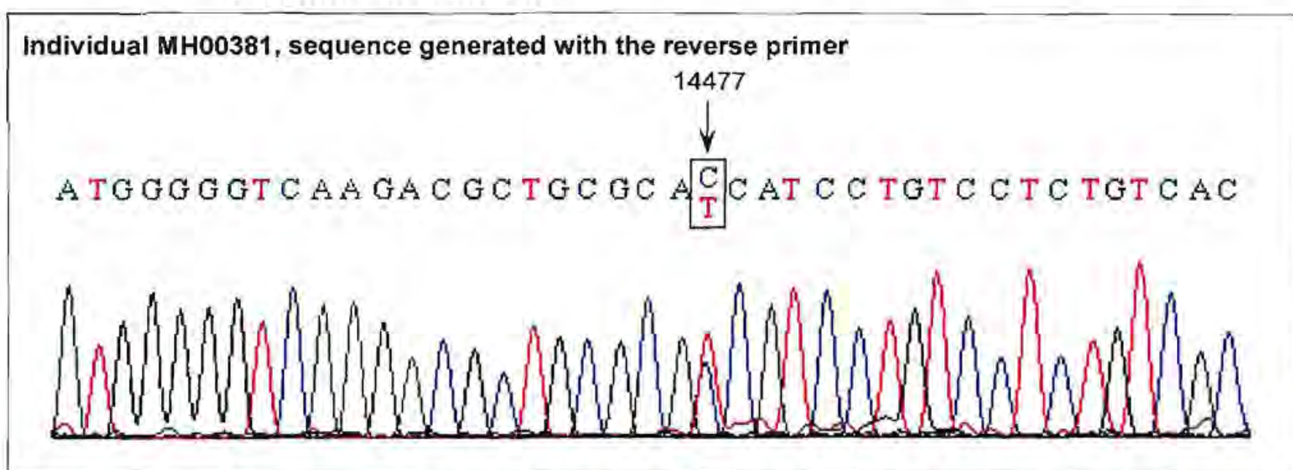
4.9.10.1 The Thr4826Ile alteration in the RYR1 gene

The Thr4826Ile alteration was detected in a single South African MH proband, MH 113-14 (MH00381) analysed during Phase 2 (Dalton, 2004). The remaining MH probands analysed did not harbour the alteration. As presented in Figure 4.88, at nucleotide position 14477, two peaks of similar amplitude, representing two different bases, i.e. C and T, were detected. The two bases correlated to distinct codons, which code for different amino acids namely, Thr and Ile, respectively.

Figure 4.88 Representative electropherogram indicating the Thr4826Ile alteration observed in exon 100



A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Thr4826Ile at nucleotide 14477.

Brown *et al.* (2000) first reported this alteration in a large Maori pedigree, of which five individuals that experienced clinical episodes of MH harboured the alteration. The author indicated that this alteration was conserved and was not detected in 220 unaffected individuals. In addition, functional characterisation studies have indicated that the alteration results in a channel that is hypersensitive (Yang *et al.*, 2003), thus the alteration meets the criteria for a causative mutation. The alteration was subsequently identified in nine unrelated MH families from the UK (Halsall and Robinson, 2004). The relatively high frequency of this mutation in two different populations and its presence in a single South African MH pedigree indicates that the third mutation hotspot of the RYR1 plays an important role and should be analysed in all MH populations in the future.

As the Thr4826Ile alteration was detected for individual MH113-14, two other family members, MH113-2 (MH00369) and MH113-11 (MH00378), were subsequently screened. The MH status of both individuals MH113-2 and MH113-11 has not been confirmed. Both

individuals yielded a positive result for this mutation and harboured the heterozygous genotype. In view of the autosomal dominant nature of this disorder, it was concluded that all three individuals are MHS. Further analysis of the remaining members of family MH113 would have to be conducted in order to determine their MH status.

4.9.11 Exon 101 of the RYR1 gene

The amplified region resides in the C-terminal of the RYR1 (representing a functionally significant domain), as discussed in Section 2.11.3.3.3 (page 58). Previously reported alterations that reside in exon 101 are listed in Table 4.10.

