCA**G**CCAC TCCGAGTCCG
43630880	GCATGTCACT ACCGGGCAGT ACCTAGC**G**CT CACCGAGGAC CAGGGCCTGG TGGTGGTTGA
43630940	CGCCAGCAAG GCTCACACCA AGGCTACCTC CTTCTGCTTC CGCATCTCCA AGgtcagtgg
43631000	ggtttgtggc gccctccctc acctgaagcc cccagtccca gcccagcctg cactctgcag
43631060	tccctcaggg gggctcccct gctaaacaca caggcagagg aggctgacct gtgtcccctg
	↓ **exon 11**
43631120	cccctgtagG AGAAGCTGGA TGTGGCCCCC AAGCGGGATG TGGAGGGCAT GGGCCCCCCT
43631180	GAGATCAAGT ACGGGGAGTC ACTGTGCTTC GTGCAGCATG TGGCCTCAGG ACTGTGGCTC
43631240	ACCTATGCTG CTCCAGACCC CAAGGCCCTG CGGCTCGGCG TGCTCAAGAA GAAGgtgggt
43631300	gtaatcccag ctactcagga ggctgaggtg ggagaatcgc ttgagtccag gaggtcaagg
43631360	ctgcagtgag ctggtgatca tgccactgta ctccagcctg ggtgacagag tgagatgggg

The partial gDNA sequence amplified for exons 10 and 11 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Several single nucleotide polymorphisms are observed in this region, the G15266C nucleotide transition is indicated in **dark purple**, the G15328A nucleotide transition is indicated in **blue**, the C15606A nucleotide transition is indicated in **orange** and the T15669C nucleotide transition is indicated in green, the C15758T nucleotide transition is indicated in purple, the T15766C nucleotide transition is indicated in **pink**. The codon that correlates to Arg316 is indicated in a **blue** dashed box (---), the codon that correlates to Arg328 is indicated in a solid box (–), the codon that correlates to Gly341 is indicated in a dashed box (---),the codon that correlates to Arg367 is indicated in a **pink** dashed box (--) and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex10F) is the single underlined sequence, while the reverse primer (RYRex10R) is the double underlined sequence; the beginnings of exons 10 and 11 are indicated with an arrow.

Both exons 10 and 11 are located in hotspot one of the RYR1 gene. Currently, one alteration, Arg316Leu, that is due to a G947T substitution, has been reported in exon 10 (Ibarra *et al.*, 2006). Exon 11 harbours four reported alterations associated with the MH phenotype. Loke *et al.* (2003) described a novel alteration, Arg328Trp that was observed to be restricted to one MHS individual in a Canadian pedigree. The alteration is due to a C982T nucleotide transition. A causative Gly341Arg alteration which is due to a G1021A transition has been described in approximately 10% of Caucasian MHS cases (Quane *et al.*, 1994a), and is the most frequently occurring alteration that has been described for European populations (Halsall and Robinson, 2004). Recently, Monnier *et al.* (2005) observed a novel base change, G1021C, which also results in the Gly341Arg alteration, which was previously not detected by RFLP. Galli *et al.* (2006) identified an Arg367Gln alteration in one MHS proband due to a G1100A substitution.

3.7.8 Detection of alterations in exon 12 of the RYR1 gene

Exon 12 is located in the first mutational hotspot of the RYR1 gene and harbours five reported alterations. The partial gDNA sequence of amplified exon 12 from the RYR1 gene is depicted in Table 3.14.

Table 3.14: Partial gDNA sequence of exon 12 of the RYR1 gene

Nucleotide number	DNA sequence: exon 12
43634180	acgtcttggg ggcatggccc tgggtggctg ggcccactcc agacctctgt ctccccactc
	↓ **exon 12**
43634240	ctagGCCATG CTGCACCAGG AGGGCCACAT GGACGACGCA CTGTCGCTGA CCCGCTGCCA
43634300	GCAGGAGGAG TCCCAGGCCG CC**CGC**ATGAT CCACAGCACC AATGGCCTAT ACAACCAGTT
43634360	CATCAAgtga gcaacctgcc ctccctgctg gggtgactcc tgtgctgtcc catgctccgg
43634420	gcatccatac acttggcctc tttcatctct acctctgttg cccacacccct tgtctaacat

The partial gDNA sequence amplified for exon 12 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Two single nucleotide polymorphisms are observed in this region, the C18654T nucleotide transition is indicated in blue; the G18682A nucleotide transition is indicated in pink and the C18760T nucleotide transition is indicated in orange. The codon that correlates to Arg401 is indicated in a solid box (–), the codon that correlates to Met402 is indicated in a dashed **blue** box (---), the codon that correlates to Ile403 is indicated in a dashed box (---) and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex12F) is the single underlined sequence, while the reverse primer (RYRex12R) is the double underlined sequence; the beginning of exon 12 is indicated with an arrow.

A C1201T nucleotide substitution that results in an Arg401Cys change was identified in three New Zealand Maori pedigrees and was not detected in 200 unrelated controls of Maori and Caucasian descent (Davis *et al.*, 2002). The Arg401His alteration that is due to a G1202A transition was observed in two MH probands from Germany (Rueffert *et al.*, 2002). In addition, Monnier *et al.* (2005) reported an Arg401Ser alteration that is due to a

C1201A nucleotide transition in one MHS family from France. Zhou *et al.* (2005) reported a G1206C alteration that resulted in a Met402Ile in one CCD patient. The Ile403Met alteration resulting from a C1209G transition was detected in one CCD pedigree of Italian descent (Quane *et al.*, 1993). Analysis of a 232 bp region was conducted in order to detect both reported and novel alterations or polymorphisms that may occur in exon 12 of the RYR1 gene.

3.7.9 Detection of alterations in exon 13 of the RYR1 gene

Analysis of a 480 bp region was conducted in order to detect both reported and novel alterations or polymorphisms that may occur in exon 13 of the RYR1 gene. The partial gDNA sequence of amplified exon 13 from the RYR1 gene is depicted in Table 3.15.

Exon 13 is located in the first mutational hotspot of the RYR1 gene and thus far, only one alteration that results in the MH phenotype has been described and two alterations have been reported in CCD patients. Gillard *et al.* (1992) reported an Arg471Cys alteration in one MHS family. The alteration was observed in the proband and her father and is due to a C1280T substitution. Both the Ser427Leu and Gln474His alterations have been reported in CCD probands and are due to nucleotide transitions C1280T and G1422T, respectively (Wu *et al.*, 2006).

Table 3.15: Partial gDNA sequence of exon 13 of the RYR1 gene

Nucleotide number	DNA sequence: exon 13
43635140	tgtgtgacct cggacaaatg cttttccctc tctgcgtctc ggtctccctc tctgtaaaac
43635200	gggtgggtct gggggagtct tgcgggagtg aggttgcggc agtgacgttg cggcagttag
	↓ exon 13
43635260	cgctcccagc cgtggctgac agctgcgagg tccctgtagG AGCCTGGACA GCTTCAGCGG
43635320	GAAGCCACGG GGCTCGGGGC CACCCGCTGG CACGGCGCTG CCCATCGAGG GCGTTATCCT
43635380	GAGCCTGCAG GACCTCATCA TCTACTTCGA GCCTCCCTCC GAGGACTTGC AGCACGAGGA
43635440	GAAGCAGAGC AAGCTGCGAA GCCTGCGCAA CCGCCAGAGC CTCTTCCAGG AGGAGgtgag
43635500	gacgtggcga gggcggagcg gggcctgtgg gcccaggggg cgggaccact gaggggcggg
43635560	gccacggcgc tgggcggggc agggcctgag ggacctgggg aagtagggtc tgagaagggg
43635620	gcggggcagg agaaggagcc ggacaggaac cccagccagt gagaacacga tggggcgggg

The partial gDNA sequence amplified for exon 13 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Three single nucleotide polymorphisms are observed in this region, the C19691T nucleotide transition is indicated in blue, the G19989A nucleotide transition is indicated in orange and the G20010del is indicated in green. The codon that correlates to Ser427 is indicated in a solid box (—), the codon that correlates to Arg471 is indicated in a dashed blue box (---), the codon that correlates to Gln474 is indicated in a dashed box (---) and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex13F) is the single underlined sequence, while the reverse primer (RYRex13R) is the double underlined sequence; the beginning of exon 13 is indicated with an arrow.

3.7.10 Detection of alterations in exons 14, 15 and 16 of the RYR1 gene

A 605 bp region was amplified via PCR and was subsequently sequenced in order to allow for the simultaneous analysis of reported and novel alterations or polymorphisms that may occur in exons 14, 15 and 16 of the RYR1 gene. All three of these exons reside in the first mutational hotspot. The partial gDNA sequence of amplified exons 14, 15 and 16 from the RYR1 gene is depicted in Table 3.16.

Table 3.16: Partial gDNA sequence of exons 14, 15 and 16 of the RYR1 gene

Nucleotide number	DNA sequence: exons 14, 15 and 16
	exon 14 ↓
43637660	aggaagggag ggcctgggtc tcctattgtg atgcctctta ttttcctcat cctagGGGAT
43637720	GCTCTCCATG GTCCTGAATT GCATAGACCG CCTAAATGTC TACACCACTG CTGCCCACTT
43637780	TGCTGAGTTT GCAGGGGAGG AGGCAGCC**GA G**TCCTGGAAA GAGATTGTGA ATCTTCTC**TA**
43637840	**T**GAACTCCTA Ggtaggggtc ccagtcctga ctcccctgag aacaccccag atccccagtc
	↓ exon 15
43637900	ctattggatc tgacacctct tccccctca gCTTCTCTAA TCCGTGGCAA T**CGT**AGCAAC
43637960	TGTGCCCTCT TCTCCACAAA CTTGGACTGG CTGGTCAGCA AGCTGGAT**CG G**CTGGAGGCC
43638020	TC**G**TCTGgta ggagaacccg ggggagtggg acagaggctt gtgggagggg atgggcatgg
	↓ exon 16
43638080	ccgcttcacc tctcattctg ggcaccctgg cagGCATCCT GGAGGTCCTG TACTGTGTCC
43638140	TCATTGAGAG TCCAGAGGTT CTGAACATCA TCCAGGAGAA TCACATCAAG TCCATCATCT
43638200	CCCTCCTGGA CAAGCATGGG AGGAACCACA AGgtcggccc ctcaccctg acctctcatc
43638260	ccctgaactc tgaatgctgg cctctcccca gggctccaga actctgctca ctccctcact

The partial gDNA sequence amplified for exons 14, 15 and 16 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Three single nucleotide polymorphisms are observed in this region, the T22112C nucleotide transition is indicated in **blue**, the G22443A nucleotide transition is indicated in **orange** and the G22476C nucleotide transition is indicated in **green**. The codon that correlates to Glu512 is indicated in a dashed **blue** box (---), the codon that correlates to Thr522 is indicated in a solid box (–), the codon that correlates to Arg533 is indicated in a **red** solid box (–), the codon that correlates to Arg552 is indicated in a dashed box (---) and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex14F) is the single underlined sequence, while the reverse primer (RYRex14R) is the double underlined sequence; the beginnings of exons 14, 15 and 16 are indicated with an arrow.

Thus far, alterations have not been reported to be associated with MHS in exon 16. However, three alterations have been reported in exon 14. The Thr522Ser alteration was detected in a single pedigree of French descent. The alteration is due to an A1565C transition, was not detected in the unaffected population and is conserved across the RYR species (Quane *et al.*, 1994b). The Tyr522Cys alteration that is due to an A1565G transition was reported in a MH proband (Yeh *et al.*, 2005). The Glu512Lys was reported in one patient diagnosed with CCD and is due to a G1534A substitution (Wu *et al.*, 2006). In addition, three alterations have been reported in exon 15, the Arg552Trp alteration has been identified in an MHS pedigree of Irish descent. The C1654T substitution gives rise to this alteration (Keating *et al.*, 1997). A single novel alteration, Arg533His, which is due to

the nucleotide transition termed G1598A, has been reported in a single MH proband (Brandt *et al.*, 1999) and Tammaro *et al.* (2003) described an Arg533Cys alteration due to a C1597T transition in one MHS family.

3.7.11 Detection of alterations in exons 17 and 18 of the RYR1 gene

The partial gDNA sequence of amplified exons 17 and 18 from the RYR1 gene is depicted in Table 3.17. Exon 17 harbours two reported alterations and is observed in hotspot one of the RYR1 gene.

Table 3.17: Partial gDNA sequence of exons 17 and 18 of the RYR1 gene

Nucleotide number	DNA sequence: exons 17 and 18
43639880	gctgtccttt cctcctggct tccctccctc ccagggttct tctgtagatc ctgccctggt
	↓ exon 17
43639940	gcctacacac cctttaacct ctgaccttga cctctagGTC CTGGACGTGC TATGCTCCCT
43640000	GTGTGTGTGT AATGGTGTGG CTGTACGCTC CAACCAAGAT CTTATTACTG AGAACTTGCT
43640060	GCCTGGCCGT GAGCTTCTGC TGCAGACAAA CCTCATCAAC TATGTCACCA Ggtctggctc
43640120	tcaacatctg accccagaac tcagaacctc tcaaccctct ccctgactta gagactccac
43640180	acccagatgg atgtcctttc cttaatctcc caccccaggg ttaacaacca gtcctcacag
43640240	atgtccactg tggccccact ctcccttggc atccactcct cttgttctgt cttcctggct
43640300	ccatttctgc ctctatctgt ttctctttct ttctccctct ccctctctct ctgttttctc
43640360	tttttatctt tgcctgtttc tgtcttgatt cttcctccat gtctttctcc ctgtctctct
43640420	cccatctctc tctctctgtc tttggatgtc tgtctctctc tggcttccca ccacttggct
	↓ exon 18
43640480	ctcctctctg cctctccgtc atccccctct cctgtcccat ctctcctgca gCATCCGCCC
43640540	CAACATCTTT GTGGGCCGAG CGGAAGGCAC CACGCAGTAC AGCAAATGGT ACTTTGAGGT
43640600	GATGGTGGAC GAGGTGACTC CATTTCTGAC AGCTCAGGCC ACCCACTTGC GGGTGGGCTG
43640660	GGCCCTCACC GAGGGCTACA CCCCCTACCC TGGGGCCGGC GAGGGCTGGG GCGGCAACGG
43640720	GGTCGGCGAT GACCTCTATT CCTACGGCTT TGATGGACTG CATCTCTGGA CAGgtacctg
43640780	acccccttcca ggggaccctc accccctgacc attgacccca gcatttctaa gtctctgacc
43640840	atacaccttg gggttctcag gatcctgact ccctgaaaag gtcaactttt gacctttag
43640900	tcctcatttc ccaagacccct aaccccagag cttctagatt cccggctctg acttgtatcc

The partial gDNA sequence amplified for exons 17 and 18 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Three single nucleotide polymorphisms are observed in this region, the T24617C nucleotide transition is indicated in **blue**, the C24993T nucleotide transition is indicated in **orange** and the C25224G nucleotide transition is indicated in **green**. The codon that correlates to Arg614 is indicated in a solid box (–) and the nucleotide positions of the mutation are indicated in **bold**. The forward primer (RYRex17F) is the single underlined sequence, while the reverse primer (RYRex17R) is the double underlined sequence; the beginnings of exons 17 and 18 are indicated with an arrow.

The Arg614Cys alteration is due to a C1840T transition and was detected in three generations of a family with MH (Moroni *et al.*, 1995), in two North German families with MH (Steinfath *et al.*, 1995), in an individual of Mennonite descent (Serfas *et al.*, 1996) and

in an MH family of Northern European descent (Hogan *et al.*, 1992). Quane *et al.* (1997) identified an Arg614Leu alteration that is due to a G1841T substitution in three unrelated MHS individuals. The alteration segregated with MHS in the families studied and the nucleotide site is conserved between different species. Exon 18 does not currently harbour any alterations associated with MHS and is observed outside hotspot one. Analysis of a 1,004 bp region was conducted in order to detect novel and reported alterations that may occur in exons 17 and 18 of the RYR1 gene, as well as to identify any novel or reported polymorphisms.

3.7.12 Detection of alterations in exon 19 of the RYR1 gene

Analysis of a 382 bp region was conducted in order to detect novel alterations associated with the MH phenotype and novel or reported polymorphisms that may occur in exon 19 of the RYR1 gene. Currently, alterations that result in the MH phenotype have not been reported for this region of the RYR1 gene. However, a single alteration associated with CCD has been reported in one family (Kossugue *et al.*, 2005). The alteration, Asn759Asp, is due to an A2274G nucleotide substitution. The partial gDNA sequence of amplified exon 19 is depicted in Table 3.18.

Table 3.18: Partial gDNA sequence of exon 19 of the RYR1 gene

Nucleotide number	DNA sequence: exon 19
43641500	gcactttcca ttagggtttc caggatgcaa tctccacagg agcctccaat atctgtccct
43641560	tttctcttgt tatcattggt tctgtgggac ctgtgacgtc tgacccatct ctggtgactg
	↓ exon 19
43641620	atgcagGACA CGTGGCACGC CCAGTGACTT CCCCAGGGCA GCACCTCCTG GCCCCTGAAG
43641680	ACGTGATCAG CTGCTGCCTG GACCTCAGCG TGCCGTCCAT CTCCTTCCGC ATCAACGGCT
43641740	GCCCCGTGCA GGGTGTCTTT GAGTCCTTCA ACCTGGACGG GCTCTTCTTC CCTGTTGTCA
43641800	GCTTCTCGGC TGGTGTCAAg tgagaacttg cccccacccc acggccagtc ctcagaccta
43641860	ggactgacct gagacagctt ccccagtcac tccatggtcc cccaggaggc caggacactg

The partial gDNA sequence amplified for exon 19 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Three single nucleotide polymorphisms are observed in this region, the T25990G nucleotide transition is indicated in **blue**, the G26120A nucleotide transition is indicated in **pink** and the C26165T nucleotide transition is indicated in **orange**. The codon that correlates to Asn759 is indicated in a solid box (–) and the nucleotide position of the mutation is indicated in **bold**. The forward primer (RYRex19F) is the single underlined sequence, while the reverse primer (RYRex19R) is the double underlined sequence; the beginning of exon 19 is indicated with an arrow.

3.7.13 Detection of alterations in exon 20 of the RYR1 gene

Thus far, exon 20 has not been reported to harbour any alterations that result in the MH phenotype. The partial gDNA sequence of amplified exon 20 is depicted in Table 3.19. In

order to identify novel alterations and novel or reported polymorphisms that may occur in exon 20, a 489 bp region was amplified of the RYR1 gene.

Table 3.19: Partial gDNA sequence of exon 20 of the RYR1 gene

Nucleotide number	DNA sequence: exon 20
43642580	gttccatgac cttgcagtca tctgaactct tctagattaa ccagagaccc tcccgctctc
43642640	aactccctgg ctcttaattc ccttatgata ttcctttgac tctagacttt cttttatgac
43642700	ttctaaatga cctccaggac actatgactg cccggtgacc tttggtctcc ccagaacttt
43642760	ccattgatcc caggactgct tccatgtccc ccactgacca cagactgtcc cccataacct
	↓ exon 20
43642820	cccctcaatg atccccattg tccttcctta cccagGGTGC GGTTCCTCCT TGGTGGCCGC
43642880	CATGGTGAAT TCAAGTTCCT GCCCCCACCT GGCTATGCTC CATGCCATGA GGCTGTGCTC
43642940	CCTCGAGAGC GACTCCATCT TGAACCCATC AAGGAGTATC GACGGGAGGG GCCCCGGGGG
43643000	CCTCACCTGG TGGGCCCCAG TCGCTGCCTC TCACACACCG ACTTCGTGCC CTGCCCTGTG
43643060	GACACTGTCC AGgtactgcc tgccctgcaa aggttttctg gcgaggcagg gtctcttagg
43643120	agtcagagag ggggcagggt gccatcgttc atgcctgtaa tcccagcact ttggatggcc

The partial gDNA sequence amplified for exon 20 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. A single nucleotide polymorphism is observed in this region, the C27208T nucleotide transition is indicated in a circle. The forward primer (RYRex20F) is the single underlined sequence, while the reverse primer (RYRex20R) is the double underlined sequence; the beginning of exon 20 is indicated with an arrow.

3.7.14 Detection of alterations in exons 21 and 22 of the RYR1 gene

A 565 bp region encompassing exons 21 and 22 of the RYR1 gene was amplified and sequenced. The partial gDNA sequence of exons 21 and 22 is represented in Table 3.20.

Both exons were simultaneously amplified and sequenced in order to identify novel alterations and novel or reported polymorphisms that may occur. To date, these exons have not been reported to harbour any alterations that result in MHS.

Table 3.20: Partial gDNA sequence of exons 21 and 22 of the RYR1 gene

Nucleotide number	DNA sequence: exons 21 and 22
43645820	tcacaggtgt tcttggaaag aggggtcatg atggaggagg gtagagggac cttggggtct
	↓ exon 21
43645880	caagaacgtc cctctgcctc tagATTGTCC TGCCGCCCCA TCTGGAGCGC ATTCGGGAGA
43645940	AGCTGGCGGA GAACATCCAC GAGCTCTGGG CGCTAACCCG CATCGAGCAG GGCTGGACCT
43646000	ACGGCCCGgt gaggggctgc ctgcagcctg cgggaggccg gctagacttg cggtgccagg
43646060	agggagagcg gctcaccggc ggagaggagg gagggaccac agggcaccag ggggtccttg
43646120	gactgagggt ggcagaacta gggttggagg tcaggggtca tagtctgggc atgtggggag
	↓ exon 22
43646180	tgggaaggaa aggggagcac atggagttga ccctgggttt tctccagGTT CGGGATGACA
43646240	ACAAGAGGCT GCACCCGTGT CTTGTGGACT TCCACAGCCT TCCAGAGCCT GAGAGGAACT
43646300	ACAACCTGCA GATGTCTGGG GAGACGCTCA Agtgagggcc caggggagcc gggggttggg
43646360	gctggctgct ggtgcggtgg gggagggagg catggagaga cagggcagga ggtagagact

The partial gDNA sequence amplified for exons 21 and 22 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Two single nucleotide polymorphisms are observed in this region, the A30257G nucleotide transition is indicated in **blue** and the C30301G nucleotide transition is indicated in **orange**. The forward primer (RYRex21F) is the single underlined sequence, while the reverse primer (RYRex21R) is the double underlined sequence; the beginnings of exons 21 and 22 are indicated with an arrow.

3.7.15 Detection of alterations in exon 23 of the RYR1 gene

A region of 256 bp containing exon 23 was amplified in order to detect novel alterations and polymorphisms that may occur in this region of the RYR1 gene. Alterations resulting in the MH phenotype and polymorphisms have not been described for exon 23. The partial gDNA sequence of amplified exon 23 is depicted in Table 3.21.

Table 3.21: Partial gDNA sequence of exon 23 of the RYR1 gene

Nucleotide number	DNA sequence: exon 23
	↓ exon 23
43647080	gaggggcgtg acctgtcgcc tccactcccc cacccccagG ACTCTGCTGG CTCTGGGCTG
43647140	CCACGTGGGC ATGGCGGATG AGAAGGCGGA GGACAACCTG AAGAAGACAA AACTCCCCAA
43647200	GACgtgagtg tgggcagcca ggtcccgtct ggggatggac tgggggctgg ggatgctgtg
43647260	ctaagggctg gggaggtcga ggggtcttgt ggggaggctg aggttaggga ggaaggagac
43647320	ttgggttagg gtgaaggtca tagggtcagg gctctggggt cagaggtgaa tgtccaggat

The partial gDNA sequence amplified for exon 23 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The forward primer (RYRex23F) is the single underlined sequence, while the reverse primer (RYRex23R) is the double underlined sequence; the beginning of exon 23 is indicated with an arrow.

3.7.16 Detection of alterations in exon 24 of the RYR1 gene

The partial gDNA sequence of amplified exon 24 is depicted in Table 3.22. Alterations associated with the MH phenotype have thus far not been reported for this region of the RYR1 gene. Therefore, a region of 475 bp containing exon 24 was amplified in order to detect novel alterations that may occur in this region. In addition, the sequence was screened in order to identify the four reported polymorphisms and was analysed for novel polymorphisms that may occur in the amplified region.

Table 3.22: Partial gDNA sequence of exon 24 of the RYR1 gene

Nucleotide number	DNA sequence: exon 24
43648460	gtcagaaacg ccgaagctgg gacaagggtc agcagtcagg gatcccatat agtgcagagc
	↓ exon 24
43648520	ccggaagtgg aggtgagggc cttgtcccat ggagccctac catgcccgca gGTATATGAT
43648580	GAGCAATGGG TACAAGCCGG CTCCGCTGGA CCTGAGCCAC GTGCGGCTGA CGCCGGCGCA
43648640	GACGACACTG GTGGACCGTC TGGCAGAAAA TGGGCACAAC GTGTGGGCCC GAGACCGCGT
43648700	GGGCCAGGGC TGGAGCTACA GCGCAGTGCA GGACATCCCA GCGCGCCGAA ACCCTCGGCT
43648760	GGTGCCCTAC CGCCTGCTGG ATGAAGCCAC CAAGCGCAGC AACCGGGACA GCCTCTGCCA
43648820	GGCCGTGCGC ACCCTCCTGG GCTACGGCTA CAACATCGAG CCTCCTGACC AGGAGCCCAg
43648880	tgagtgctca cccctggccc tggccctgac tcctacccca actctgaccc cagccccgat
43648940	ccctgatctc tgacctgact cagcccccaa atgggctata tctttttttt tttttttttt

The partial gDNA sequence amplified for exon 24 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Four single nucleotide polymorphisms are observed in this region, the G33064A nucleotide transition is indicated in blue, the C33100T nucleotide transition is indicated in orange, the G33163A nucleotide transition is indicated in green and the A33400G nucleotide transition is indicated in pink. The forward primer (RYRex24F) is the single underlined sequence, while the reverse primer (RYRex24R) is the double underlined sequence; the beginning of exon 24 is indicated with an arrow.

3.7.17 Detection of alterations in exon 25 of the RYR1 gene

Alterations resulting in the MH phenotype have not been reported for exon 25. A region of 402 bp containing exon 25 was amplified in order to detect both novel alterations and novel or reported polymorphisms that may occur in this region of the RYR1 gene. The partial gDNA sequence of amplified exon 25 is depicted in Table 3.23.

Table 3.23: Partial gDNA sequence of exon 25 of the RYR1 gene

Nucleotide number	DNA sequence: exon 25
43649960	aaattgaccc tcttccaaca gttccccaaa gcccttactg tcccccaaag cctgtcttct
43650020	accaacttct cgatgtcttg ggatccacat cttccctagc ctctctgact ctgcctggcc
	↓ exon 25
43650080	tcatttatag GTCAGGTGGA GAACCAGTCT CGTTGTGACC GGGTGCGCAT CTTCCGGGCA
43650140	GAGAAATCCT ATACAGTGCA GAGCGGCCGC TGGTACTTCG AGTTTGAAGC AGTCACCACA
43650200	GGCGAGATGC GCGTGGGCTG GGCGAGGCCC GAGCTGAGGC CTGATGTAGA GCTGGGAGCT
43650260	GACGAGCTGG CCTATGTCTT CAATGGGCAC CGCgtgggta cctccctggg caccattctg
43650320	ccaggtcctg tggtctctcc cacagcttgt ctactctggc cctgcctctg tctgtgcctc
43650380	actctgtccc cacttcatgc atctactgta ccactcatcc acccatctat ccatccattc

The partial gDNA sequence amplified for exon 25 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. A single nucleotide polymorphism is observed in this region, the G34658A nucleotide transition is indicated in a circle. The forward primer (RYRex25F) is the single underlined sequence, while the reverse primer (RYRex25R) is the double underlined sequence; the beginning of exon 25 is indicated with an arrow.

3.7.18 Detection of alterations in exons 26 and 27 of the RYR1 gene

A 668 bp region encompassing exons 26 and 27 of the RYR1 gene was amplified. The partial gDNA sequence of amplified exons 26 and 27 from the RYR1 gene is represented in Table 3.24.

Table 3.24: Partial gDNA sequence of exons 26 and 27 of the RYR1 gene

Nucleotide number	DNA sequence: exons 26 and 27
43651340	gacacttcga atgtctgtct tcatatatct ctccctccct gcttccttat ctctccattt
	↓ exon 26
43651400	ctctgtgtgt ctccccacac catgtcttct ctggctgtcc tcacagGGCC AGCGCTGGCA
43651460	CTTGGGCAGT GAACCATTTG GGCGCCCCTG GCAGCCGGGC GATGTCGTTG GCTGTATGAT
43651520	CGACCTCACA GAGAACACCA TTATCTTCAC CCTCAATGGC GAGGTCCTCA TGTCTGACTC
43651580	AGGCTCCGAA ACAGCCTTCC GGGAGATTGA GATTGGGGAC Ggtgagggct gagacccctt
43651640	cacatgccct ttcttgtttt cctctgtctc tcccaaccct gcactgccct tctgcctcca
43651700	actctcccat ccctacctcc tcccctcggc tccctctgcc ctgcccacct gccctcaccc
	↓ exon 27
43651760	ctgcccatcc atcccctccc accagGCTTC CTGCCCGTCT GCAGCTTGGG ACCTGGCCAG
43651820	GTGGGTCATC TGAACCTGGG CCAGGACGTG AGCTCTCTGA GGTTCTTTGC CATCTGTGGC
43651880	CTCCAGGAAG GCTTCGAGCC ATTTGCCATC AACATGCAGC GCCCAGTCAC CACCTGGTTC
43651940	AGCAAAGGCC TGCCCCAGTT TGAGCCAGTG CCCCTTGAAC ACCCTCACTA TGAGgtaagg
43652000	actgagcccc tcaatgcctt ctcatctgcc tccaaagctc cttccttcca cagtgctctt

The partial gDNA sequence amplified for exons 26 and 27 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Three single nucleotide polymorphisms are observed in this region, the C35941T nucleotide transition is indicated in **blue**, the C36132T nucleotide transition is indicated in **red** and the del36158G nucleotide transition is indicated in green. The forward primer (RYRex26F) is the single underlined sequence, while the reverse primer (RYRex26R) is the double underlined sequence; the beginnings of exons 26 and 27 are indicated with an arrow.

To date, these exons have not been reported to harbour any alterations that result in the MHS. However, three SNPs have been observed in this region of the RYR1 gene.

3.7.19 Detection of alterations in exon 28 of the RYR1 gene

In order to identify novel alterations as well as novel and reported polymorphisms that may occur in exon 28 of the RYR1 gene, a 551 bp region was amplified. To date, this exon has not been reported to harbour any alterations that result in MHS. The partial gDNA sequence of amplified exon 28 is depicted in Table 3.25.

Table 3.25: Partial gDNA sequence of exon 28 of the RYR1 gene

Nucleotide number	DNA sequence: exon 28
43655780	tgtgtgacca ggtgtaggac caacggcctg gcctagcccg cctgcccagc ccagtactcc
	↓ exon 28
43655840	attccctgcc acctcagGTA TCCCGAGTGG ACGGCACTGT GGACACGCCC CCCTGCCTGC
43655900	GCCTGACCCA CCGCACCTGG GGCTCCCAGA ACAGCCTGGT GGAGATGCTT TTCCTGCGGC
43655960	TGAGCCTCCC AGTCCAGTTC CACCAGCACT TCCGCTGCAC TGCAGGGGCC ACCCCGCTGG
43656020	CACCTCCTGG CCTGCAGCCC CCCGCCGAGG ACGAGGCCCG GGCGGCGGAA CCCGACCCTG
43656080	ACTACGAAAA CCTGCGCCGC TCAGCTGGGG GCTGGAGCGA GGCAGAGAAC GGCAAAGAAG
43656140	GGACTGCGAA GGAGGGCGCC CCCGGGGGCA CCCCGCAGGC GGGGGAGAG GCGCAGCCCG
43656200	CCAGGGCGGA GAATGAGAAG GATGCCACCA CCGAGAAGAA CAAGAAGAGA GGgtgagtcg
43656260	agggggGccc agagtgggga ttgggggctg ccttgggacc cccaagtagg caaccacagt
43656320	aacctgagaa acccccattg taccccaaag tagacccata tatgctaagt ggagtaagaa

The partial gDNA sequence amplified for exon 28 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Five single nucleotide polymorphisms are observed in this region, the T40370C nucleotide transition is indicated in blue, the A40536G nucleotide transition is indicated in red, the G40602T nucleotide transition is indicated in pink, the C40619T nucleotide transition is indicated in orange and the T40786G nucleotide transition is indicated in green. The forward primer (RYRex28F) is the single underlined sequence, while the reverse primer (RYRex28R) is the double underlined sequence; the beginning of exon 28 is indicated with an arrow.

3.7.20 Detection of alterations in exon 29 of the RYR1 gene

A 258 bp region of exon 29 was amplified in order to identify novel alterations and novel or reported polymorphisms that may occur in this region of the RYR1 gene. This exon has not been reported to harbour any alterations that result in the MH phenotype. The partial gDNA sequence of amplified exon 29 is depicted in Table 3.26.

Table 3.26: Partial gDNA sequence of exon 29 of the RYR1 gene

Nucleotide number	DNA sequence: exon 29
43657700	gcagggtgta tccaagctgg atgtgggggc atgaatattg cggtgggagg gctgggcttg
	↓ exon 29
43657760	aaagctggct ctcatggcgc ctctcctccc actaccagCT TCTTATTCAA GGCCAAGAAG
43657820	GTCGCCATGA TGACCCAGCC ACCGGCCACC CCCACGCTGC CCCGACTCCC TCACGACGTG
43657880	GTGCCTGCAG ACAACCGCGA TGACCCCGAG ATCATCCTCA ACACCACCAC Ggtgtggacc
43657940	agtaaccctc aattttgggg tccccccgca tagcataggc actcctgaat ttccaagttt

The partial gDNA sequence amplified for exon 29 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. A single nucleotide polymorphism is observed in this region, the C42315T nucleotide transition is indicated in a circle. The forward primer (RYRex29F) is the single underlined sequence, while the reverse primer (RYRex29R) is the double underlined sequence; the beginning of exon 29 is indicated with an arrow.

3.7.21 Detection of alterations in exon 30 of the RYR1 gene

Exon 30 was amplified and sequenced as a 356 bp region in order to identify novel alterations and novel or reported polymorphisms that may occur in this region of the RYR1 gene. This exon has not been reported to harbour any alterations that result in the MH phenotype. The partial gDNA sequence of amplified exon 30 is depicted in Table 3.27.

Table 3.27: Partial gDNA sequence of exon 30 of the RYR1 gene

Nucleotide number	DNA sequence: exon 30
43660040	acataaccag ggggtggggg actcagatcc aacaacttcc tgttaaactc ccagaggacc
43660100	caacagtcca gggaaaccaa tttcgagtcc cagggagccc gagtccctga cttccagact
	↓ exon 30
43660160	gaccactagt tcccctcctt gtgtcaccag TACTATTACT CCGTGAGGGT CTTTGCTGGA
43660220	CAGGAGCCCA GCTGCGTGTG GGCGGGCTGG GTCACCCCTG ACTACCATCA GCACGACATG
43660280	AGCTTCGACC TCAGCAAGGT CCGGGTCGTG ACGGTGACCA TGGGGGATGA ACAAGGCAAC
43660340	GTCCACAGCA Ggtgccgggg ctgggggag gtgggaggtg cagggtgggg agggcaggag
43660400	gcagtcagag ctcccgacac cagctctgtg gctgcctggt tgtgggacct aggaactttc

The partial gDNA sequence amplified for exon 30 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Two single nucleotide polymorphisms are observed in this region, the G44800A nucleotide transition is indicated in **blue** and the T44877C nucleotide transition is indicated in **orange**. The forward primer (RYRex30F) is the single underlined sequence, while the reverse primer (RYRex30R) is the double underlined sequence, the beginning of exon 30 is indicated with an arrow.

3.7.22 Detection of alterations in exon 31 of the RYR1 gene

In order to identify novel alterations and novel or reported polymorphisms that may occur in exon 31, a region of 309 bp was amplified and subsequently sequenced. This exon has not been reported to harbour any alterations that result in the MH phenotype. The partial gDNA sequence of amplified exon 31 is depicted in Table 3.28.

Table 3.28: Partial gDNA sequence of exon 31 of the RYR1 gene

Nucleotide number	DNA sequence: exon 31
43660820	tgaggttgtg tgtttccggg agcttgggga agggggtgtc cagggtccag agctactcac
	↓ exon 31
43660880	atgaggagtg cagtgaccgc ttctgtctcc tgcagCCTCA AGTGTAGCAA CTGCTACATG
43660940	GTGTGGGGCG GAGACTTTGT GAGTCCCGGG CAGCAGGGCC GGATCAGCCA CACGGACCTT
43661000	GTCATTGGGT GCCTGGTGGA CTTGGCCACT GGCTTAATGA CCTTTACAGC CAATGGCAAA
43661060	GAGAGCAACA CCTTTTTCCA Ggtgagtcca ggccacagca atttagcgag agcatcatgt
43661120	cccagcatcc caggacagct cttatagatg tcccctgagg ccagacctca gagatggaac

The partial gDNA sequence amplified for exons 30 and 31 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. A single nucleotide polymorphism is observed in this region, the G45347A nucleotide transition is indicated in a circle. The forward primer (RYRex31F) is the single underlined sequence, while the reverse primer (RYRex31R) is the double underlined sequence; the beginning of exon 31 is indicated with an arrow.

3.7.23 Detection of alterations in exons 32 and 33 of the RYR1 gene

Analysis of a 599 bp region was conducted in order to detect both novel and reported alterations and polymorphisms that occurred in exons 32 and 33 of the RYR1 gene. The partial gDNA sequence of amplified exons 32 and 33 is depicted in Table 3.29.

Table 3.29: Partial gDNA sequence of exons 32 and 33 of the RYR1 gene

Nucleotide number	DNA sequence: exons 32 and 33
43665440	gaggtccaga gtcaaccctc cctccagccc acccgtttgc tcacctcgtc ctcttctcct
	↓ exon 32
43665500	ctgccagGTG GAACCCAACA CTAAGCTATT TCCTGCCGTC TTCGTCCTGC CCACCCACCA
43665560	GAACGTCATC CAGTTTGAGC TGGGGAAGCA GAAGgtacaa gtgcagtgat gggggcacta
43665620	atggggccag gctgaggcag gagatgtggg gaggccaggc gggcagagcc actgaagggg
43665680	agggggcaat ccaagaggtc tccctggaag tggtgtggtg ggacagaggg ggctggccat
	↓ exon 33
43665740	cttgacccat gtgtgtctct ctgccctcag AACATCATGC CGTTGTCAGC CGCCATGTTC
43665800	CAAAGCGAGC GCAAGAACCC GGCCCCGCAG TGCCCACCGC GGCTGGAGAT GCAGATGCTG
43665860	ATGCCAGTGT CCTGGAGCCG CATGCCCAAC CACTTCCTGC AGGTGGAGAC GAGGCGTGCC
43665920	GGCGAGCGGC TGGGCTGGGC CGTGCAGTGC CAGGAGCCGC TGACCATGAT GGCGCTGCAC
43665980	ATCCCCGAGG AGAACCGgtc agggccagcc cagctatgca ggggtgggca ggtgttgcaa
43666040	gccctctggg gtctgggtcc cactcagtgc ccctcctcaa cacaaccccg ggattccaga

The partial gDNA sequence amplified for exons 32 and 33 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. A single nucleotide polymorphism is observed in this region, the G50202A nucleotide transition is indicated in a circle. The codon that correlates to Pro1592 is indicated in a solid box (--) and the nucleotide position of the mutation is indicated in **bold**. The forward primer (RYRex32F) is the single underlined sequence, while the reverse primer (RYRex32R) is the double underlined sequence; the beginnings of exons 32 and 33 are indicated with an arrow.

To date, exon 32 has not been reported to harbour any alterations that result in MHS. Exon 33 however, harbours one alteration, the Pro1592Leu alteration, which is due to a C4775T substitution, was observed in one MHS family (Ibarra *et al.*, 2006).

3.7.24 Detection of alterations in exons 34 of the RYR1 gene

The partial gDNA sequence of amplified exon 34 from the RYR1 gene is depicted in Table 3.30. The amplified region was subsequently sequenced in order to identify reported and novel alterations or polymorphisms.

Table 3.30: Partial gDNA sequence of exon 34 of the RYR1 gene

Nucleotide number	DNA sequence: exon 34
43667900	catcttctcc caggatgggt gaattgatag atggaatggt aggggtttga aggaaagacg
43667960	aatgaataaa tgggtggata gtgatgaagg aaatggagga agagatggtg gcttgactga
	↓ exon 34
43668020	tgcaggaggc tcattcatct gtccctgtct gtttcccacc tctgctgcag GTGCATGGAC
43668080	ATCCTGGAGC TGTCGGAGCG CCTGGACCTG CAGCGCTTCC ACTCGCACAC CCTGCGCCTC
43668140	TACCGCGCTG TGTGCGCCCT GGGCAACAAT CGCGTGGCGC ACGCTCTGTG CAGCCACGTA
43668200	GACCAAGCTC AGCTGCTGCA CGCCCTGGAG GACGCGCACC TGCCAGGCCC ACTGCGCGCA
43668260	GGCTACTATG ACCTCCTCAT CAGCATCCAC CTCGAAAGTG CCTGCCGCAG CCGCCGCTCC
43668320	ATGCTCTCTG AATACATCGT GCCCCTCACG CCTGAGACCC GCGCCATCAC GCTCTTCCCT
43668380	CCTGGAAGGA GCACAGAAAA TGGTCACCCC CGGCATGGCC TGCCGGGAGT TGGAGTCACC
43668440	ACTTCGCTGA GGCCCCCGCA TCATTTCTCG CCCCCCTGTT TCGTGGCCGC TCTGCCAGCT
43668500	GCTGGGGCAG CAGAGGCCCC GGCCCGCCTC AGCCCTGCCA TCCCGCTGGA GGCCCTGCGG
43668560	GACAAGGCAC TGAGGATGCT GGGGGAGGCG GTGCGCGACG GTGGGCAGCA CGCTCGCGAC
43668620	CCCGTCGGGG GCTCCGTGGA GTTCCAGTTT GTGCCTGTGC TCAAGCTCGT GTCCACCCTG
43668680	CTGgtaatgg cttcctcctg ctttcctctg tcccattctt ctcccacatt cccaaaactc
43668740	cagagataca tgcatcaatc ctcctctatt tatgcatcca accacccatt cattccctcc

The partial gDNA sequence amplified for exon 34 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Three single nucleotide polymorphisms are observed in this region, the T52438G nucleotide transition is indicated in **blue**, the C52668T nucleotide transition is indicated in **red** and the G52890T nucleotide transition is indicated in **green**. The codon that correlates to Arg1667 is indicated in a solid box (–), the codon that correlates to Ser1728 is indicated in a dashed box (---), the codon that correlates to Pro1773 is indicated in a solid **red** box (–), the codon that correlates to Leu1786 is indicated in a dashed **red** box (---), the codon that correlates to Pro1787 is indicated in a solid **blue** box (–) and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex34F) is the single underlined sequence, while the reverse primer (RYRex34R) is the double underlined sequence; the beginning of exon 34 is indicated with an arrow.

A Ser1728Phe that is due to a T5182C nucleotide transition has been reported to occur in this exon. This alteration was detected outside the mutational hotspots and was observed in one MH individual from North America. The alteration was not detected in 100 unrelated control North Americans samples and the nucleotide site was highly conserved among different species of RYR1 (Sambuughin *et al.*, 2005). The Arg1667Cys alteration was

observed in three MH families, and is due to a C499T nucleotide transition (Ibarra *et al.*, 2006). In addition, Ibarra *et al.* (2006) observed a Pro1773Ser alteration in one MHS patient from Japan, which is due to a C5317T substitution. Gillard *et al.* (1992) identified two alterations, a Leu1786Pro and Pro1787Leu in exon 34, which are due to nucleotide transitions, T5357T and C5360T, respectively. Both alterations were detected in single families but did not segregate with the MH phenotype.

3.7.25 Detection of alterations in exon 35 of the RYR1 gene

To date, exon 35 has not been reported to harbour any alterations that result in the MH phenotype. The partial gDNA sequence of amplified exon 35 is depicted in Table 3.31. A 416 bp region of exon 35 was amplified and subsequently sequenced. The exon was analysed in order to identify novel alterations and novel or reported polymorphisms that may occur in this region of the RYR1 gene.

Table 3.31: Partial gDNA sequence of exon 35 of the RYR1 gene

Nucleotide number	DNA sequence: exon 35
43671560	tggcatgtgc atgaggggca ggtctggaga atgaggccag ggcctgatga tggaggcctt
43671620	↓ exon 35 gcaggccaca gtgaagaacc gagactttgt cctgtagGTG ATGGGCATCT TTGGCGATGA
43671680	GGATGTGAAA CAGATCTTGA AGATGATTGA GCCTGAGGTC TTCACTGAGG AAGAAGAGGA
43671740	GGAGGACGAG GAGGAAGAGG GTGAAGAGGA AGATGAGGAG GAGAAGGAGG AGGATGAGGA
43671800	GGAAACAGCA CAGGAAAAGG AAGATGAGGA AAAAGAGGAA GAGGAGGCAG CAGAAGGGGA
43671860	GAAAGAAGAA GGCTTGGAGG AAGGGCTGCT CCAGATGAAG TTGCCAGAGT CTGTGAAGTT
43671920	ACAGgtgggc tgctgcttcc tgcttttcgg cctctgtcca tctgggctgg gagacacagg
43671980	gtaggtggga tgtgagtctg gacttcgtcc tcaggcagtg gggagctgtg gaaatgcata

The partial gDNA sequence amplified for exon 35 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Two single nucleotide polymorphisms are observed in this region, the A56152G nucleotide transition is indicated in **red** and the C56379G nucleotide transition is indicated in **blue**. The forward primer (RYRex35F) is the single underlined sequence, while the reverse primer (RYRex35R) is the double underlined sequence; the beginning of exon 35 is indicated with an arrow.

3.7.26 Detection of alterations in exons 36 and 37 of the RYR1 gene

In order to identify both novel alterations and novel or reported polymorphisms of the RYR1 gene, exons 36 and 37 were simultaneously amplified as a 773 bp region. To date, both these exons have not been reported to harbour any alterations that result in MHS. The partial gDNA sequence of amplified exons 36 and 37 is depicted in Table 3.32.

Table 3.32: Partial gDNA sequence of exons 36 and 37 of the RYR1 gene

Nucleotide number	DNA sequence: exons 36 and 37
43672460	agggccatgg agaggggaga ggaagcaaga gaagtttcaa ggaagtcctg atggtctcac
	↓ exon 36
43672520	ctccatctct cctcccacac ggctgtcctt ccacagATGT GCCACCTGCT GGAGTATTTC
43672580	TGTGACCAAG AGCTGCAGCA CCGTGTGGAG TCCCTGGCAG CCTTTGCGGA GCGCTATGTG
43672640	GACAAGCTCC AGGCCAACCA GCGGAGCCGC TATGGCCTCC TCATAAAAGC CTTCAGCATG
43672700	ACCGCAGCAG AGACTGCAAG ACGTACCCGC GAGTTCCGCT CCCCACCCCA GGAACAGgtc
43672760	atctgacccc tgacgctggc cacttttact gtctaaaccc caacctcaac atctcctgac
43672820	tctgatcact gaggaccctc aacctctaaa cccgtgcttg accccctgacc ctagtgatac
43672880	atttatctcc tactctctga atcaacctga cctctgagtc acctcagact gatgctgact
43672940	cttttcaaac ctctggccct agtctcccaa atagtcttca ttaactcaca cttcgactca
43673000	tgaccttaga catggactaa caattgcatc ttctatctct gatctcagag ttcctgcttt
	↓ exon 37
43673060	gggatctcag accctcattc taatctttga ccttccccta gATCAATATG CTATTGCAAT
43673120	TCAAAGATGG TACAGATGAG GAAGACTGTC CTCTCCCTGA AGAGATTCGA CAGGATTTGC
43673180	TTGACTTTCA TCAAGACCTG CTGGCACACT GTGgtaagga gtggggatca gagagtcctc
43673240	cccatgctaa ctttctctcg agacctctcc agaagtttcc ctaagatttc ctgacaaccc

The partial gDNA sequence amplified for exons 36 and 37 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Two single nucleotide polymorphisms are observed in this region, the C57264T nucleotide transition is indicated in **blue** and the A57545G nucleotide transition is indicated in **orange**. The forward primer (RYRex36F) is the single underlined sequence, while the reverse primer (RYRex36R) is the double underlined sequence; the beginnings of exons 36 and 37 are indicated with an arrow.

3.7.27 Detection of alterations in exon 38 of the RYR1 gene

A 342 bp region of exon 38 was amplified in order to identify novel and reported alterations as well as to detect polymorphisms that may occur in this region of the RYR1 gene. The partial gDNA sequence of amplified exon 38 from the RYR1 gene is represented in Table 3.33. Gillard *et al.* (1992) identified a Gly2060Cys alteration that is due to a G6178T transition in exon 38. The alteration was detected in a single family but did not segregate with the MH phenotype.

Table 3.33: Partial gDNA sequence of exon 38 of the RYR1 gene

Nucleotide number	DNA sequence: exon 38
43674860	aaatgaaaaa ctccatgcat gcatgcacat atgcacaaat aaatgagtgt gtaagcaggt
43674920	↓ exon 38 gaataagcaa actaatgaat gacatttccc gccttcttga ccacttccag GAATTCAGCT
43674980	AGATGGAGAG GAGGAGGAAC CAGAGGAAGA GACCACCCTG GGCAGCCGCC TCATGAGCCT
43675040	GTTGGAGAAA GTGCGGCTGG TGAAGAAGAA GGAAGAGAAA CCTGAGGAGG AGCGGTCAGC
43675100	AGAGGAGAGC AAACCCCgtg aggactgggg tcactgggga gagggcaggg gtggggtggg
43675160	tagccccatg cctgcggagc ctctgggtcc caaagagggc atgaggacag atgcaaggga
43675220	ggggtagata ggcaggagtg agaggggaag agtggcgggc aaagtggaag caggcgtggt

The partial gDNA sequence amplified for exon 38 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. A single nucleotide polymorphism is observed in this region, the C59592T nucleotide transition is indicated in a circle. The codon that correlates to Gly2060 is indicated in a solid box (—) and the nucleotide position of the mutation is indicated in bold. The forward primer (RYRex36F) is the single underlined sequence, while the reverse primer (RYRex36R) is the double underlined sequence; the beginning of exon 38 is indicated with an arrow.

3.7.28 Detection of alterations in exon 39 of the RYR1 gene

The partial gDNA sequence of amplified exon 39 is depicted in Table 3.34. Exon 39 is observed in mutation hotspot two of the RYR1 gene. A 519 bp PCR product of exon 39 was amplified in order to identify novel and reported alterations as well as detect polymorphisms that may occur in this region of the RYR1 gene. Manning *et al.* (1998a) provided the first report of three novel alterations that clustered in the central portion (6400 - 6700) of the RYR1 gene. The alterations, Arg2163Cys, Arg2163His and Val2168Met, result from the transitions C6487T, G6488A and G6502A, respectively. The transitions occur in exon 39 of the RYR1 gene and all three alterations are causative, as the amino acids that are affected are conserved in the three isoforms of RYR. Arg2163His was observed in an individual from a single family that had both MH and CCD. However, the daughter of the proband had the mutation but was asymptomatic for CCD. Tammaro *et al.* (2003) observed two novel alterations in exon 39 in two MH families. The Val2117Leu is due to a G6349C transition and the Met2101Lys alteration is due to a A6302C transition. An alteration, Asp2129Glu, which is due to a C6387G nucleotide transition, was observed in a single MH family (Rueffert *et al.*, 2001). Fortunato *et al.* (2000) observed a G6488C transition that results in an Arg2163Pro alteration in one MH family. A single alteration, Ile2182Phe, was observed in a single family diagnosed with MH and is due to an A6544T nucleotide transition (Rueffert *et al.*, 2002).

Table 3.34: Partial gDNA sequence of exon 39 of the RYR1 gene

Nucleotide number	DNA sequence: exon 39
43676652	aagaaaaaaa ggaaaacaat ctgctagaat ctgcctgctc ccagcaggtg gagggcgcag
43676712	gtggtagtaa ctgggaaaac ttctggaaca gggggcccct tccacattgt tctggtccaa
43676772	ggccccatgt gccgacctgc cctgcatggt gctccaagcc ttgcattgtc tccttcccag
	↓**Exon 39**
43676832	GGTCCCTGCA GGAGCTGGTG TCCCAC**A**TGG TGGTGCGCTG GGCCCAAGAG GACTTCGTGC
43676892	AGAGCCCCGA GCTG**G**TGCGG GCCATGTTCA GCCTCCTGCA CCGGCAGTAC GACGGGCTGG
43676952	GTGAGCTGCT GCGTGCCCTG CCGCGGGCGT ACACCATCTC ACCGTCCTCC GTGGAAGACA
43677012	CCATGAGCCT GCTCGAGTGC CTCGGCCAGA TC**C**GCTCGCT GCTCATC**G**TG CAGATGGGCC
43677072	CCCAGGAGGA GAACCTCATG ATCCAGAGC**A** TCGGgtgaga caccgccctt cccttactt
43677132	tgcatatccc cttgggtaat gaatacccte aggatacaat aacattccct tccccaactt
43677192	ctggcccatc ctctgggtga tctcagtctc tcgatggcta gctcacctcc tgggtaatga

The partial gDNA sequence amplified for exon 39 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The codon that correlates to Met2101 is indicated in a dashed **red** box (—), the codon that correlates to Val2117 is indicated in a solid **red** box (–), the codon that correlates to Asp2129 is indicated in a solid **blue** box (–), the codon that correlates to Arg2163 is indicated in a solid box (–), the codon that correlates to Val2168 is indicated in a dashed box (—), the codon that correlates to Ile2182 is indicated in a dashed **blue** box (—), and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex39F) is the single underlined sequence, while the reverse primer (Val2168Met) is the double underlined sequence; the beginning of exon 39 is indicated with an arrow.

3.7.29 Detection of alterations in exon 40 of the RYR1 gene

A 229 bp PCR product of exon 40 from hotspot two was amplified in order to identify novel and reported alterations and polymorphisms that may occur in this region of the RYR1 gene. The partial gDNA sequence of amplified exon 40 is depicted in Table 3.35.

Table 3.35: Partial gDNA sequence of exon 40 of the RYR1 gene

Nucleotide number	DNA sequence: exon 40
	↓ **exon 40**
43678652	gacctgggcc cctggtgacc ccgcacactc tgcccgtgca cagGAACATC ATGAACAACA
43678712	AAGTCTTCTA CCAACACCCG AACCTGATGA GG**G**CGCTGGG CATGCACGAG A**C**GGTCATGG
43678772	AG**G**TCATG**G**T CAAC**G**TCCTC GGGGGCGGCG AGTCCAAGGt gagggcccag gcaggtgctg
43678832	gggagctcag gggaggcagc cacagagggc aggccctgac caccctgcct gtcccaggag

The partial gDNA sequence amplified for exon 40 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The codon that correlates to Ala2200 is indicated in a solid box (–), the codon that correlates to Thr2206 is indicated in a dashed box (---), the codon that correlates to Val2210 is indicated in a solid **blue** box (–), the codon that correlates to Val2212 is indicated in a dashed **pink** box (—), the codon that correlates to Val2214 is indicated in a solid **red** box (–) and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRE40F) is the single underlined sequence, while the reverse primer (RYRE40R) is the double underlined sequence; the beginning of exon 40 is indicated with an arrow.

Six alterations associated with the MH phenotype have been reported in exon 40. Halsall and Robinson (2004) identified an Ala2200Val alteration that results from a C6599T transition in the RYR1 gene in one of 434 UK families. Manning *et al.* (1998a) provided the

first report of a novel alteration, Thr2206Met, which clustered in the central portion (6400 - 6700) of the RYR1 gene. The alteration Thr2206Met is a result of a nucleotide transition C6617T. Brandt *et al.* (1999) observed the Thr2206Arg alteration of exon 40 in a single MH pedigree that was due to nucleotide transition C6617G. In addition, Sambuughin *et al.* (2005) identified a Val2210Phe alteration due to a G6628T nucleotide transition in one MH individual from North America. The nucleotide site was conserved through the RYR1 evolution and across RYR1 species and was not detected in 200 unaffected chromosomes. Recently, a Val2212Asp alteration was observed in a single MH proband from Italy due to a T6635A nucleotide transition (Galli *et al.*, 2006). A novel Val2214Ile alteration due to a G6640A substitution was observed in one North American MH pedigree and was not detected in 158 unaffected chromosomes (Sambuughin *et al.*, 2001b).

3.7.30 Detection of alterations in exons 41 and 42 of the RYR1 gene

A 613 bp PCR product of exons 41 and 42 from hotspot two was amplified in order to identify novel and reported alterations and polymorphisms that may occur in this region of the RYR1 gene. The partial gDNA sequence of amplified exons 41 and 42 is depicted in Table 3.36.

Table 3.36: Partial gDNA sequence of exons 41 and 42 of the RYR1 gene

Nucleotide number	DNA sequence: exons 41 and 42
43678820	aggcaggtgc tggggagctc aggggaggca gccacagagg gcaggccctg accaccctgc
	↓ exon 41
43678880	ctgtcccagG AGATCCGCTT CCCCAAGATG GTGACAAGCT GCTGCCGCTT CCTCTGCTAT
43678940	TTCTGCCGAA TCAGCCGGCA GAACCAGCGC TCCATGTTTG ACCACCTGAG CTACCTGCTG
43679000	GAGAACAGTG GCATCGGCCT GGgtgagaac ccccgagccc aggggctgtc ccccagaacc
43679060	cactcctggc accccgtcca ggcctgcccc actttccacc agctcactca ttcaacaaac
43679120	actccctctc aactgtggtt ctggccctgt aatgagtaat gctggggaca caatagtgac
43679180	cccaatagtg acagcccaga gtggtcagag cttggatgag ggaagtacag accagaggag
43679240	gcacctgatc caggctggaa aaagggtggt cagggagggc ttcccagagg @ggcgagaca
	↓ exon 42
43679300	agcaggagtg agatgttctc cccacctctc gcccctgcag GCATGCAGGG CTCCACGCCC
43679360	CTGGACGTGG CTGCTGCCTC CGTCATTGAC AACAATGAGC TGGCCTTGGC ATTGCAGGAG
43679420	CAGGACCTGG AAAAGgtgtg gagggcaggg ctgggcccca ggcctaaggg aggaaatcgg

The partial gDNA sequence amplified for exons 41 and 42 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. A single nucleotide polymorphism is observed in this region, the A63711G nucleotide transition is indicated in a circle. The codon that correlates to Val2280 is indicated in a solid box (–), the codon that correlates to Asn2283 is indicated in a dashed box (---), and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex41F) is the single underlined sequence, while the reverse primer (RYRex41R) is the double underlined sequence; the beginnings of exons 41 and 42 are indicated with an arrow.

Thus far, alterations resulting in the MH phenotype have not been reported for exon 41. However, two alterations have been reported to occur in exon 42, in a family diagnosed with MH and a family diagnosed with CCD. Galli *et al.* (2002) reported a Val2280Ile alteration due to a G6838A substitution and Zhou *et al.* (2005) reported an Asn2283His alteration, which is due to the presence of a A6847C transition.

3.7.31 Detection of alterations in exon 43 of the RYR1 gene

A region of 238 bp of the RYR1 gene was analysed in order to identify novel and reported alterations and polymorphisms in the PCR product of exon 43, which resides in the second mutational hotspot. Thus far, two alterations resulting in the MH phenotype has been reported for this exon. The Asn2342Ser alteration occurs due to an A7025G nucleotide substitution and was identified in two UK MH families (Halsall and Robinson, 2004). In addition, Galli *et al.* (2006) reported an Arg2336Gln alteration in two MH families from Italy. The alteration is due to a G7007A nucleotide substitution. The partial gDNA sequence of amplified exon 43 from the RYR1 gene is depicted in Table 3.37.

Table 3.37: Partial gDNA sequence of exon 43 of the RYR1 gene

Nucleotide number	DNA sequence: exon 43
43681520	cagagggctg agccccagga ggaaggtggc atgggtctgg tctctgactg agccccttct
	↓ exon 43
43681580	gcccccagGT TGTGTCCTAC CTGGCAGGCT GTGGCCTCCA GAGCTGCCCC ATGCTTGTGG
43681640	CCAAAGGGTA CCCAGACATT GGCTGGAACC CCTGTGGTGG AGAGCGCTAC CTGGACTTCC
43681700	TGCGCTTTGC TGTCTTCGTC AACGgtgagg agggggtggc agtggcagag cgggaagtat
43681760	ggagtcactg gtcacacacc tccctcgaga tgactgctcg caccctgagc cacagatggg

The partial gDNA sequence amplified for exon 43 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Three single nucleotide polymorphisms are observed in this region, the A66078G nucleotide transition is indicated in **red**, the C66171T nucleotide transition is indicated in **blue** and the G66207A nucleotide transition is indicated in **orange**. The codon that correlates to Asn2342 is indicated in a solid box (–), the codon that correlates to Arg2336 is indicated in a **pink** dashed box (---) and the nucleotide position of the mutation is indicated in **bold**. The forward primer (RYRE43F) is the single underlined sequence, while the reverse primer (RYRE43R) is the double underlined sequence; the beginning of exon 43 is indicated with an arrow.

3.7.32 Detection of alterations in exons 44 and 45 of the RYR1 gene

To detect 18 known alterations in exon 44 to exon 45, a region of 936 bp was amplified and subsequently sequenced. The partial sequence of the amplified exons is depicted in Table 3.38 and both exons reside in the second mutational hotspot.

Table 3.38: Partial gDNA sequence of exons 44 and 45 of the RYR1 gene

Nucleotide number	DNA sequence: exons 44 and 45
	↓ Exon 43
43681587	gGTTGTGTCC TACCTGGCAG GCTGTGGCCT CCAGAGCTGC CCCATGCTTG TGGCCAAAGG
43681647	GTACCCAGAC ATTGGCTGGA ACCCCTGTGG TGGAGAGCGC TACCTGGACT TCCTGCGCTT
43681707	TGCTGTCTTC GTCAACGgtg aggaggggt ggcagtggca gagcgggaag tatggagtca
43681767	ctggtcacac acctccctcg agatgactgc tcgcaccctg agccacagat ggggtccagg
43681827	caggaatccc ttccagcagg cctggggctg gcaggggcct gtgttacccc tggaggtgtt
43681887	gggtcctgtg gctggcagtg ttggatcctg gggctggcgg gagcctggtg ttacccctag
43681947	aggtgttggg tcctggggct ggcaggggcc tggtgttacc tctggaggtg ttgggtcctg
43682007	gagctggatg ggacctgtgt taccoctgga ggtgttgggt cctggggctg catggggagg
	↓ Exon 44
43682067	tctctgatgg tggctcatga gaccccttt ccccatgcgg gtggccagGC GAGAGCGTGG
43682127	AGGAGAACGC CAATGTGGTG GTGCGGCTGC TCATCCGGAA GCCTGAGTGC TTCGGACCCG
43682187	CCCTGCGGGG TGAGGGTGGC TCAGGGCTGC TGGCTGCCAT CGAAGAGGCC ATCCGCATCT
43682247	CCGAGGACCC TGCGAGGGAT GGCCCAGGCA TCCGCAGGGA CCGGCGGCGC GAGCAgtgag
43682307	tctcccggcc ccctcctcaa tagggcaacc cgccctccct ggcccctggc tgcctcccca
	↓ Exon 45
43682367	acccacccac cttccctgca gCTTTGGTGA GGAACCGCCT GAAGAAAACC GGGTGCACCT
43682427	GGGACACGCC ATCATGTCCT TCTATGCCGC CTTGATCGAC CTGCTCGGAC GCTGTGCACC
43682487	AGAGATGCAT gtgagaccct gagccagggc aggatgggaa gggagggcag gcacagccgc
43682547	tttgaacgcc ctcatgcagg cactcggtga cacggagtga gctcccatat gtgggtggtc

The partial gDNA sequence amplified for exons 43, 44 and 45 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Six single nucleotide polymorphisms are observed in this region, the A66078G nucleotide transition is indicated in **red**, the C66171T nucleotide transition is indicated in **blue**, the G66207A nucleotide transition is indicated in **orange**, the C66597T nucleotide transition is indicated in **dark purple**, the C66606T nucleotide transition is indicated in **green** and the C66854T nucleotide transition is indicated in **pink**. The codon that correlates to Glu2344 is indicated in a solid box (–), the codon that correlates to Val2346, is indicated in a dashed box (---), the codon that correlates to Glu2348 is indicated in a solid **blue** box (–), the codon that correlates to Ala2350 is indicated in a solid **red** box (–), the codon that correlates to Arg2355 is indicated in a **blue** dashed box (---), the codon that correlates to Glu2362 is indicated in a **pink** dashed box (---), the codon that correlates to Phe2364 is indicated in a **red** dashed box (---), the codon that correlates to Pro2366 is indicated in a light blue box (–), the codon that correlates to Ala2367 is indicated in a **green** dashed box (---), the codon that correlates to Gly2375 is indicated in a solid purple box (–), the codon that correlates to Met2423 is indicated in a light blue dashed box ( ), the codon that correlates to Ala2428 is indicated in a **pink** dashed box (---), the codon that correlates to Asp2431 is indicated in a purple dashed box (---), the codon that correlates to Gly2434 is indicated in a solid **green** box (–) the codon that correlates to Arg2435 is indicated in a solid **pink** box (–), the codon that correlates to Ala2436 is indicated in a solid **red** box (–), the codon that correlates to Glu2439 is indicated in a **red** dashed box (---) and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex43F) is the single underlined sequence, while the reverse primer (Glu2434R) is the double underlined sequence; the beginnings of exons 43, 44 and 45 are indicated with an arrow.

Ten alterations have been identified in exon 44. A Glu2344Asp alteration was observed due to a G7032C transition in one Italian individual that was diagnosed as MH due to an observed clinical crisis. The Ala2350Thr missense alteration was first identified by Sambuughin *et al.* (2001a) and is due to a nucleotide transition of G7048A. In addition, Val2346Met, Glu2348Gly and Phe2364Val were demonstrated by Halsall and Robinson (2004). Val2346Met and Glu2348Gly were described in a single UK family and were due to a G7036A and A7043G transition, respectively. The alteration Glu2362Gly was identified in an MH proband from Italy (Galli *et al.*, 2006) whereas the alteration

Phe2364Val was identified in a UK family. It is due to a T7090G transition. Lastly, an Ala2367Thr alteration that is due to nucleotide transition G7099A has been reported in one North American MHS individual (Sambuughin *et al.*, 2001b). The Arg2355Cys alteration was first reported by McWilliams *et al.* (2002) in a large Brazilian MH family and was subsequently reported in six MH families from the UK (Halsall and Robinson, 2004).

Alteration Gly2375Ala was observed in one family with MH and is due to nucleotide transition G7124C. The alteration was investigated in a myotube derived from a mutation carrier and altered Ca^{2+} homeostasis as it displayed higher sensitivity to RyR agonists (Wehner *et al.*, 2004). The Arg2355Cys alteration was first reported by McWilliams *et al.* (2002) in a large Brazilian MH family and was subsequently reported in six MH families from the UK (Halsall and Robinson, 2004). A novel alteration, Pro2366Arg, was observed in a single MH family from Japan. The alteration results from a C7097G substitution and is conserved among RYR1 isoforms (Ibarra *et al.*, 2006).

Thus far, a total of eight alterations have been reported to be associated with MH in exon 45. An Ala2428Thr alteration, which is due to a G7282A transition, has been observed in one MHS individual from France (Monnier *et al.*, 2005). Zhou *et al.* (2005) observed a Met2423Lys alteration in one family diagnosed with MH. The alteration is due to a T7268A nucleotide transition. One novel alteration was detected with a frequency of one MHS individual from North America. The alteration Asp2431Asn is due to G7291A transition, and was not detected in 134 - 158 unaffected chromosomes. The alteration was also conserved among different RYR isoforms (Sambuughin *et al.*, 2001b).

Alterations Gly2434Arg, Arg2435His and Arg2435Leu, detected in this region, are currently being used in the genetic diagnosis of MHS in Europe (Ørding *et al.*, 1997). In four families with MH, Keating *et al.* (1994) observed the Gly2433Arg alteration, adjacent to an Arg2434His alteration. The amino acid numbering was altered according to corrected sequence data for the human RYR1 provided by Phillips *et al.* (1996) and the alterations were renamed Gly2434Arg and Arg2435His in a study conducted by Richter *et al.* (1997). These mutations are due to G7300A and G7304A transitions, respectively. Arg2435Leu was identified in a single UK family by Halsall and Robinson (2004) and is caused by a G7304T transition. In addition, alterations Ala2436Val and Glu2439Asp were identified each in a single family from Italy. They are due to nucleotide substitutions C7310T and G7317C, respectively (Galli *et al.*, 2006).

3.7.33 Detection of alterations in exon 46 of the RYR1 gene

Sequencing was used to screen mutations observed in exon 46, which resides in hotspot two. The partial sequence of amplified exon 46 is depicted in Table 3.39. Chamley *et al.* (2000) observed a C7354T transition that resulted in an Arg2452Trp alteration in exon 46 in a 6-month-old child with MH. In addition, Ibarra *et al.* (2006) observed an Arg2452Gln alteration due to a G7355A substitution. The alteration is conserved and was detected in one Japanese MH family. A recently observed alteration, Ile2453Thr, which results from a T7358C substitution, was observed in a patient with spondylocostal dysostosis who developed an MH reaction during anaesthesia. The alteration segregated in the mother who was diagnosed with both CCD and MH and was absent in 82 unaffected individuals (Rueffert *et al.*, 2004). The alteration Arg2454Cys that was first reported in a single MH pedigree, results in a C7360T nucleotide substitution (Brandt *et al.*, 1999). An Arg2454His alteration that is due to a G7361A substitution was observed in one family with MH (Barone *et al.*, 1999). The alteration was absent in 50 unaffected chromosomes and was conserved across species and related isoforms (Barone *et al.*, 1999). Alterations Arg2458Cys and Arg2458His were both first reported by Manning *et al.* (1998). The two novel mutations were due to a C7372T and G7373A transition, respectively. Both alterations occur at a CpG dinucleotide in the central portion of the RYR1 gene.

Table 3.39: Partial gDNA sequence of exon 46 of the RYR1 gene

Nucleotide number	DNA sequence: exon 46
43682979	aaagaggcct gctctaccct cctgtgtggt aagggaggga gcagagcagt cactgagtgg
	↓ Exon 46
43683039	ggcaccagcg cctgatgagt gcccctctcc ctccctctac tccccagCTA ATCCAAGCCG
43683099	GCAAGGGTGA GGCCCTG**CG**G **A**TC**CG**CGCCA TCCTC**CG**CTC CCTTGTGCCC TTGGAGGACC
43683159	TTGTGGGCAT CATCAGCCTC CCACTGCA**G**A TTCCCACCCT GGGCAAAGgt gcagagggat
43683219	ggaacttggc gaaggagta tgctggggag ggtggtccgc aggcatcccc gaacccaccc

The partial gDNA sequence amplified for exon 46 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Two single nucleotide polymorphisms are observed in this region, the C67488T nucleotide transition is indicated in **blue** and the G67608A nucleotide transition is indicated in **red**. The codon that correlates to Arg2452 is indicated in a solid box (–), the codon that correlates to Ile2453 is indicated in a dashed box (---), the codon that correlates to Arg2454 is indicated in a solid **blue** box (–) and the codon that correlates to Arg2458 in a solid **red** box (–) respectively and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYR46F) is the single underlined sequence, while the reverse primer (RYR46R) is the double underlined sequence; the beginning of exon 46 is indicated with an arrow.

3.7.34 Detection of alterations in exon 47 of the RYR1 gene

A region of 308 bp of exon 47 was analysed in order to identify novel and reported alterations as well as detect polymorphisms that may occur in this region of the RYR1

gene. The partial gDNA sequence of amplified exon 47 from the RYR1 gene is depicted in Table 3.40.

Table 3.40: Partial gDNA sequence of exon 47 of the RYR1 gene

Nucleotide number	DNA sequence: exon 47
43683200	GGCAAAGgtg cagaggggat ggaacttggc gaaggagtga tgctggggag ggagcggctg
	↓ exon 47
43683260	ggtccgcagg gcatccccga acccaccctc cctgcctgca gATGGGGCTC TGGTGCAGCC
43683320	AAAGATGTCA GCATCCTTCG TGCCGGACCA CAAGGCGTCC ATGGTGCTCT TCCTGGACCG
43683380	TGTGTATGGC ATCGAGAACC AGGACTTCTT GCTGCACGTG CTGGACGTGG GGTTCCTGCC
43683440	CGACATGAGG GCAGCCGCCT CGCTGGACAC Ggtgagcaac cctgcccagc ctggccaccc
43683500	tccccacttc cacagaggga caggagatgg gtcacggtag agcagcagca gctgcttttg

The partial gDNA sequence amplified for exon 47 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Five single nucleotide polymorphisms are observed in this region, the G67777A nucleotide transition is indicated in **blue**, the G67804A nucleotide transition is indicated in **orange**, the C67861T nucleotide transition is indicated in green and the C67901G nucleotide transition is indicated in **pink**. The codon that correlates to Pro2496 is indicated in a solid black box (–), the codon that correlates to Arg2508 is indicated in a dashed black box (---) and the nucleotide position of the mutation is indicated in **bold**. The forward primer (RYRex47F) is the single underlined sequence, while the reverse primer (RYRex47R) is the double underlined sequence; the beginning of exon 47 is indicated with an arrow.

To date, two alterations associated with MH have been reported to occur in exon 47. This exon resides outside the second mutational hotspot and harbours the recently reported Arg2508His alteration, that was identified in one MH family from Italy (Galli *et al.*, 2006) and the Pro2496Leu alteration, that was reported in one MH family from Japan (Ibarra *et al.*, 2006). In addition, three alterations were identified in patients diagnosed with CCD. The Arg2508Cys, Arg2508His and Arg2508Gly are due to nucleotide transitions C7522T, C7522G and G7523A, respectively (Ibarra *et al.*, 2006; Wu *et al.*, 2006).

3.7.35 Detection of alterations in exons 48 and 49 of the RYR1 gene

The gDNA sequence of amplified exons 48 and 49 from the RYR1 gene is represented in Table 3.41. A 624 bp PCR product encompassing both these exons was amplified.

Analysis of the PCR product encompassing exons 48 and 49 was subsequently sequenced in order to identify both novel and reported alterations. To date, two alterations in these exons have been reported to be associated with MHS. The Arg2591Gly alteration was reported in two MH families from Italy and is due to a C7771G substitution and the Val2627Leu alteration was reported in one family and is due to the nucleotide transition G7888C (Galli *et al.*, 2006). In addition, a single alteration Glu2454Asp has been reported in one patient diagnosed with CCD (Wu *et al.*, 2006).

Table 3.41: Partial gDNA sequence of exons 48 and 49 of the RYR1 gene

Nucleotide number	DNA sequence: exons 48 and 49
43684880	ggggagtcat cagaagcttg gatcctttgg ccacagtccc tcaagacagg tgccagagca
	↓ exon 48
43684940	gccccagggg tgtgcagcgg gcctgatgtc ctcaccctgc gccctagGCC ACTTTCAGCA
43685000	CCACCGAGAT GGCGCTGGCG CTGAACCGCT ACCTGTGCCT GGCCGTGCTG CCGCTCATCA
43685060	CCAAGTGTGC GCCGCTCTTT GCGGGCACAG AACACCGCGC CATCATGGTG GACTCTATGC
43685120	TGCATACCGT GTACCGCCTG TCTCGGGGTC GTTCGCTCAC CAAGGCGCAG CGTGACGTCA
43685180	TCGAGGACTG CCTCATGTCG CTCTGCAGgt ggagcggggc aggcttcagg gtggggcagg
43685240	ggcaggggca ggggcagggg caggggcagg ggcaggggca ggggcagggg gaggagcagg
43685300	ggcaggggca gcagagcggg cctggacggg ggattctaca tcttgtgcat tgtcccgcag
	↓ exon 49
43685360	GTACATCCGC CCGTCGATGC TGCAGCACCT GTTGCGCCGC CTGGTGTTCG ACGTGCCCAT
43685420	CCTCAACGAG TTCGCCAAGA TGCCACTCAA Ggtgagggca agcgctcttt agcatctcat
43685480	ttccaggccg cacccactgg tttgctcttc cctcctactg cggggctcat ttgtgtcggc

The partial gDNA sequence amplified for exons 48 and 49 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Seven single nucleotide polymorphisms are observed in this region, the A69633G nucleotide transition is indicated in **blue**, the G69655C nucleotide transition is indicated in green, the G69711C nucleotide transition is indicated in **pink**, the A69715G nucleotide transition is indicated in purple, the C69808T is indicated in **orange**, the C69817T nucleotide transition is indicated in **red** and the A69899G nucleotide transition is indicated in dark green. The codon that correlates to Glu2454Asp, is indicated in a dashed **red** box (—), the codon that correlates to Arg2591, is indicated in a solid box (–), the codon that correlates to Val2627, is indicated in a dashed box (---) and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex48F) is the single underlined sequence, while the reverse primer (RYRex48R) is the double underlined sequence; the beginnings of exons 48 and 49 are indicated with an arrow.

3.7.36 Detection of alterations in exons 50, 51 and 52 of the RYR1 gene

A region of 943 bp was amplified and harbours exon 50, 51 and 52 of the RYR1 gene. This region was analysed in order to identify reported and novel alterations as well as detect polymorphisms that may occur in this region of the RYR1 gene. The partial gDNA sequence of amplified exon 50 is depicted in Table 3.42.

A single alteration, Arg2676Trp, which is due to a C8026T transition has been reported in a family susceptible to MH that was also diagnosed with MmD (Guis *et al.*, 2004). Exon 51 has been reported to harbour a Gly2733Asp alteration that is due to a G8198A transition (Sambuughin *et al.*, 2005). In addition, Ibarra *et al.* (2006) identified an Asp2730His alteration in one MHS proband that was due to a G8188C nucleotide transition. Thus far a single alteration has been reported to occur in exon 52. Galli *et al.* (2006) identified a Glu2764Lys alteration that is due to a G8290A nucleotide transition in one MH family from Italy.

Table 3.42: Partial gDNA sequence of exons 50, 51 and 52 of the RYR1 gene

Nucleotide number	DNA sequence: exons 50, 51 and 52
43686620	tgtgtctctc tgggccttcg tctgcctgcc attcgctggt gcccccctca tttgtgtgtc
	↓ exon 50
43686680	cccctcttgt tcccacccag CTCCTCACCA ACCACTATGA GCGCTGTTGG AAGTACTACT
43686740	GCCTACCCAC GGGCTGGGCC AACTTCGGGG TCACCTCAGA GGAGGAGCTG CACCTCACAC
43686800	GGAAACTCTT CTGGGGCATC TTTGACTCTC TGGCCCATAA Ggtctgggca gcagggagcc
43686860	ccaaaatggc ctatgtggag ggtttggggc ccaaaattgg gggtccagag tgaaatccct
43686920	caattttggg gggttcaagg aggagaaggt tctgcaagtt tggatctagg aggatctatg
43686980	ggttgaggct tcgatttgga ggttatgaaa gagggggtgg acctctagtt tgggagcttg
43687040	gagagggcaa tatggggatg atttgagcat acaattggga ctgacatttg ggtttcaagg
43687100	agagggacca taattcaggt ttggggttca gggaggaggg ctgatgattg cagtgtgtga
43687160	gtttgaggtc ctgggggtca gtaaggctta tagcgacct**c** **ct**acccctgc ttcacccggt
	↓ exon 51
43687220	tttcccagAA ATACGACCCG GAGCTGTACC GCATGGCCAT GCCTTGTCTG TGCGCCATTG
43687280	CCGGGGCTCT GCCCCCCGAC TATGTGGATG CCTCATACTC ATCTAAGGCA GAGAAAAAGG
43687340	CCACAGTGGA TGCTGAAGGC AACTTTGATC CCCGGCCTGT GGAGACCCTC AAgtgaggcc
43687400	tgggggctgg gagacagaga ggaagatttc aggggtggag ggaaccccag ctccaacatc
	↓ exon 52
43687460	tgctgaccct gtgcccccaa cagTGTGATC ATCCCGGAGA AGCTGGACTC CTTCATTAAC
43687520	AAGTTTGCGG AGTACACACA CGAGAAGTGG GCCTTCGACA AGgttggcct cagggtcctc
43687580	ctatccaaga aaccctcaag accccagctt tccccccgac ctggttcttc cctgaggccc

The partial gDNA sequence amplified for exons 50, 51 and 52 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Six single nucleotide polymorphisms are observed in this region, the G71171A nucleotide transition is indicated in red, the G71413A nucleotide transition is indicated in light blue, the A71494G nucleotide transition is indicated in green, the CCT71619del is indicated in blue, the T76199C nucleotide transition is indicated in orange and the T71771C nucleotide transition is indicated in purple. The codon that correlates to Arg2676 is indicated in a dashed box (---), the codon that correlates to Asp2730 is indicated in a solid red box (–), the codon that correlates to Gly2733 is indicated in a solid box (–), the codon that correlates to Glu2764 is indicated in a red dashed box (---) and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex50F) is the single underlined sequence, while the reverse primer (RYRex50R) is the double underlined sequence; the beginnings of exons 50, 51 and 52 are indicated with an arrow.

3.7.37 Detection of alterations in exons 53 and 54 of the RYR1 gene

Exons 53 and 54 were analysed in order to identify both novel and reported alterations and polymorphisms that may occur. The partial gDNA sequence of amplified exon 54 from the RYR1 gene is depicted in Table 3.43.

A region of 814 bp was amplified and thus far one alteration has been reported in exon 54. The Arg2840Trp alteration is due to a C8518T nucleotide transition and was reported in one MHS family from Japan (Ibarra *et al.*, 2006). A single alteration has been reported in exon 53. Monnier *et al.* (2005) observed a Thr2787Ser alteration, which is due to a C8360G nucleotide transition, in an MHS individual from France.

Table 3.43: Partial gDNA sequence of exons 53 and 54 of the RYR1 gene

Nucleotide number	DNA sequence: exons 53 and 54
43687700	caggattctc tgtcctcggc tcctccaggg tcgccccgtg tgtccccaac tgctgcctcc
	↓exon 53
43687760	ccctcaccct gcctcccctc catctctagA TCCAGAACAA CTGGTCCTAT GGAGAGAACA
43687820	TAGACGAGGA GCTGAAGACC CACCCCATGC TGAGGCCCTA CAAGACCTTT TCAGAGAAGg
43687880	tgaccaggcc ttggggccca gcattgaggg tcaaaatgaa acccccaaat ttgaggattc
43687940	ggggaggagt gaggcaattt cacatgtttg catctaggtg gatctgtggg ttaggtctcc
43688000	ccattcatgg actttgcctt ctctcaaact tggtagagtg ggtagagact ccgagagagt
43688060	gggtttgatt ccttggctgt agtaagactt ctcggagact caagtgtcta atgggataag
43688120	gagattgggt ttgggaggct ctgttacaga gcaggtaaga gacttgagtt ggaatccaga
43688180	ctggaccatt gcctagccac atggtcaggg ttttctcctt tggggtcctt cctccacccc
	↓exon 54
43688240	tctctcatcc cattccacca actccccacc ctcctgtcca ccccagGACA AAGAGATTTA
43688300	CCGCTGGCCC ATCAAGGAGT CCCTGAAGGC CATGATTGCC TGGGAATGGA CGATAGAGAA
43688360	GGCCAGGGAG GGTGAGGAGG AGAAGACGGA AAAGAAAAAA ACGCGGAAGA TATCACAAAG
43688420	TGCCCAGgtg aaggcggggc ctgggtggag ggcaggggca cgatgggggg agggtctaga
43688480	acaaggggca tggccagaca gggaagggat ggagaggaga ggggcccagg gaggtaggtg

The partial gDNA sequence amplified for exon 53 and 54 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Five single nucleotide polymorphisms are observed in this region, the G72236A nucleotide transition is indicated in purple, the A72327G nucleotide transition is indicated in green and the C72360T nucleotide transition is indicated in red, the C72881T nucleotide transition is indicated in blue and the T72884G nucleotide transition is indicated in orange. The codon that correlates to Thr2787 is indicated in a solid box (–), the codon that correlates to Arg2840 is indicated in a dashed box (---) and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex53F) is the single underlined sequence, while the reverse primer (RYRex53R) is the double underlined sequence; the beginnings of exons 53 and 54 are indicated with an arrow.

3.7.38 Detection of alterations in exons 55, 56 and 57 of the RYR1 gene

Sequencing was conducted in order to analyse exons 55, 56 and 57 simultaneously for novel and reported alterations and polymorphisms that may occur in this region. The partial gDNA sequence of amplified exons 55, 56 and 57 from the RYR1 gene is represented in Table 3.44.

A PCR product of 830 bp was amplified and thus far alterations associated with MHS have only been reported in exon 55. The Leu2867Gly alteration has been identified in one individual with MH and is due to a T8600A nucleotide substitution (Galli *et al.*, 2006). However, Jungbluth *et al.* (2005) identified an Arg2939Lys alteration in one family diagnosed with centronuclear myopathy (CNM). The alteration is due to a G8816A nucleotide transition. In addition, Zhou *et al.* (2005) observed the Arg2939Ser alteration that is due to an A8817C substitution in one family diagnosed with CCD.

Table 3.44: Partial gDNA sequence of exons 55, 56 and 57 of the RYR1 gene

Nucleotide number	DNA sequence: exons 55, 56 and 57
43688720	gggctggcct gggcttcctg ctagcccatc agcccacctc ccatcttccc cttgtcctct
	↓ exon 55
43688780	cagACCTATG ATCCTCGAGA AGGCTACAAC CCTCAGCCCC CCGACCTTAG **T**GCTGTTACC
43688840	CTGTCCCGGG AGCTGCAGgt gagagccctg atccttttgg ggggacatag ggtgtctttg
	↓ exon 56
43688900	gggggctgg catcctctga atctagccct tgactctgca tccactccca gGCCATGGCA
43688960	GAACAACTGG CAGAAAATTA CCACAACACG TGGGGACGGA AGAAGAAGCA GGAGCTGGAA
43689020	GCCAAAGgtg agggcgccca tgccgccccc acgctacccc cgtggattca ccgtgtggtt
43689080	ttgctgattg ccttcatgcc cctgaaactc ggtttctcca tctgtagatg ggaataataa
43689140	cagcgtttac caccatgggg taatgagatg agcaccagca agcaatgttt ccgttattcg
43689200	tatcttcttt atcaccataa ttacgcatgc cgggcactgc aggaaccact tcagtgagag
	↓ exon 57
43689260	tggcccgggt cttccccaga gccctgattt ctggtctttg cctccccagG CGGTGGGACC
43689320	CACCCCCTGC TGGTCCCCTA CGACACGCTC ACGGCCAAGG AGAAGGCACG AGATCGAGAG
43689380	AAGGCCCAGG AGCTACTGAA ATTCCTGCAG ATGAATGGCT ACGCGGTTAC AAGGCACGCG
43689440	Ggttggggct cccgcggaag agcagcaggc agaacacacc cggcaaaggc tggaaggggc
43689500	ggggccagag aggggtggag ccgagaggaa cggggcctga ggagcaaaga tggaaccaga
43689560	ggggaggagc taagggagtg gggcctggac acagaggcgg ggccagatgg ggaggagttc

The partial gDNA sequence amplified for exons 55, 56 and 57 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Nine single nucleotide polymorphisms are observed in this region, the T73251C is indicated in **blue**, the C73337T nucleotide transition is indicated in **orange**, the T73475G nucleotide transition is indicated in **green**, the T73584C nucleotide transition is indicated in **pink**, the G73720C nucleotide transition is indicated in purple, the T73870A nucleotide transition is indicated in **light green**, the A73896C nucleotide transition is indicated in **green**, the G73940T nucleotide transition is indicated in **light blue** and the C74024A nucleotide transition is indicated in **dark purple**. The codon that correlates to Leu2867 is indicated in a solid box (–), the codon that correlates to Arg2939 is indicated in a dashed box (---) and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex55F) is the single underlined sequence, while the reverse primer (RYRex55R) is the double underlined sequence; the beginnings of exons 55, 56 and 57 are indicated with an arrow.

3.7.39 Detection of alterations in exon 58 of the RYR1 gene

A PCR product of 209 bp was amplified in order to detect novel alterations that may occur in exon 58 as well as novel polymorphisms in this region. To date, alterations associated with the MHS have not been reported in this exon. The partial gDNA sequence of amplified exon 58 from the RYR1 gene is depicted in Table 3.45.

Table 3.45: Partial gDNA sequence of exon 58 of the RYR1 gene

Nucleotide number	DNA sequence: exon 58
43690100	cggggccagc aggagcagag gcggacctga gaagggtggg aaactgtagg gccggcgtct
	↓ exon 58
43690160	gggctgatcc ttctctccac atctccatgc agagGCCTTA AGGACATGGA ACTGGACTCG
43690220	TCTTCCATTG AAAAGCGGTT TGCCTTTGGC TTCCTGCAGC AGCTGCTGCG CTGGATGGAC
43690280	ATTTCTCAGG AGTTCATTGC CCACCTGGgt acggagaaat acccccgct tatgcccgcc

The partial gDNA sequence amplified for exon 58 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The forward primer (RYRex58F) is the single underlined sequence, while the reverse primer (RYRex58R) is the double underlined sequence; the beginning of exon 58 is indicated with an arrow.

3.7.40 Detection of alterations in exons 59 and 60 of the RYR1 gene

The partial gDNA sequence of exons 59 and 60 from the RYR1 gene is depicted in Table 3.46. The amplified region encompassing both exons does not harbour MHS alterations. In order to detect novel alterations and polymorphisms in exons 59 and 60, a region of 429 bp was amplified and subsequently analysed. To date, this region has not been reported to harbour any polymorphisms.

Table 3.46: Partial gDNA sequence of exons 59 and 60 of the RYR1 gene

Nucleotide number	DNA sequence: exons 59 and 60
	exon 59 ↓
43692920	aacaccctgg gttccccagc cttgaaccca ctgtgaaccc tatttgccct ccctacagAG
43692980	GCTGTGGTCA GCAGTGGGCG AGTGGAAAAG TCCCCACATG AACAGGAGAT TAAATTCTTT
43693040	GCCAAGgtga gaggtgggct tagaagctgg agggcgctgg ggactcatag gctctcccca
	↓ exon 60
43693100	cccctcattg gaccctttat ctcccccaac ccgtctccag ATCCTGCTCC CTTTGATCAA
43693160	CCAGTACTTC ACCAACCACT GCCTCTATTT CTTGTCCACT CCGGCTAAAG TGCTGGGCAG
43693220	CGGTGGCCAC GCCTCTAACA AGGAGAAGGA AATGATCACC AGgtgggccg cctgtgaccc
43693280	tggacccagc cccctgacct cacaggattg taatcctttg cccatggagg ctctgtcctg
43693340	ggtgctgggt gttgggtact gaccgtctcc tgcaaaggcg attgaagggt gaccagccat

The partial gDNA sequence amplified for exons 59 and 60 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The forward primer (RYRex59F) is the single underlined sequence, while the reverse primer (RYRex59R) is the double underlined sequence; the beginnings of exons 59 and 60 are indicated with an arrow.

3.7.41 Detection of alterations in exon 61 of the RYR1 gene

A 226 bp region was analysed in order to screen for novel alterations and novel or reported polymorphisms in exon 61. The partial gDNA sequence of amplified exon 61 from the RYR1 gene is depicted in Table 3.47. To date, this region has not been reported to harbour any alterations associated with MHS.

Table 3.47: Partial gDNA sequence of exon 61 of the RYR1 gene

Nucleotide number	DNA sequence: exon 61
43693940	ttctctgtcc ctgtctcctc taattgggtc acgctgtcct ©gtctccttg gcctcctcac
43694000	tcgctgtttc tcctgccttc tgtccctttc tctttcttca ↓ exon 61 gCCTCTTCTG CAAACTTGCT
43694060	GCTCTCGTCC GCCACCGAGT CTCTCTCTTT Ggtaagtggc tccacacctt cggtcttcct
43694120	ccctaatctt tctcttcccc accctgaaga aatagctccc aggttctgcc ttaatttgaa

The partial gDNA sequence amplified for exon 61 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. One single nucleotide polymorphism is observed in this region, the C78401T nucleotide transition is indicated in a circle. The forward primer (RYRex61F) is the single underlined sequence, while the reverse primer (RYRex61R) is the double underlined sequence; the beginning of exon 61 is indicated with an arrow.

3.7.42 Detection of alterations in exons 62 and 63 of the RYR1 gene

A region of 566 bp was analysed in order to screen for novel alterations and novel or reported polymorphisms that may occur in either exon 62 or 63. The partial gDNA sequence of amplified exons 62 and 63 is depicted in Table 3.48. An Arg3119His alteration that has been reported in exon 63 in one MH family is due to a G9356A substitution (Ibarra *et al.*, 2006).

Table 3.48: Partial gDNA sequence of exons 62 and 63 of the RYR1 gene

Nucleotide number	DNA sequence: exons 62 and 63
43694480	tggatgtaga gggaggcact gtcctctgtc ctcttagcca tggcatcccc ccggcccatc
43694540	ttcctctccc ↓ exon 62 agGGACAGAC GCCCCAGCTG TGGTCAACTG TCTTCACATC CTGGCCCGCT
43694600	CCCTGGATGC CAGgtagggc cataggcagt ggcgcccact cccaccatca tcgggccccc
43694660	accccaaccc ctggtctcct agactctccg attccagagc tgatgttccc ccgctgccct
43694720	↓ exon 63 tctagGACAG TGATGAAGTC AGGCCCTGAG ATCGTGAAGG CTGGCCTCCG CTCCTTCTTC
43694780	GAGAGTGCCT CGGAGGACAT CGAGAAGATG GTGGAGAACC TGCGGCTGGG CAAGGTGTCG
43694840	CAGGCGCGCA CCCAGGTGAA AGGCGTGGGC CAGAACCTCA CCTACACCAC TGTGGCACTG
43694900	CTGCCGGTCC TCACCACCCT CTTCCAGCAC ATCGCCCAGC ACCAGTTCGG AGATGACGTC
43694960	ATCCgtaagg gcgcctgacc caagggcagg ttgcggggag tcagtgtggc caacaccacc
43695020	catccgggtg cctgtgagag tccctgggtg tttgaatgtg tggatttctt gctgtaagca

The partial gDNA sequence amplified for exons 62 and 63 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Three single nucleotide polymorphisms are observed in this region, the C78952T nucleotide transition is indicated in **blue**, the A78986G nucleotide transition is indicated in **orange** and the C79265T nucleotide transition is indicated in **green**. The codon that correlates to Arg3119 is indicated in a dashed box (---) and the nucleotide position of the mutation is indicated in **bold**. The forward primer (RYRex62F) is the single underlined sequence, while the reverse primer (RYRex62R) is the double underlined sequence; the beginnings of exons 62 and 63 are indicated with an arrow.

3.7.43 Detection of alterations in exon 64 of the RYR1 gene

The partial gDNA sequence of amplified exon 64 from the RYR1 gene is depicted in Table 3.49. A region of 355 bp region was analysed in order to screen for novel alterations and novel or reported polymorphisms that may occur in exon 64. Thus far, this region has not been reported to harbour any alterations associated with the MHS.

Table 3.49: Partial gDNA sequence of exon 64 of the RYR1 gene

Nucleotide number	DNA sequence: exon 64
43697300	ataactatcc ccatgttaca ggtagggaag ctgaggctga gagagagttg gtaacttgct
43697360	caagtcacaa gactcgtaca tggaaggctg gctgtacatc tgcttgctct tccccactgc
43697420	atgggcctat ttgagacaag ggaggtgggg tggggagggc ttgtcttgtg agcgcatgcc
	↓ exon 64
43697480	gcagcctcgc cccctgtctc cctcagTGGA CGACGTCCAG GTCTCTTGCT ACCGAACGCT
43697540	GTGCAGTATC TACTCCCTGG GAACCACCAA GAACACTTAT GTGGAAAAgt aaggagaggg
43697600	agccatcgtt tggggctggg tggggctgga ggggaaggga gggagcaggg gaagaagatg
43697660	gggtgggtga aaagcagggt agatgttaag aattttcccg cacacggcgg cagccgcggt

The partial gDNA sequence amplified for exon 64 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. A single nucleotide polymorphism is observed in this region, the C81823T nucleotide transition is indicated in a circle. The forward primer (RYRex64F) is the single underlined sequence, while the reverse primer (RYRex64R) is the double underlined sequence; the beginning of exon 64 is indicated with an arrow.

3.7.44 Detection of alterations in exon 65 of the RYR1 gene

The partial gDNA sequence of amplified exon 65 is depicted in Table 3.50. Sequencing was conducted in order to screen for novel alterations that may occur in the 349 bp region. Thus far, this region has not been reported to harbour any alterations associated with MH.

Table 3.50: Partial gDNA sequence of exon 65 of the RYR1 gene

Nucleotide number	DNA sequence: exon 65
43698440	acacatggat gaatggcagc tctgtcccaa agggttctgg gaggagccgt ttctatggag
43698480	atggggctgg gacccaggac cccaaagagg gggacacgtg gcagctaaac acagccccgt
	↓ exon 65
43698540	cttccagGCT TCGGCCAGCC CTCGGGGAGT GCCTGGCCCG TCTGGCAGCA GCCATGCCGG
43698620	TGGCGTTCCT GGAGCCGCAG CTGAACGAGT ACAACGCCTG CTCCGTGTAC ACCACCAAGT
43698680	CTCCGCGGGA GCGGGCCAgt aagctgtgtg gggcgggagc agtgctggga gtccaaatct
43698740	ccccagcaca gggccttggg gagaccctaa tttgggggta gtgtggctgg gctgggctgt

The partial gDNA sequence amplified for exon 65 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. A single nucleotide polymorphism is observed in this region, the C82975T nucleotide transition is indicated in a circle. The forward primer (RYRex65) is the single underlined sequence, while the reverse primer (RYRex65R) is the double underlined sequence; the beginning of exon 65 is indicated with an arrow.

3.7.45 Detection of alterations in exon 66 of the RYR1 gene

Analysis of a 500 bp region was conducted in order to screen for novel alterations and novel or reported polymorphisms that may occur in exon 66. Thus far, this region has not been reported to harbour any alterations associated with MHS. The partial gDNA sequence of amplified exon 66 from the RYR1 gene is represented in Table 3.51.

Table 3.51: Partial gDNA sequence of exon 66 of the RYR1 gene

Nucleotide number	DNA sequence: exon 66
43699700	agatgggaag aatagggttt gggagactgt ttaaggggg tggcaattca atggtgtctg
43699760	atgtattgcg ggggaggcca gggcactgag gtctgggggt gatggcttga cattccctgc
	↓ exon 66
43699820	ccccgtccct gtaccccagT CCTGGGGCTC CCCAACAGTG TGGAGGAGAT GTGTCCCGAC
43699880	ATCCCGGTGC TGGAGCGGCT CATGGCAGAC ATTGGGGGGC TGGCCGAGTC AGGTGCCCGC
43699940	TACACAGAGA TGCCGCATGT CATCGAGATC ACGCTGCCCA TGCTATGCAG CTACCTGCCC
43700000	CGATGGTGGG AGCGCGGGCC CGAGGCACCC CCTTCCGCCC TGCCCGCCGG CGCCCCCCCA
43700060	CCCTGCACAG CTGTCACCTC TGACCACCTC AACTCCCTGC TGGGGAATAT CCTGAGAATC
43700120	ATCGTCAACA ACCTGGGCAT TGACGAGGCC TCCTGGATGA AGCGGCTGGC TGgtgggtcg
43700180	gggggcactg ggcctctgag gggtgggtca gcagcctggg ctcccttggc agatggtctg

The partial gDNA sequence amplified for exon 66 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. One single nucleotide polymorphism is observed in this region, the G84264A nucleotide transition is indicated in a circle. The forward primer (RYRex66F) is the single underlined sequence, while the reverse primer (RYRex66R) is the double underlined sequence; the beginning of exon 66 is indicated with an arrow.

3.7.46 Detection of alterations in exon 67 of the RYR1 gene

Analysis of a 382 bp region was conducted in order to screen for novel and reported alterations and polymorphisms that may occur in exon 67. The partial gDNA sequence of amplified exon 67 is depicted in Table 3.52.

A single alteration, Arg3348His that is due to a G10043A transition, has been reported in an MH family from North America. This nucleotide site is evolutionarily conserved and the alteration was not detected in 100 unrelated North American control individuals. Arg3348His does not occur in a mutational hotspot and is located between the central and C-terminal region of the RYR1 (Sambuughin *et al.*, 2005). In addition, the Lys3367Arg alteration has been identified in a single CCD proband. The alteration is due to a A10100G nucleotide transition (Wu *et al.*, 2006).

Table 3.52: Partial gDNA sequence of exon 67 of the RYR1 gene

Nucleotide number	DNA sequence: exon 67
43701560	caaggttagg gtcaggctgg ggtcaaatgg cagctgctag gttggagatg ctgtttggga
43701620	gtcgggctgg gaacggagtt tggggcctgt gtcagaggcc ggaggtggca tcagagccca
	↓ exon 67
43701680	tcgcacccct gcagTGTTCG CACAGCCCAT TGTGAGCCGT GCACGGCCGG AGCTCCTGCA
43701740	GTCCCACTTC ATCCCAACTA TCGGGCGGCT GCGCAAGAGG GCAGGGAAGG TGGTGTCCGA
43701800	GGAGGAGCAG CTGCGCCTGG AGGCCAAGGC GGAGGCCCAG GAGGGCGAGC TGCTGGTGCG
43701860	GGACGAGTTC TCTGTGCTCT GCCGGGACCT CTACGCCCTG TATCCGCTGC TCATCCGCTA
43701920	CGTGGACAAC AACAGgtcag cggggccccg ctgtccccat gccctccgcc ccgacctccc
43701980	acgtcctcca gccccatctg atctccgcct cctgactggc tagaaacttc ttccaatgct

The partial gDNA sequence amplified for exon 67 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. One single nucleotide polymorphism is observed in this region, the C86284T nucleotide transition is indicated in a circle. The codon that correlates to Arg3348 is indicated in a solid box (–), the codon that correlates to Lys3367 is indicated in a dashed box (---) and the nucleotide transition for the mutation is indicated in **bold**. The forward primer (RYRex67F) is the single underlined sequence, while the reverse primer (RYRex67R) is the double underlined sequence; the beginning of exon 67 is indicated with an arrow.

3.7.47 Detection of alterations in exons 68 and 69 of the RYR1 gene

Analysis of a 437 bp region was conducted in order to screen simultaneously for novel alterations and novel or reported polymorphisms that may occur in both exons 68 and 69. The partial gDNA sequence of amplified exons 68 and 69 of the RYR1 is depicted in Table 3.53.

Table 3.53: Partial gDNA sequence of exons 68 and 69 of the RYR1 gene

Nucleotide number	DNA sequence: exons 68 and 69
43705400	tgactggatg tctcctggtc cccatctcct cctccaaggt ctctctctgg catccccctt
	↓ exon 68
43705460	cgcctgggat ccccaccccc tccctcacct cccctccgct gaccccagGG CGCAGTGGCT
43705520	GACGGAGCCG AATCCCAGCG CGGAGGAGCT GTTCAGGATG GTGGGCGAGA TCTTCATCTA
43705580	CTGGTCCAAG TCCCACgtga gtgcccaccc caaccgccct ccccacaacc agaggagccg
	exon 69 ↓
43705640	cagcccacag gcgccggcct tcacctgtcc ggtctgcaac actgcttccc ccaccagAAC
43705700	TTCAAGCGCG AGGAGCAGAA CTTTGTGGTC CAGAATGAGA TCAACAACAT GTCCTTCCTG
43705760	ACTGCTGACA ACAAAAGCAA AATGGCTAAG gtcggggctt ggttctggga ggagcacttg
43705820	gcagagaggg cggg agcacc ctctaggact tcctacctgg cctgtcctca cccagccagc

The partial gDNA sequence amplified for exons 68 and 69 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. One single nucleotide polymorphism is observed in this region, the T90076G nucleotide transition is indicated in a circle. The codon that correlates to Ser3446 is indicated in a dashed box (---) and the nucleotide transition for the mutation is indicated in bold. The forward primer (RYRex68F) is the single underlined sequence, while the reverse primer (RYRex68R) is the double underlined sequence; the beginnings of exons 68 and 69 are indicated with an arrow.

Currently, no alterations associated with MHS have been reported for this region of the RYR1 gene. However, Zhou *et al.* (2005) identified a Ser3446Phe alteration in one family diagnosed with CCD. The alteration is due to a C10337T nucleotide transition.

3.7.48 Detection of alterations in exon 70 of the RYR1 gene

Analysis of a 486 bp region was conducted in order to screen for novel alterations and polymorphisms that may both occur in exon 70. The partial gDNA sequence of amplified exon 70 of the RYR1 is depicted in Table 3.54. Currently, no alterations associated with MHS have been reported for this region of the RYR1 gene.

Table 3.54: Partial gDNA sequence of exon 70 of the RYR1 gene

Nucleotide number	DNA sequence: exon 70
43706000	tctccatctc cctcttctct catctctgtc tccttcctcc tcctgtatct tctccctcct
43706060	cccatttccc tcctctccat tttcctcttc tccaagcctc tctctcctcc catttccctc
43706120	ctcctcctcc tccccatttt ccccctcttc catttctttc ttcctctccc cattacccca
43706180	tttcctgctt tcctccctat ccttctcagc actgcccttc tcaggtctca gagaacgacc
43706240	ccccaccccg agccaaggcc tggaaatgcc cagctagaga atacatggcg ggtggggcag
43706300	aggaggtggg gtgctggcaa cttggagttg ggcctgggct tctctgcggg gctggggtaa
	↓ exon 70
43706360	cccttcttgt ctctgtctgc ggtccggtga agcagGCGGG AGATATACAG gtcagcccca
43706420	catctgggac cttccgcatg tctcttggct aatgccctct tcccccagcc tctgcacgcc
43706480	cccgcctcga gaaaacccct gcttctgttc cccaccccog tcctcccctc ccagccccca

The partial gDNA sequence amplified for exon 70 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The forward primer (RYRex70F) is the single underlined sequence, while the reverse primer (RYRex70R) is the double underlined sequence; the beginning of exon 70 is indicated with an arrow.

3.7.49 Detection of alterations in exon 71 of the RYR1 gene

Although no alterations associated with the MH phenotype have thus far been identified for exon 71, a region of 478 bp was amplified in order to identify novel alterations and polymorphisms that may occur. The partial gDNA sequence of amplified exon 71 from the RYR1 gene is depicted in Table 3.55. However, the Pro3527Ser alteration has been observed in one patient diagnosed with CCD. The alteration is due to a C10579T nucleotide transition (Zhou *et al.*, 2005).

Table 3.55: Partial gDNA sequence of exon 71 of the RYR1 gene

Nucleotide number	DNA sequence: exon 71
43707620	ggtctccggt catggctgtg ggcctgaagt gtagagtcag caagtctggg gtggaaattg
43707680	aggtgtcgtc ggcagttggg gagggagtgc ctggtgtcca gactggggcc tggggtgtgg
43707740	atgatggccg cgggttgggg ctgaggcatg ggattggggc ttgggctggt gctgagccct
	↓ exon 71
43707800	gtgtccccac agTCCGGTGG CTCGGACCAG GAACGCACCA AGAAGAAGCG CCGGGGGGAC
43707860	CGGTACTCTG TGCAGACGTC ACTGATCGTG GCCACACTGA AGAAGATGCT GCCCATCGGC
43707920	CTGAATATGT GTGCGCCCAC CGACCAAGAC CTCATCACGC TGGCCAAGAC CCGTTACGCC
43707980	CTGgtgcctg cccagccccg tcctcggaac cttccaggat gccgcccagc acccactgaa
43708040	cccctgggac cttagggaac aaccacaatg ccactgagcc ccccaggtcc ctgggagcct
43708100	tcccttcaga ccccactgag ttcttttccg ggatgctgag gactcactgt gcccctggga

The partial gDNA sequence amplified for exon 71 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The codon that correlates to Pro3527 is indicated in a dashed box (---) and the nucleotide transition for the mutation is indicated in **bold**. The forward primer (RYRex71F) is the single underlined sequence, while the reverse primer (RYRex71R) is the double underlined sequence; the beginning of exon 71 is indicated with an arrow.

3.7.50 Detection of alterations in exon 72 of the RYR1 gene

The partial gDNA sequence of amplified exon 72 from the RYR1 gene is represented in Table 3.56. Thus far, alterations associated with the MH phenotype have not been reported for exon 72. Therefore, a region of 215 bp was amplified in order to identify novel alterations and polymorphisms that may occur in this region.

Table 3.56: Partial gDNA sequence of exon 72 of the RYR1 gene

Nucleotide number	DNA sequence: exon 72
43709360	accccagaaa aacctcttca gttcctgggg tgctgggcct ggaaggaaag ggttgtgggt
	↓ exon 72
43709420	caggaaggag gatgggacct ccagagtgac ccagcctggc tctgtctccc cagAAAGACA
43709480	CAGATGAGGA GGTCCGGGAA TTTCTGCACA ACAACCTTCA CCTTCAGGGA AAGgtatgcc
43709540	tccttcctct gcaagcaaaa gaagcaagtc agaaagtaac cacaatatta gtgaaggttt
43709600	gagcatttac caaagaccag gcattgaaga aagacctcaa aggtcaggag tttgagacca

The partial gDNA sequence amplified for exon 72 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The forward primer (RYRex72) is the single underlined sequence, while the reverse primer (RYRex72R) is the double underlined sequence; the beginning of exon 72 is indicated with an arrow.

3.7.51 Detection of alterations in exon 73 of the RYR1 gene

The partial gDNA sequence of amplified exon 73 from the RYR1 gene is represented in Table 3.57. Thus far alterations associated with the MH phenotype have not been reported for exon 73. Wu *et al.* (2006) observed a Leu3606Pro alteration in one CCD

family due to a T10817C nucleotide transition. A region of 259 bp was amplified in order to identify novel alterations and polymorphisms that may occur in this region.

Table 3.57: Partial gDNA sequence of exon 73 of the RYR1 gene

Nucleotide number	DNA sequence: exon 73
43710020	tcaacatgca ctcacccatt gagtcctccc aaaaacggaa aggggacatc cggcggacac
	↓ exon 73
43710080	tgtgggaagg gtccctcacg ccggccactc cttcttcctc ccttcagGTC GAAGGCTCCC
43710140	CGTCTCTGCG CTGGCAGATG GCTCTGTACC GGGGCGTCCC GGGTCGCGAG GAGGACGCCG
43710200	ATGACCCCGA GAAAATCGTG CGCAGAGTCC AGGAAGTGTC AGCCGTGCTC TACTACCTGG
43710260	ACCAGgtggg tggggccgga ggggtctttc tactgggcgg caggtgggag cctggcgggg

The partial gDNA sequence amplified for exon 73 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The codon that correlates to Leu3606 is indicated in a dashed box (---) and the nucleotide transition for the mutation is indicated in bold. The forward primer (RYRex73) is the single underlined sequence, while the reverse primer (RYRex73R) is the double underlined sequence; the beginning of exon 73 is indicated with an arrow.

3.7.52 Detection of alterations in exons 74, 75 and 76 of the RYR1 gene

In order to identify novel alterations and novel and reported polymorphisms, a 918 bp PCR product was amplified and subsequently sequenced. Thus far alterations associated with the MH phenotype have not been reported for exons 74, 75 and 76. The partial gDNA sequence of amplified exons 74, 75 and 76 from the RYR1 gene is depicted in Table 3.58.

Table 3.58: Partial gDNA sequence of exons 74, 75 and 76 of the RYR1 gene

Nucleotide number	DNA sequence: exons 74, 75 and 76
43710620	aggcggagtc aggaccctga ctctgttggt ggagacaagg ttttccttct gccgtgtgag
43710680	tcttaacctg aatatggact tcgacacagc tgggcagttt catccagggc tgggagtgag
	↓ exon 74
43710740	aggggcaggg tctggggatg tgactgtcct gctatcccct ccccagACCG AGCACCCTTA
43710800	CAAGTCTAAG AAGGCCGTGT GGCACAAGCT TTTGTCCAAA CAGCGCCGGC GGGCAGTCGT
43710860	GGCCTGTTTC CGTATGACGC CCCTGTACAA CCTGCCCACg taaggccccc agggacaagg
43710920	gaagcgtgaa gggctgcgga gaaagggtgg ctggagagtc tggagaatgg agggccaacg
43710980	tgatggggcc ttgagggtgg ttgggggctg caggcgcatg ggaggtcggg aagcacggag
	↓ exon 75
43711040	gagggcgcgt cccagtgacg tcacacctct cccctgcagG CACCGGGCAT GTAACATGTT
43711100	CCTGGAGAGC TACAAGGCTG CATGGATCCT GACTGAAGAC CACAGTTTTG AGGACCGCAT
43711160	GATAGATGAC CTTTCAgtga gctgggaccc gcctggggga gtgggggcg agctggatag
43711220	ggctggggcg gaggccaccc ttggcacacc tccaggggtc ggccctccac atcaaggggt
43711280	atagaaatgc cagctcctgg cttgagtaga accaaagtag ggcgcaggat gtgggaaaag
43711340	agaaaaaaaa atccagacca acagggacat gggggcagtg acaggagggg actctagaaa
	↓ exon 76
43711400	ccctctcccc aagtctcccct ctcccacca gAAAGCTGGG GAGCAGGAGG AGGAGGAGGA
43711460	AGAGGTGGAA GAGAAGAAGCC AGACCCCCT GCACCAGTTG GTCCTGCACT TCAGCCGCAC
43711520	TGCCCTGACG GAAAAGAGgtg aagactctt gccagggccc cagaaatgcc cccaaggtcc
43711580	tggggccacc cccagcccag cagcttccct gtgcctcagg agaggccctc caggtcctgg

The partial gDNA sequence amplified for exons 74, 75 and 76 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Two single nucleotide polymorphisms are observed in this region, the G95358A nucleotide transition is indicated in **blue** and the A95414G nucleotide transition is indicated in **orange**. The forward primer (RYRex74F) is the single underlined sequence, while the reverse primer (RYRex74R) is the double underlined sequence; the beginnings of exons 74, 75 and 76 are indicated with an arrow.

3.7.53 Detection of alterations in exons 77 and 78 of the RYR1 gene

A region of 351 bp encompassing exons 77 and 78 was amplified in order to identify novel alterations and polymorphisms that may occur in this region. The partial gDNA sequence of amplified exons 77 and 78 from the RYR1 gene is represented in Table 3.59. Alterations associated with MHS have not been reported for exons 77 and 78.

Table 3.59: Partial gDNA sequence of exons 77 and 78 of the RYR1 gene

Nucleotide number	DNA sequence: exons 77 and 78
	↓ exon 77
43714940	tcctgaccac tcccctgctt acttccccag CAAACTGGAT GAGGATTACC TGTACATGGC
43715000	CTATGCTGAT ATCATGGCAA Aggtgaggcc ctaccccct cttctggggc agatttccct
43715060	ctccccacct gcagtgcttg tccacaaaaa gggctggggc gggatggagg ggtctgcttt
	↓ exon 78
43715120	gttcatccct taactgatgc cccctcccca gAGCTGCCAC CTGGAGGAGG GAGGGGAGAA
43715180	CGGTGAAGCT GAAGAGGAGG TTGAGGTCTC CTTTGAGgta ggtgggctca ggaggtcctg
43715240	gagggaaggg atgggggacc ctgactgcag tcatctccca ttcattcagc atgtgttcac

The partial gDNA sequence amplified for exons 77 and 78 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The forward primer (RYRex77F) is the single underlined sequence, while the reverse primer (RYRex77R) is the double underlined sequence; the beginnings of exons 77 and 78 are indicated with an arrow.

3.7.54 Detection of alterations in exons 79, 80 and 81 of the RYR1 gene

Sequencing was conducted in order to screen a region of 805 bp encompassing exons 79, 80 and 81 in order to identify novel alterations and novel and reported polymorphisms that may occur. The partial gDNA sequence of amplified exons 79, 80 and 81 from the RYR1 gene is depicted in Table 3.60. Currently, alterations associated with MHS have not been reported for exons 79, 80 and 81.

Table 3.60: Partial gDNA sequence of exons 79, 80 and 81 of the RYR1 gene

Nucleotide number	DNA sequence: exons 79, 80 and 81
43717100	ggatgtggct ggaacaggga gggcagaagt gagaatgtga gggggaaagg ctgggctgga
	↓exon 79
43717160	aagcctggac ttgccttcat gtgtctgcct cccttcccag GAGAAACAGA TGGAGAAGCA
43717220	GAGGCTCTTG TACCAGCAAG CACGGCTGCA CACCCGGGGG GCGGCCGAGA TGGTGCTGCA
43717280	GATGATCAGT GCCTGCAAAG gtgcccctca catgtgcact ggactcttcc gagtgcactc
43717340	atcctaacct cactcctcct ggctcgccca ttccctgtgg acccatttgc cgcccctcaa
43717400	tgcctgtggt ttgcacgcac accccagccc ttgcagcttc cccttgtata cctgccttgc
43717460	aatttctcac tcatccttca atcacgatca cccctgcatg cacaccttgg cacactctta
43717520	aatccccttc tctcacatcc cttgggatgg ctgttttctg gtgggtggaa cacactgcct
	↓exon 80
43717580	tccaactggg tggaccatct tttttctccc actccctcca gGAGAGACAG GTGCCATGGT
43717640	GTCCTCCACC CTGAAGCTGG GCATCTCCAT CCTCAATGGA GGCAATGCTG AGGTCCAGCA
43717700	Ggtaacagag gcaaagggac ttcagaagaa ggcaaggagg gatgagaggtt cctgtgtga
	↓exon 81
43717760	ctcccagttt ctcctcccct gcctcgccct ctgcagAAAA TGCTGGATTAT CTTAAGGAC
43717820	AAGAAGGAAG TTGGCTTCTT CCAGAGTATC CAGGCACTGA TGCAAACATGC AGgtaggtt
43717880	cgagtggacc tcttcttgtt aagctgtgtt tggtgccact gccaccccact cctggtacc

The partial gDNA sequence amplified for exons 79, 80 and 81 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Two single nucleotide polymorphisms are observed in this region, the T101602G nucleotide transition is indicated in blue and the C101627G nucleotide transition is indicated in orange. The forward primer (RYRex79F) is the single underlined sequence, while the reverse primer (RYRex79R) is the double underlined sequence; the beginnings of exons 79, 80 and 81 are indicated with an arrow.

3.7.55 Detection of alterations in exon 82 of the RYR1 gene

A region of 365 bp of exon 82 was analysed in order to identify novel alterations and novel and reported polymorphisms that may occur. The partial gDNA sequence of amplified exon 82 from the RYR1 gene is depicted in Table 3.61. Ibarra *et al.* (2006) reported a Val3840Ile alteration, which is due to a G11518A substitution, in one MH family.

Table 3.61: Partial gDNA sequence of exon 82 of the RYR1 gene

Nucleotide number	DNA sequence: exon 82
43718300	gcccaaccat atgtcctagc ttctgccaac tcttcatttc tgcttcctct tgatttcagc
43718360	gcatagatgg tttactgtgg ctctccaggc taccatggtg gggagctgcc aggccctggg
	exon 82 ↓
43718420	agagaggagg gcagaggctt catcacatac ccctatctt tcctttcttt tcctcagCGT
43718480	CCTGGATCTC AATGCCTTTG AGAGACAGAA CAAGGCCGAG GGGCTGGGCA TGGTGAATGA
43718540	GGATGGCACT Ggtgaggccc tcccttgggc ttcccacccc ctgagacatc ttcctttggg
43718600	attcctccca ccccaccccc acccgccatt gcccagagct cccttcctcc aagacactgg
43718660	ttcccaaggg ctccctcggg tccctccctc tgctgtggtc ccccagtccc accccctcca

The partial gDNA sequence amplified for exon 82 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Two single nucleotide polymorphisms are observed in this region, the C102984T nucleotide transition is indicated in **blue** and the C103014T nucleotide transition is indicated in **orange**. The codon that correlates to Val3840 is indicated in a dashed box (—) and the nucleotide transition for the mutation is indicated in **bold**. The forward primer (RYRex73F) is the single underlined sequence, while the reverse primer (RYRex73R) is the double underlined sequence; the beginning of exon 82 is indicated with an arrow.

3.7.56 Detection of alterations in exon 83 of the RYR1 gene

The partial gDNA sequence of amplified exon 83 from the RYR1 gene is depicted in Table 3.62. Thus far no alterations associated with the MH phenotype have been identified for this exon. Therefore a region of 252 bp was analysed in order to identify novel alterations and polymorphisms that may occur in exon 83.

Table 3.62: Partial gDNA sequence of exon 83 of the RYR1 gene

Nucleotide number	DNA sequence: exon 83
43719080	ctgcctttct ctctgtgggt tgttcctctc tctctgtgtg tctttctgtg tctctgtccc
43719140	tgactgtcat tgtgtgtgtt tgcggtctgt ctccctctct ttttctctct tttctcctgc
	↓ exon 83
43719200	ttcctcctcc catcctgttg gctgccccag TCATCAATCG CCAGAACGgt aattccccca
43719260	gcccaccccc gtgctgtgct gctgtcaccc acccctccta accccgtcca cccctccacc
43719320	taggggctga ggatctggga cgtggagggg agggagggac ccttcagcag gtgcaccctg

The partial gDNA sequence amplified for exon 83 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The forward primer (RYRex83F) is the single underlined sequence, while the reverse primer (RYRex83R) is the double underlined sequence; the beginning of exon 83 is indicated with an arrow.

3.7.57 Detection of alterations in exon 84 of the RYR1 gene

The partial gDNA sequence of amplified exon 84 from the RYR1 gene is depicted in Table 3.63. A PCR product of 431 bp was amplified to determine if any novel alterations or novel and reported polymorphisms occur in exon 84. Thus far no alterations associated with the MHS have been identified for this exon.

Table 3.63: Partial gDNA sequence of exon 84 of the RYR1 gene

Nucleotide number	DNA sequence: exon 84
43720100	agaaagacac ttgaggcccc agcttcatga ctcagggccc cttgggtctc cgtctgctga
43720160	tgtcatgggc tctgaggtca agaccgcctg gcgtc(a)tgga tgcccatggc cctcacagtg
43720220	tctttggagt ggcagctgct cctcccagca cccccatgct ttgtgcatgc gtgtgcagtg
43720280	tgcatgggcc ttgtgcatgt gtgcgctgtg tcttggcgca tctgacccct cctgggccct
	↓ exon 84
43720340	gtcccctccc ttccacctag GAGAGAAGGT CATGGCGGAT GATGAATTCA CACAAGACCT
43720400	GTTCCGATTC CTACAATTGC TCTGTGAGGG GCACAATAAT Ggtgaggagg aggggtgtgg
43720460	ggtggagggg aagccgaggt ttggggctgg tacggaaggg ttgactgaat tagtttccgc
43720520	ccctcctcct cccccggtca atgatggcca ctctccagcc agtgtcaatc cacctagctt

The partial gDNA sequence amplified for exon 84 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. A single nucleotide polymorphism is observed in this region, the A104616G nucleotide transition is indicated in a circle. The forward primer (RYRex84F) is the single underlined sequence, while the reverse primer (RYRex84R) is the double underlined sequence; the beginning of exon 84 is indicated with an arrow.

3.7.58 Detection of alterations in exons 85, 86 and 87 of the RYR1 gene

The partial gDNA sequence of amplified exons 85, 86 and 87 is depicted in Table 3.64. A PCR product of 662 bp was amplified and subsequently sequenced in order to identify reported or novel alterations and polymorphisms that may occur in the amplified region. Thus far, a single alteration associated with MHS in one French pedigree has been reported. The Ile3916Met alteration occurs in exon 85 and is due to a T11748G transition (Monnier *et al.*, 2005). In addition, Galli *et al.* (2006) identified an Arg3903Gln alteration in three MH families from Italy. The alteration is due to a G11708A nucleotide substitution and is observed in exon 85.

Table 3.64: Partial gDNA sequence of exons 85, 86 and 87 of the RYR1 gene

Nucleotide number	DNA sequence: exons 85, 86 and 87
43725740	gagtattgca taaatgaata aatgacccac tgttcatctc ccctagcaca tgggaggtgc
	↓ exon 85
43725800	tggataaatg acttttcatc tccccagATT TCCAGAACTA CCTACGGACA CAGACAGGGA
43725860	ACACGACCAC TATTAACATC ATCATTTGCA CTGTGGACTA CCTCCTGCGG CTGCAGgtga
43725920	ggacgtgaga cggttcaggt gtgacttggg tcgggggctg cagggccatg gtcggcccca
	↓ exon 86
43725980	gcacccccctc acaccctacc cgcccccacc agGAATCCAT CAGCGACTTC TACTGGTACT
43726040	ACTCGGGCAA GGATGTCATT GAAGAGCAGG GCAAGAGGAA CTTCTCCAAA GCCATGTCGG
43726100	TGGCTAAGCA GGTGTTCAAC AGCCTCACTG AGTACATCCA Ggtagggcgc tccccctggg
43726160	gcgggagtgg gaagggaggg ggtcccgcat cgtgatccct gatcccttct cggggattcc
	↓ exon 87
43726220	cttcccccc acacggcact ctgcctccca gGGTCCCTGC ACCGGGAACC AGCAGAGCCT
43726280	GGCGCACAGT CGCCTATGGG ACGCAGTGGT GGGATTCCTG CACGTGTTCG CCCACATGAT
43726340	GATGAAGCTC GCTCAGgttc gagcccctct ggtctccatc cacctgcttc cgggcgtccc
43726400	ccaagtggtc catttccaag tcttgcccct ttggtcagtt tgtcacccga gtgctcccgg

The partial gDNA sequence amplified for exons 85, 86 and 87 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Three single nucleotide polymorphisms are observed in this region, the C110647G nucleotide transition is indicated in **blue**, the C110649T nucleotide transition is indicated in **orange** and the A110658G nucleotide transition is indicated in green. The codon that correlates to Arg3903 is indicated in a dashed box (—), the codon that correlates to Ile3916 is indicated in a solid box (–) and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex85F) is the single underlined sequence, while the reverse primer (RYRex85R) is the double underlined sequence; the beginnings of exons 85, 86 and 87 are indicated with an arrow.

3.7.59 Detection of alterations in exon 88 of the RYR1 gene

A region of 278 bp was amplified in order to identify novel alterations or polymorphisms that may occur in exon 88. The partial gDNA sequence of amplified exon 88 from the RYR1 gene is depicted in Table 3.65.

Table 3.65: Partial gDNA sequence of exon 88 of the RYR1 gene

Nucleotide number	DNA sequence: exon 88
43728860	ggagaagcaa cagaggtggg ggaggtgtat gctgagacca gccctcaccg agctgggatc
	↓ Exon 88
43728920	tctagGACTC AAGCCAGATC GAGCTGCTGA AGGAGCTGCT GGATCTGCAG AAGGACATGG
43728980	TGGTGATGTT GCTGTCGCTA CTAGAAGgta aacacccagg agtgagggtg agggaacagt
43729040	aaagaggttc agagaagcct gagaatggcc ccctgagacc cgggagaccc taatcctcaa
43729100	ccccatctga catcgtgtca ggatccatgg gttgatgaag aagccataat aggccagatg

The partial gDNA sequence amplified for exon 88 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The forward primer (RYRex88F) is the single underlined sequence, while the reverse primer (RYRex88R) is the double underlined sequence; the beginning of exon 88 is indicated with an arrow.

3.7.60 Detection of alterations in exon 89 of the RYR1 gene

The partial gDNA sequence of amplified exon 89 from the RYR1 gene is depicted in Table 3.66. A region of 359 bp was amplified in order to identify reported or novel alterations and novel polymorphisms that occur in exon 89. Thus far, two alterations that may be associated with the MHS phenotype have been identified in this exon. The Arg4041Trp alteration is due to a C12121T nucleotide transition and was reported in a single MH pedigree from Italy (Galli *et al.*, 2006). The Thr4081Met alteration was reported in a single MH family from Japan. It is due to a C12242T substitution (Ibarra *et al.*, 2006).

Table 3.66: Partial gDNA sequence of exon 89 of the RYR1 gene

Nucleotide number	DNA sequence: exon 89
43730600	tggccaggga cactccagca gcgtggtggc tcctgggctg gaaagagagg caagcctggt
	↓ exon 89
43730660	ggggccccag aagggagtgt tcaccggcca cactgacctg gggctgcctg cagGGAACGT
43730720	GGTGAACGGC ATGATCGCCC GGCAGATGGT GGACATGCTC GTGGAATCCT CATCCAATGT
43730780	GGAGATGATC CTCAAGTTCT TCGACATGTT CCTGAAACTC AAGGACATTG TGGGCTCTGA
43730840	AGCCTTCCAG GACTACGTAA CGGATCCCCG TGGCCTCATC TCCAAGAAGG ACTTCCAGAA
43730900	Ggtgggtgtg ggacatcgtg tgggcccagg acttgggtgg ggttgccaag ggccagccat
43730960	acccttctgg ctggctgcct caggcaagag acatctctgc aagcctcact ttccttgttt

The partial gDNA sequence amplified for exon 89 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The codon that correlates to Arg4041 is indicated in a dashed box (---), the codon that correlates to Thr4081 is indicated in a solid box (–) and the nucleotide positions of the mutations are indicated in bold. The forward primer (RYRex89F) is the single underlined sequence, while the reverse primer (RYRex89R) is the double underlined sequence; the beginning of exon 89 is indicated with an arrow.

3.7.61 Detection of alterations in exon 90 of the RYR1 gene

The partial gDNA sequence of amplified exon 90 is depicted in Table 3.67. A 470 bp product of exon 90 was amplified in order to identify reported or novel alterations and novel polymorphisms. Exon 90 of the RYR1 gene occurs in hotspot three. Thus far, two alterations have been observed to occur in this exon. Sambuughin *et al.* (2005) reported an Asn4119Tyr alteration due to an A12355T nucleotide transition in one MHS individual from North America. The site at which the alteration occurs is conserved throughout RYR1 evolution and among RYR1 species and was not detected in 50 unaffected chromosomes. In addition, another mutation was observed in exon 90, namely the Arg4136Ser mutation that is due to a C12406A nucleotide transition (Galli *et al.*, 2002).

Table 3.67: Partial gDNA sequence of exon 90 of the RYR1 gene

Nucleotide number	DNA sequence: exon 90
43743500	gacacagcga gaccttgtct taaaaaaaaa aaaaaaaaga gagagaattg aggctctcca
	↓ exon 90
43743560	ggtcaccccca ctgacctccc tgcccgcccc cagGCCATGG ACAGCCAGAA GCAGTTCAGC
43743620	GGTCCAGAAA TCCAGTTCCT GCTTTCGTGC TCCGAAGCGG ATGAGAACGA AATGATCAAC
43743680	TGCGAAGAGT TCGCCAACCG CTTCCAGGAG CCAGCACGCG ACATCGGCTT CAACGTGGCG
43743740	GTGCTGCTGA CCAACCTGTC GGAGCATGTG CCGCATGACC CTCGCCTGCA CAACTTCCTG
43743800	GAGCTGGCCG AGAGCATCCT TGAGTACTTC CGCCCCTACC TGGGCCGCAT CGAGATCATG
43743860	GGCGCGTCAC GCCGCATCGA GCGCATCTAC TTCGAGATCT CAGAGACCAA CCGCGCCCAG
43743920	TGGGAGATGC CCCAGgtcag ggaacccgcg cgcgtgcaag ctcgcctcct ggggcttcgg
43743980	gcatgcgggt gctcacttcc tgcaccctca gaccccacgg gggctgtgcg tgcctcgcat

The partial gDNA sequence amplified for exon 90 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The codon that correlates to Asn4119 is indicated in a solid box (–) and the codon that correlates to Arg4136 is indicated in a dashed box (---) and the nucleotide positions for the mutations are indicated in **bold**. The forward primer (RYRex90F) is the single underlined sequence, while the reverse primer (RYRex90R) is the double underlined sequence; the beginning of exon 90 is indicated with an arrow.

3.7.62 Detection of alterations in exon 91 of the RYR1 gene

The partial gDNA sequence of amplified exon 91, from the RYR1 gene, is depicted in Table 3.68. The region was amplified and subsequently sequenced in order to identify reported or novel alterations and polymorphisms. Thus far, two alterations associated with MH have been detected in exon 91 of the RYR1 gene. A Val4234Leu, which is due to a G12700C nucleotide transition, was observed in one MHS family (Galli *et al.*, 2002). In one family from Japan, Ibarra *et al.* (2006) identified the Glu4283Val alteration that is due to an A12848T substitution. In addition, Monnier *et al.* (2001) reported a 12,640 - 12,648del alteration in exon 91 in one family with CCD. The deletion resulted in the loss of three residues i.e. Arg4214, Gln4215 and Phe4216. All three residues were conserved among RYR isoforms and segregated with the disorder in the family.

Table 3.68: Partial gDNA sequence of exon 91 of the RYR1 gene

Nucleotide number	DNA sequence: exon 91
43747340	cgcctgccgc ggtgacccct tgtagctgcc actgcgctgt cgctgctgtc cgagcccccg
	↓ exon 91
43747400	ctgacggcgc cctatcctgt ctgccgcccc tcgcttcagG TGAAGGAGTC CAAGCGCCAG
43747460	TTCATCTTCG ACGTGGTGAA CGAGGGCGGC GAGGCTGAGA AGATGGAGCT CTTCGTGAGT
43747520	TTCTGCGAGG ACACCATCTT CGAGATGCAG ATCGCCGCGC AGATCTCGGA GCCCGAGGGC
43747580	GAGCCGGAGA CCGACGAGGA CGAGGGCGCG GGCGCGGCGG AGGCGGGCGC GGAAGGCGCG
43747640	GAGGAGGGCG CGGCGGGGCT CGAGGGCACG GCGGCCACGG CGGCGGCGGG GGCGACGGCG
43747700	CGGGTTGTGG CGGCCGCAGG CCGGGCCCTG CGAGGCCTCA GCTACCGCAG CCTGCGGCGG
43747760	CGCGTGCGGC GGCTGCGGCG GCTTACGGCC CGCGAGGCGG CCACCGCAGT GGCGGCGCTG
43747820	CTCTGGGCAG CAGTGACGCG CGCTGGG**G**CC GCTGGCGCGG GGGCGGCGGC GGGCGCGCTG
43747880	GGCCTGCTCT GGGGCTCGCT GTTCGGCGGC GGCCTGGTGG AGGGCGCCAA GAAGGTGACG
43747940	GTGACCGAGC TCCTGGCAGG CATGCCCGAC CCCACCAGCG ACGAGGTGCA CGGCGAGCAG
43748000	CCGGCCGGGC CGGGCGGAGA CGCAGACGGC GAGGGTGCCA GCGAGGGCGC TGGAGACGCC
43748060	GCGGAGGGCG CTGGAGACGA GGAGGAGGCG GTGCACGAGG CCGGGCCGGG CGGTGCCGAC
43748120	GGGGCGGTGG CCGTGACCGA TGGGGGCCCC TTCCGGCCCG AAGGGGCTGG CGGTCTCGGG
43748180	GACATGGGGG ACACGACGCC TGCGGAACCG CCCACACCCG AGGGCTCTCC CATCCTCAAG
43748240	AGGAAATTGG GGgtgagaga gcaggcgggg ttttggggtt ttggaaagat ggggg<u>attgg</u>
43748300	<u>agggaggaag agagccc</u>ggc tgggtggaga cacacacaga ggagagaact ggctaggggg

The partial gDNA sequence amplified for exon 91 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. A single nucleotide polymorphism is observed in this region, the G132268C nucleotide transition is indicated in **blue**. The codon that correlates to Val4234 is indicated in a solid box (–) and the codon that correlates to Glu4283 is indicated in a dashed box (---) and the nucleotide positions of the mutations are indicated in **bold**. The codons that correlate to 12640del9nt are indicated in a **red** solid box (–). The forward primer (RYRex91cF) is the single underlined sequence, while the reverse primer (RYRex91cR) is the double underlined sequence; the beginning of exon 91 is indicated with an arrow.

3.7.63 Detection of alterations in exon 92 of the RYR1 gene

The partial gDNA sequence of amplified exon 92 is depicted in Table 3.69. To date this region has not been reported to harbour any alterations associated with MHS. A 166 bp region containing exon 92 was amplified in order to identify novel alterations as well as to detect novel and reported polymorphisms. The amplified region harbours two known polymorphisms, which occur in the third mutational hotspot.

Table 3.69: Partial gDNA sequence of exon 92 of the RYR1 gene

Nucleotide number	DNA sequence: exon 92
43749320	agaggagcag gcaggcagcc tgagaagcgc ttagggtgag gactcagccc tgatgcttgc
	↓ exon 92
43749380	cctgtcccta gGTGGATGGA GTGGAGGAGG AGCTCCCGCC AGAGCCAGAG CCCGAGCCGG
43749440	AACCAGAGCT GGAGCCGGAG AAAGCCGAgt gagtggcctt ggggctgagg ggcctagccc
43749500	ctatcactgc ctccctccta gagtaggagc ctccagaggt caggccccaa ggctgtcctg

The partial gDNA sequence amplified for exon 92 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Two single nucleotide polymorphisms are observed in this region, the G133838A nucleotide transition is indicated in **blue** and the G133877A nucleotide transition is indicated in **orange**. The forward primer (RYRex92F) is the single underlined sequence, while the reverse primer (RYRex92R) is the double underlined sequence; the beginning of exon 92 is indicated with an arrow.

3.7.64 Detection of alterations in exon 93 of the RYR1 gene

The partial gDNA sequence of amplified exon 93 from the RYR1 gene is depicted in Table 3.70. A 381 bp fragment of exon 93 was amplified in order to identify reported or novel alterations and polymorphisms. Alterations associated with MHS have thus far not been reported for this exon, even though the exon resides in hotspot three of the RYR1. However, a single alteration has been reported in a family diagnosed with CCD. The Arg4549Gln alteration is due to an A13645C nucleotide substitution (Kossugue *et al.*, 2005).

Table 3.70: Partial gDNA sequence of exon 93 of the RYR1 gene

Nucleotide number	DNA sequence: exon 93
43750100	ggcaagtcct gattatctca tcatcccatg tacccagtac catccaaacc tgggccaggc
43750160	acagggcggg cccttggtga atggttttga atgaatgaac tcatgcattg cctgcccagg
	↓ exon 93
43750220	cacctcctga cctctctctg tcctgccctg cagTGCCGAG AATGGGGAGA AGGAAGAAGT
43750280	TCCCGAGCCC ACACCAGAGC CCCCCAAGAA GCAAGCACCT CCCTCACCCC CTCCAAAGAA
43750340	GGAGGAAGCT GGAGGCGAAT TCTGGGGAGA ACTGGAGGTG CAGAGGGTGA AGTTCCTGGt
43750400	aaggatccag ccaggtcacc tgaaccttct tctccccggg agccccacct ctggtgccca
43750460	cctctcctgc tcaccttgtt cttgagttca tctgttcaag ctcgtctcta agaagaaccc

The partial gDNA sequence amplified for exon 93 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. A single nucleotide polymorphism is observed in this region, the C134776T nucleotide transition is indicated in a circle. The codon that correlates to Arg4549 is indicated in a solid box (–) and the nucleotide position of the mutation is indicated in **bold**. The forward primer (RYRex93F) is the single underlined sequence, while the reverse primer (RYRex93R) is the double underlined sequence; the beginning of exon 93 is indicated with an arrow.

3.7.65 Detection of alterations in exon 94 of the RYR1 gene

A region of 224 bp of exon 94 was amplified and analysed in order to detect either of the novel alterations associated with MHS or novel and reported polymorphisms. No alterations in this region of the RYR1 have been reported to be associated with MH and the partial gDNA sequence of amplified exon 94 from the RYR1 gene is represented in Table 3.71. Exon 94 resides in the third hotspot of the gene. A single alteration, Leu4568Pro, has been reported to be associated in one family diagnosed with CCD. The alteration is due to a T13703C nucleotide transition (Wu *et al.*, 2006)

Table 3.71: Partial gDNA sequence of exon 94 of the RYR1 gene

Nucleotide number	DNA sequence: exon 94
	↓ Exon 94
43753040	tgggggctg tctgtggcgc tttctctttt tttctcttct ctctcagAAC TACCTGTCCC
43753100	GGAACTTTTA CACCCTGCGG TTCCTTGCCC TCTTCTTGGC ATTTGCCATC AACTTCATCT
43753160	TGCTGTTTTA TAAGgtgctg gtcctgaagg gctgggaggg tcaggccctt ttccatgctg
43753220	tgggatggga ggctcagccc ctatcagaat ttcagggttc ctccactgaa gggataaggt

The partial gDNA sequence amplified for exon 94 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. A single nucleotide polymorphism is observed in this region, the C137519G nucleotide transition is indicated in a circle. The codon that correlates to Leu4568 is indicated in a solid box (–) and the nucleotide position of the mutation is indicated in **bold**. The forward primer (RYRex94F) is the single underlined sequence, while the reverse primer (RYRex94R) is the double underlined sequence; the beginning of exon 94 is indicated with an arrow.

3.7.66 Detection of alterations in exon 95 of the RYR1 gene

A region of 473 bp of exon 95 was sequenced to detect the alteration Gly4638Met, which is due to a G13913A transition (Halsall and Robinson, 2004) and the Arg4645Gln alteration, which is due to a G13934A substitution (Ibarra *et al.*, 2006), as well as to detect novel alterations or novel and reported polymorphisms that may occur in this region. The partial sequence of amplified exon 95 is indicated in Table 3.72 and the exon resides in hotspot three of the RYR1 gene.

Both the Arg4645Gln and Gly4638Met have been reported in MHS families. The Gly4638Met alteration has been observed in two UK families with MH (Halsall and Robinson, 2004). The nucleotide substitution can also result in a Gly4638Asp alteration and has been reported in two families from the UK with CCD (Shepherd *et al.*, 2004). In addition, eight alterations that occur in exon 95 have been reported in association with CCD. The Tyr4631Asn, Glu4634Lys, Thr4637Ala, Thr4637Ile, Gly4638Ser, Gly4638Asp, His4651Pro and Leu4665Pro have all been reported in single CCD families and are due

to T13891A, G13900A, A13909G, C13910T, G13912A, G13913A, A13952C and T13994C nucleotide transitions respectively (Scacheri *et al.*, 2000; Davis *et al.*, 2003; Shepherd *et al.*, 2004; Zhou *et al.*, 2005; Wu *et al.*, 2006). Monnier *et al.* (2001) reported a 13938 - 13943 deletion that included the deletion of six nucleotides. The deletion resulted in the loss of two amino acids, Leu4647 and Ser4748, in exon 95. The deletion was observed in one family with CCD and segregated with the phenotype in the family.

Table 3.72: Partial gDNA sequence of exon 95 of the RYR1 gene

Nucleotide number	DNA sequence: exon 95
43754373	tgattgacag ccacaccaag actgtatctg gtatggtccc agtccaatct cgggaatgga
43754433	ggctcaattt tgtgagtggg ctctgcatgt ggcagaccca cagatgaatc tctgtcccca
	↓ **Exon 95**
43754493	tttcagGTCT CAGACTCTCC ACCAGGGGAG GACGACATGG AAGGCTCAGC TGCTGGGGAT
43754553	GTGTCAGGTG CAGGCTCTGG TGGCAGCTCT GGCTGGGGCT TGGGGGCCGG AGAGGAGGCA
43754613	GAGGGCGATG AGGATGAGAA CATGGTGTAC **T**ACTTCCTG**G** AGGAAAGC**AC** A**GG**CTACATG
43754673	GAACCCGCCC TG**C**GGTGTCT GAGCCTCCTG **CA**TACACTGG TGGCCTTTCT CTGCATCATT
43754733	GGCTATAATT GT**CT**CAAGgt gggcccatgg ccatggttct ggggcaaggg cttattggct
43754793	gggtgggggt gggggcagtg ctggagcact ggctggggct gggggcctc aaagtggttg
43754853	ggacagaggg ggcctagggt tggggtgagg gctggggaac tgggtacagg attggggtct

The partial gDNA sequence amplified for exon 95 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The codon that correlates to Tyr4631 is indicated in a solid box (–), the codon that correlates to Glu4634 is indicated in a dashed box (—), the codon that correlates to Thr4637 is indicated in a red solid box (–), the codon that correlates to Gly4638 is indicated in a red dashed box (—), the codon that correlates to Arg4645 is indicated in a blue solid box (–), the codon that correlates to His4651 is indicated in a blue dashed box (—), the codon that correlates to Leu4665 is indicated in a green solid box (–) and the nucleotide positions of the mutations are indicated in **bold**. The codons that correlate to 13938del6nt are indicated in a pink solid box (–). The forward primer (RYRex95F) is the single underlined sequence, while the reverse primer (RYRex95bR) is the double underlined sequence; the beginning of exon 95 is indicated with an arrow.

3.7.67 Detection of alterations in exon 96 of the RYR1 gene

In order to detect novel or reported alterations and polymorphisms in exon 96, a region of 218 bp was analysed. To date, two alterations resulting in MH have been detected in exon 96, which occurs in hotspot three of the RYR1 gene. Oyamada *et al.* (2002) reported a Pro4668Ser alteration in one MH family that was due to a C14002T transition and Monnier *et al.* (2005) reported a Phe4684Ser alteration in one MHS family that was due to a T14501C substitution. In addition, a single alteration Thr4709Met, has been observed in a single CCD family (Zhou *et al.*, 2005). The partial sequence of amplified exon 96 is indicated in Table 3.73.

Table 3.73: Partial gDNA sequence of exon 96 of the RYR1 gene

Nucleotide number	DNA sequence: exon 96
43755560	tcacacacag accccagcaa gatgtcatgg cttctgctga gactatggtc cagccaaggt
	↓ Exon 96
43755620	gcctgacgcc caccttggc ctcctcccac tatccagGTG CCCCTGGTAA TCTTTAAGCG
43755680	GGAGAAGGAG CTGGCCCGGA AGCTGGAGTT TGATGGCCTG TACATCACGG AGCAGCCTGA
43755740	GGACGATGAC GTGAAGGGGC AGTGGGACCG ACTGGTGCTC AACACGCCgt aaggacccag
43755800	cccccacctc agggtggcag caggaggga cctgggtttc cacccagtcc aggcctggac

The partial gDNA sequence amplified for exon 96 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The codon that correlates to Pro4668 is indicated in a solid box (–), the codon that correlates to Phe4684 is indicated in a dashed box (---), the codon that correlates to Thr4709 is indicated in a red solid box (–) and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex96F) is the single underlined sequence, while the reverse primer (RYRex96R) is the double underlined sequence; the beginning of exon 96 is indicated with an arrow.

3.7.68 Detection of alterations in exon 97 of the RYR1 gene

The partial sequence of amplified exon 97 from the RYR1 gene is indicated in Table 3.74. In order to detect novel alterations associated with the MH phenotype as well as novel polymorphisms in exon 97, a region of 228 bp was analysed. To date, alterations resulting in MH have not been detected in exon 97 of the RYR1 gene, even though the exon resides in hotspot three. However, a single alteration that results in CCD susceptibility has been reported. The Lys4724Gln alteration is due to an A14170C nucleotide transition and has thus far been reported in one CCD family (Zhou *et al.*, 2005).

Table 3.74: Partial gDNA sequence of exon 97 of the RYR1 gene

Nucleotide number	DNA sequence: exon 97
43758260	gcaacagagt gagactccat ctcaaaaaaa aaggaaaaag aaaaacagtg gagtttcagc
43758320	caaccctgtc gtggctgaca gctctgatcc ctctggccct aacatcttat actcacgctt
	↓ Exon 97
43758380	tctctctctc tctctgcagG TCTTTCCCTA GCAACTACTG GGACAAGTTT GTCAAGCGCA
43758440	AGgtgagagg acatggatgc cctgggtcct ggattgggtc cctgcctgcc accaggccat
43758500	cacaggcctg ccaagcactt gcttggtgtg tgaccttggg caagaggttt ttttgccttt

The partial gDNA sequence amplified for exon 97 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The codon that correlates to Lys4724 is indicated in a solid box (–) and the nucleotide position of the mutation is indicated in **bold**. The forward primer (RYRex97F) is the single underlined sequence, while the reverse primer (RYRex97R) is the double underlined sequence; the beginning of exon 97 is indicated with an arrow.

3.7.69 Detection of alterations in exons 98 and 99 of the RYR1 gene

The partial sequence of amplified exon 98 and exon 99 is indicated in Table 3.75. In order to detect novel and reported alterations as well as polymorphisms in exon 98 and 99, a region of 347 bp was analysed. Thus far, one alteration, Arg4737Gln, which is due to a G14210A transition in exon 98, has been reported in one MHS individual from France (Monnier *et al.*, 2005). In addition, Sambuughin *et al.* (2005) reported a Tyr4733Asp alteration in one MHS family and Galli *et al.* (2002) reported a Arg4737Trp alteration in two MHS families. The alterations are due to nucleotide substitutions T14197G and C14209T, respectively. Alterations resulting in the MH phenotype have not been reported for exon 99. Both exons 98 and 99 reside in the third mutational hotspot of the RYR1 gene.

Table 3.75: Partial gDNA sequence of exons 98 and 99 of the RYR1 gene

Nucleotide number	DNA sequence: exons 98 and 99
	↓ exon 98
43760360	ccagctgtgt ctacacagcc tgatgctctc ttgtgcagGT CCTGGACAAA CATGGGGACA
43760420	TCTACGGGCG GGAGCGGATT GCTGAGCTAC TGGGCATGGA CCTGGCCACA CTAGAGATCA
43760480	CAGCCCACAA TGAGCGCAAG CCCAACCCGC CGCCAGGGCT GCTGACCTGg tgagcccagg
43760540	acacccctgc acaggcctgg ggcatgcagg ggaggtgact ggagtctgac actcaagcat
	↓ exon 99
43760600	ctctccccac ccccgccccc acagGCTCAT GTCCATCGAT GTCAAGTACC AGATCTGGAA
43760660	GTTCGGGGTC ATCTTCACAG ACAACgtgag caggggccca cagactgggg agggactctg

The partial gDNA sequence amplified for exons 98 and 99 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. One single nucleotide polymorphism is observed in this region, the A144902C nucleotide transition is indicated in a circle. The codon that correlates to Tyr4733 is indicated in a dashed box (---), the codon that correlates to Arg4737 is indicated in a solid box (–) and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex98F) is the single underlined sequence, while the reverse primer (RYRex98R) is the double underlined sequence; the beginnings of exons 98 and 99 are indicated with an arrow.

3.7.70 Detection of alterations in exon 100 of the RYR1 gene

The partial sequence of amplified exon 100 is indicated in Table 3.76. In order to detect reported or novel alterations and polymorphisms in exon 100 in hotspot three, a region of 274 bp was analysed. Alterations Leu4814Phe, Ile4817Phe and Leu4824Pro were observed in UK pedigrees with MH. The mutations are due to nucleotide substitutions C14440T, A14449T and T14471C, respectively. Two alterations, Leu4814Phe and Ile4817Phe, were observed in one family, while the Leu4824Pro alteration was observed in three families. The alteration Thr4826Ile was first described by Brown *et al.* (2000) in a large Maori pedigree. It occurs in the C-terminal region/transmembrane loop of exon 100 and is due to a C14477T transition. In addition, four alterations have been reported to be

associated with CCD. The Leu4793Pro alteration (Monnier *et al.*, 2001), Tyr4796Cys (Monnier *et al.*, 2000), Phe4808Asn (Davis *et al.*, 2003), and Arg4825Cys (Monnier *et al.*, 2001) are due to nucleotide transitions T14378C, A14387G, T14422A and C14473T.

Table 3.76: Partial gDNA sequence of exon 100 of the RYR1 gene

Nucleotide number	DNA sequence: exon 100
43762400	ctccagagtg ctcctcgtgt gtccctgcct tccccctgac ccctggccct gtgtgcccac
	↓ **Exon 100**
43762460	agTCCTTCCT GTACCTGGGC TGGTATATGG TGATGTCCCT CTTGGGACAC TACAACAAC**T**
43762520	TCTTCTTTGC TGCCCATCTC CTGGACATCG CCATGGGGGT CAAGACGCTG CGCACCATCC
43762580	TGTCCTCTGT CACCCACAAT GGGAAACAGg tgtggggagg acctggctgt ggggcgtggg
43762640	ccagcaggga ccagcgtggc agtgggtggt gaagggataa gggccgggca gctgggctga

The partial gDNA sequence amplified for exon 100 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The codon that correlates to Leu4793 is indicated in a solid box (–), the codon that correlates to Tyr4796 is indicated in a dashed box (---), the codon that correlates to Phe4808 is indicated in a **red** solid box (–), the codon that correlates to Leu4814 is indicated in a **red** dashed box (---), the codon that correlates to Ile4817 is indicated in a **blue** solid box (–), the codon that correlates to Leu4824 is indicated in a **blue** dashed box (---), the codon that correlates to Arg4825 is indicated in a **green** solid box (–), the codon that correlates to Thr4826 is indicated in a **green dashed** box (---) and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex100aF) is the single underlined sequence, while the reverse primer (RYRex100aR) is the double underlined sequence; the beginning of exon 100 is indicated with an arrow.

3.7.71 Detection of alterations in exon 101 of the RYR1 gene

The partial sequence of amplified exon 101 is indicated in Table 3.77. Sequencing was conducted in order to detect reported or novel alterations and polymorphisms in exon 101, which resides in the third mutational hotspot. Alterations Leu4838Val and Val4849Ile were observed in UK families (Halsall and Robinson, 2004). The mutations are due to nucleotide transitions in the RYR1 gene, C14512G and G14545A, respectively. Alteration Leu4838Val was observed in a single pedigree and Val4849Ile was observed in four pedigrees. The Arg4861His alteration on exon 101, which results from substitution, G14582A, was first described by Monnier *et al.* (2001). The mutation was detected in three unrelated CCD pedigrees. The Arg4861His alteration was also detected in a single CCD pedigree. Three members of the family were also subsequently diagnosed as MHS via an IVCT (Davis *et al.*, 2003). In addition, Sambuughin *et al.* (2005) detected a Lys4876Arg alteration that is due to an A14627G nucleotide transition in one MHS individual and identified the nucleotide transition T14639C, which results in a Met4880Thr alteration in one MHS individual from North America. In addition, four alterations have been reported in CCD families. Kossugue *et al.* (2005) observed an Ala4846Val alteration that is due to a C14537T substitution, Wu *et al.* (2006) detected alterations Asn4858Asp and Arg4861Cys, which are due to A14572G and C14581T transitions respectively and Zorzato *et al.* (2003) reported a Tyr4864Cys alteration, which is due to a A14591G

nucleotide substitution. In addition, Monnier *et al.* (2001) reported a Phe4860del in one CCD family. The deletion was due to 14578delttc and was only observed in the proband of the family, indicating that the alteration may be sporadic.

Table 3.77: Partial gDNA sequence of exon 101 of the RYR1 gene

Nucleotide number	DNA sequence: exon 101
	Exon 100
43762468	CTGTACCTGG GCTGGTATAT GGTGATGTCC CTCTTGGGAC ACTACAACAA CTTCTTCTTT
43762528	GCTGCCCATC TCCTGGACAT CGCCATGGGG GTCAAGACGC TGCGCACCAT CCTGTCCTCT
43762588	GTCACCCACA ATGGGAAACA Ggtgtgggga ggacctggct gtggggcgtg ggccagcagg
43762648	gaccagcgtg gcagtgggtg gtgaagggat aagggccggg cagctgggct gaggaggggc
43762708	aaggccaggt gcgctgagcc gggggtgtgt ggggcagcaa ggtagagcca cagggactga
43762768	accggggcca ggacccagca tgggcagggt gggggaggg caagcccagg gcggagctga
	↓ **Exon 101**
43762828	cctggcccca tcctgccccc ag**CTG**GTGAT GACCGTGGGC CTTCTG**GC**G TGGTC**GTC**TA
43762888	CCTGTACACC GTGGTGGCCT TC**AAC**TTCTT C**CGC**AAGTTC **TAC**AACAAGA GCGAGGATGA
43762948	GGATGAACCT GACATG**AAG**T GTGATGAC**AT** GATGACGgtg agcccctccc ctagcactct
43763008	gggaccctte cttctcgcat ctgttgaagg agttaataat ggtacctcca ggccgggcgt

The partial gDNA sequence amplified for exon 101 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The codon that correlates to Leu4838 is indicated in a solid box (–), the codon that correlates to Ala4846 is indicated in a dashed box (---), the codon that correlates to Val4849 is indicated in a red solid box (–), the codon that correlates to Asn4858 is indicated in a blue solid box (–), the codon that correlates to Arg4861 is indicated in a blue dashed box (---), the codon that correlates to Tyr4864 is indicated in a green solid box (–), the codon that correlates to Lys4876 is indicated in a green dashed box (---), the codon that correlates to Met4880 is indicated in a pink solid box (–) and the nucleotide positions of the mutations are indicated in **bold**. The codon that correlates to 14578del3nt is indicated in a pink dashed box (---). The forward primer (RYRex100F) is the single underlined sequence, while the reverse primer (RYRex100R) is the double underlined sequence; the beginning of exon 101 is indicated with an arrow.

3.7.72 Detection of alterations in exon 102 of the RYR1 gene

The partial sequence of amplified exon 102 from the RYR1 gene is indicated in Table 3.78. Sequencing was conducted in order to detect novel or reported alterations and polymorphisms in exon 102. Thus far, one alteration resulting in the MH phenotype has been reported, Ibarra *et al.* (2006) reported the Ala4894Thr alteration in one MH family. It is due to a G14680A nucleotide transition. However, 13 alterations resulting in CCD have been observed in this exon, which resides in hotspot three of the RYR1 gene. Alterations Gly4891Arg (Tilgen *et al.*, 2001), Arg4893Trp (Monnier *et al.*, 2001), Arg4893Gln (Davis *et al.*, 2003), Arg4893Pro (Wu *et al.*, 2006), Gly4897Val (Kossugue *et al.*, 2005), Ile4898Thr (Lynch *et al.*, 1999), Gly4899Arg (Tilgen *et al.*, 2001), Gly4899Glu (Monnier *et al.*, 2001), Ala4906Val (Tilgen *et al.*, 2001), Arg4914Gly (Monnier *et al.*, 2001), Arg4914Thr (Davis *et al.*, 2003), Thr4920Asn and Phe4921Ser (Wu *et al.*, 2006) have all been reported associated with the CCD phenotype.

Table 3.78: Partial gDNA sequence of exon 102 of the RYR1 gene

Nucleotide number	DNA sequence: exon 102
43767260	ggctgttggt ccctgtctga tgccgtatct gtgagccctt tgagggcagg gcccagggct
43767320	gtctcagtcg ttaccatgtc ttcagccctg cctatcccgg ggccttggct ggtactcagt
	↓ exon 102
43767380	gaatgtcgaa tgaatgagtg accagtgtgc tcccctccct cagTGTTACC TGTTTCACAT
43767440	GTACGTGGGT GTCCGGGCTG GCGGAGGCAT TGGGGACGAG ATCGAGGACC CCGCGGGTGA
43767500	CGAATACGAG CTCTACAGGG TGGTCTTCGA CATCACCTTC TTCTTCTTCG TCATCGTCAT
43767560	CCTGTTGGCC ATCATCCAGG gtcagtgctg ggagtgggcg ctcagggccc ggaggcaggc
43767620	tagctccatg gctaagaatg caggcccagg atccagtcgg cctgcattca taccccatct
43767680	ctacctctcg ctactgtgag accttgggca agtcacctct cggggcctcc gtttctccat

The partial gDNA sequence amplified for exon 102 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. One single nucleotide polymorphism is observed in this region, the C151784T nucleotide transition is indicated in **blue**. The codon that correlates to Gly4891 is indicated in a solid box (–), the codon that correlates to Arg4893 is indicated in a dashed box (---), the codon that correlates to Ala4894 is indicated in a **red** solid box (–), the codon that correlates to Gly4897 is indicated in a **red** dashed box (---), the codon that correlates to Ile4898 is indicated in a **blue** solid box (–), the codon that correlates to Gly4899 is indicated in a **blue** dashed box (---), the codon that correlates to Ala4906 is indicated in a **green** solid box (–), the codon that correlates to Arg4914 is indicated in a **green** dashed box (---), the codon that correlates to Thr4920 is indicated in a **pink** solid box (–), the codon that correlates to Phe4921 is indicated in a **pink** dashed box (---) and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex102F) is the single underlined sequence, while the reverse primer (RYRex102R) is the double underlined sequence; the beginning of exon 102 is indicated with an arrow.

3.7.73 Detection of alterations in exon 103 of the RYR1 gene

A region of 147 bp of exon 103, which occurs in hotspot three, was sequenced to detect alterations Ile4938Met and Asp4939Glu, which have previously been observed in single UK families (Halsall and Robinson, 2004). The mutations are due to nucleotide transitions in the RYR1 gene, C14814G and C14817A, respectively. In addition, an Ala4940Thr alteration has been reported in one MHS individual from North America. The alteration is due to a G14818A nucleotide transition (Sambuughin *et al.*, 2005). Galli *et al.* (2002) reported a Gly4942Val alteration associated with the MH phenotype in one MHS family. The alteration is due to a G14825T nucleotide transition. In addition to analysing reported alterations, the sequence was also screened for novel alterations and polymorphisms. The partial sequence of amplified exon 103 is indicated in Table 3.79.

Table 3.79: Partial gDNA sequence of exon 103 of the RYR1 gene

Nucleotide number	DNA sequence: exon 103
43768385	↓ exon 103 gtcgggcact gacttgtgtc ctgccacccc agGTCTGATC ATCGACGCTT TTGGTGAGCT
43768445	CCGAGACCAA CAAGAGCAAG TGAAGGAGGA TATGGAGgta ggtcatgtct gggggtgacc
43768505	cagagggatt acgggattca gggggtcaag tgggcctcca ctctgatgtc tcttgccact

The partial gDNA sequence amplified for exon 103 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The codon that correlates to Ile4938 is indicated in a solid box (–), the codon that correlates to Asp4939 is indicated in a dashed box (—), the codon that correlates to Ala4940 is indicated in a red solid box (–), the codon that correlates to Gly4942 is indicated in a red dashed box (—) and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex103F) is the single underlined sequence, while the reverse primer (RYRex103R) is the double underlined sequence; the beginning of exon 103 is indicated with an arrow.

3.7.74 Detection of alterations in exons 104 and 105 of the RYR1 gene

A region of 643 bp encompassing exons 104 and 105 was analysed in order to detect novel and reported mutations and polymorphisms that may occur in this region of the RYR1 gene. The partial sequence of amplified exons 104 and 105 is indicated in Table 3.80.

Table 3.80: Partial gDNA sequence of exons 104 and 105 of the RYR1 gene

Nucleotide number	DNA sequence: exons 104 and 105
43768460	exon 103 AGCAAGTGAA GGAGGATATG GAGgtaggtc atgtctgggg gtgacccaga gggattacgg
43768520	↓ exon 104 gattcagggg gtcaagtggg cctccactct gatgtctctt gccactcaca gACCAAGTGC
43768580	TTCATCTGTG GAATCGGCAG TGACTACTTT GATACGACAC CGCATGGCTT CGAGACTCAC
43768640	ACGCTGGAGG AGCACAACCT GGCCAATTAC ATgtgagcag acacactggc cagtcaggag
43768700	ggtggggggc atggctgcca atagccagca gtggggtact tagctttggc cagttaggaa
43768760	agggggtgta gtgtccatgt gggcagattc cctgccagcc aatcagaagg taagggtggg
43768820	gccccgcaag atggttcaca cctgtaagcc cagcactttg ggaggccaag tggggaggat
43768880	tacttgaggc caggagttcg agaccagctt gggcaacata gcaagacttc ctctctacta
43768940	taaatgaaaa ataaaataag gtaagggtgg ttctgacttg tctcctgtgg tcctctcacc
43769000	↓ exon 105 ctcagGTTTT TCCTGATGTA TTTGATAAAC AAGGATGAGA CAGAACACAC GGGTCAGgta
43769060	agggggtgtt aatgggagga cagtgggcag gacgtggagc cctttaacat aaggccagtc

The partial gDNA sequence amplified for exons 104 and 105 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. Two single nucleotide polymorphisms are observed in this region, the C153015A nucleotide transition is indicated in **blue** and the G153471A nucleotide transition is indicated in red. The codon that correlates to Phe4960 is indicated in a solid box (–), the codon that correlates to Pro4973 is indicated in a dashed box (—) and the nucleotide positions of the mutations are indicated in **bold**. The forward primer (RYRex104F) is the single underlined sequence, while the reverse primer (RYRex104R) is the double underlined sequence; exon 103 is indicated in bold and the beginnings of exons 104 and 105 are indicated with an arrow.

Thus far two alterations have been reported to be associated with the MH phenotype. Monnier *et al.* (2002) identified a Pro4973Leu alteration in exon 104 that is due to a

C14918T nucleotide transition in a single MH pedigree from France. The alteration was detected in four MHS individuals in the family and was not observed in 100 unaffected controls obtained from the general population. Ibarra *et al.* (2006) reported a Phe4960Tyr alteration in one MH family from Japan, which is due to a T14879A nucleotide substitution. Exon 104 occurs in the third mutational hotspot of the RYR1 gene, however, exon 105 occurs outside this mutational hotspot.

3.7.75 Detection of alterations in exon 106 of the RYR1 gene

A region of 375 bp encompassing exon 106 was analysed in order to detect novel mutations and polymorphisms that may occur in this region of the RYR1 gene. The partial sequence of amplified exon 106 is indicated in Table 3.81.

Table 3.81: Partial gDNA sequence of exon 106 of the RYR1 gene

Nucleotide number	DNA sequence: exon 106
43769720	aacaccctgt ctaaaaatat atatatatat atgtctcaag ggtttgaaga tgtgaccaat
	↓ exon 106
43769880	gaactctttc tatccccaat cctagGAGTC TTATGTCTGG AAGATGTACC AAGAGAGATG
43769940	TTGGGATTTC TTCCCAGCTG GTGATTGTTT CCGTAAGCAG TATGAGGACC AGCTTAGCTG
43770000	ACACACCCCC AGCTGGCCCT CCACCCCCAC CTCAAGTGCC TTATTCTCAC AGCAAGCCCC
43770060	TTAGTCCCCA AGCCCCTCCC CCTAAGGCAG CTGGGGGAGA GGTGACCTAG TACtggaaaa
43770120	taaatctgtg ctacgccccc cagcatcact gtgttggcct gctgaaattt tggaggagtg
43770180	gacatccagg aattgtttcc cccaagaaaa acaagatgac agcagaggct aaagtcatgt

The partial gDNA sequence amplified for exon 106 was obtained from Ensembl (v.36) with accession number AC011469.6.1.110569. The exon sequence is indicated in UPPER case and the intron sequence is indicated in lower case. The forward primer (RYRex106F) is the single underlined sequence, while the reverse primer (RYRex106R) is the double underlined sequence; the beginning of exon 106 is indicated with an arrow.