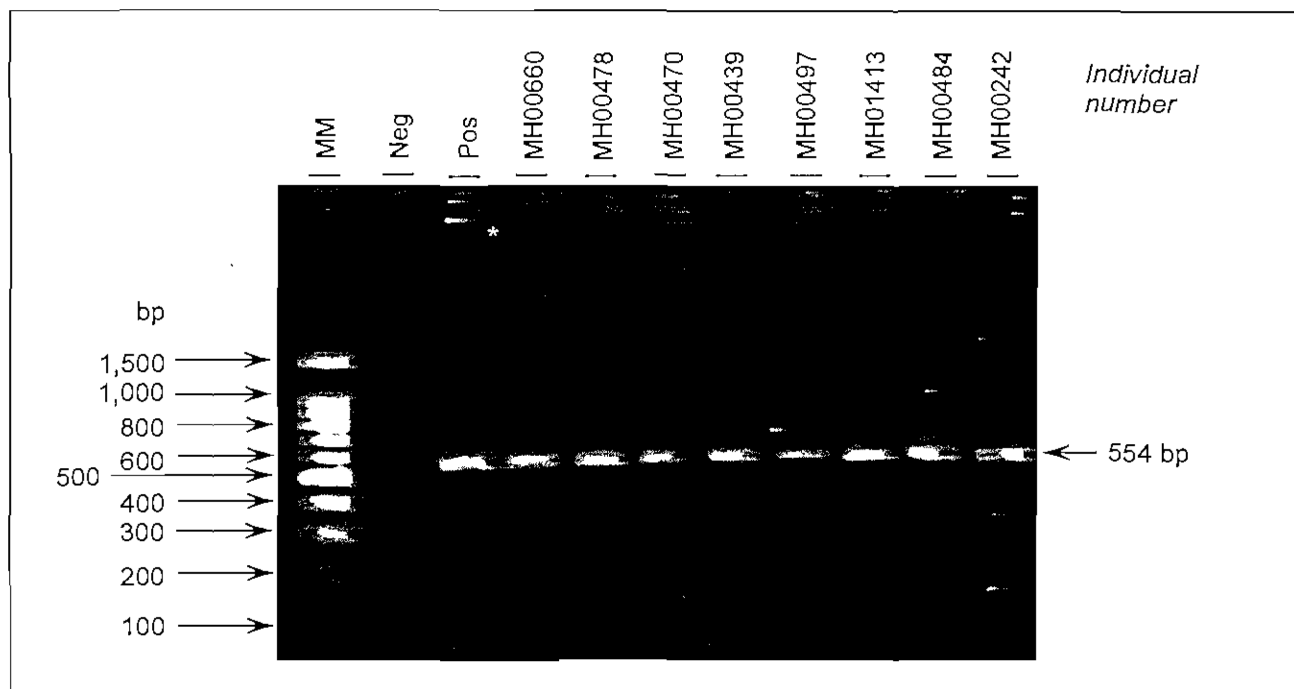
Table 4.10: Reported alterations in exon 101 of the RYR1 gene

Amino acid change	Nucleotide change	Reference	Amino acid change	Nucleotide change	Reference
Leu4838Val	C14512G	Halsall and Robinson, 2004	Arg4861Cys[1]	C14581T	Wu *et al.*, 2006
Ala4846Val[1]	C14537T	Kossugue *et al.*, 2005	Arg4861His[1]	G14582A	Monnier *et al.*, 2001
Val4849Ile	G14545A	Halsall and Robinson, 2004	Tyr4864Cys[1]	A14591G	Zorzato *et al.*, 2003
Asn4858Asp[1]	A14572G	Wu *et al.*, 2006	Lys4876Arg	A14627G	Sambuughin *et al.*, 2005
Phe4860del[1]	14578del	Monnier *et al.*, 2001	Met4880Thr	T14639C	Sambuughin *et al.*, 2005

del = deletion; [1] = indicates alterations observed in probands clinically diagnosed with CCD.

PCR was used to amplify a 554 bp product of exon 101 for 39 MH probands in Phase 2. In the Phase 3 study, an additional two MH probands (MH01626 and MH01394) were amplified and analysed. PCR conditions were optimised as discussed in Section 4.2 (page 159) and the results are illustrated in Figure 4.89.

Figure 4.89: Photographic representation of amplified PCR products encompassing exon 101



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Several artefacts including an artefact in the gel matrix, as indicated by the white asterisk (*), fragment distortion, variation in amplification efficiency and MM overloading were observed, as discussed in Sections 4.2 and 4.3.

The 41 successfully amplified samples obtained via PCR for Phase 2 and Phase 3 were subsequently sequenced using the standard protocol. A representative electropherogram obtained for individual MH01394, depicting the nucleotide positions of reported alterations in exon 101 of the RYR1 gene, is presented in Figure 4.90.

Figure 4.90: Representative electropherogram of exon 101 indicating the nucleotide positions of the Leu4838Val, Ala4846Val, Val4849Ile, Asn4858Asp, Phe4860del, Arg4861Cys, Arg4861His, Tyr4864Cys, Lys4876Arg and Met4880Thr alterations

Individual MH01394

A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Leu4838Val at nucleotide 14512; Ala4846Val at nucleotide 14537; Val4849Ile at nucleotide 14545; Asn4858Asp at nucleotide 14572; Phe4860del at nucleotides 14578-14580; Arg4861Cys at nucleotide 14581; Arg4861His at nucleotide 14482; Tyr4864Cys at nucleotide 14591; Lys4876Arg at nucleotide 14627 and Met4880Thr at nucleotide 14639.

None of the South African MH individuals that were sequenced in Phase 2 and Phase 3 harboured any reported alterations, or presented with any novel alterations. The Leu4838Val alteration was reported in a single UK pedigree with MH (Halsall and Robinson, 2004). The Lys4876Arg alteration has thus far only been identified in one MHS individual from France (Monnier *et al.*, 2005), and the Met4880Thr alteration was only

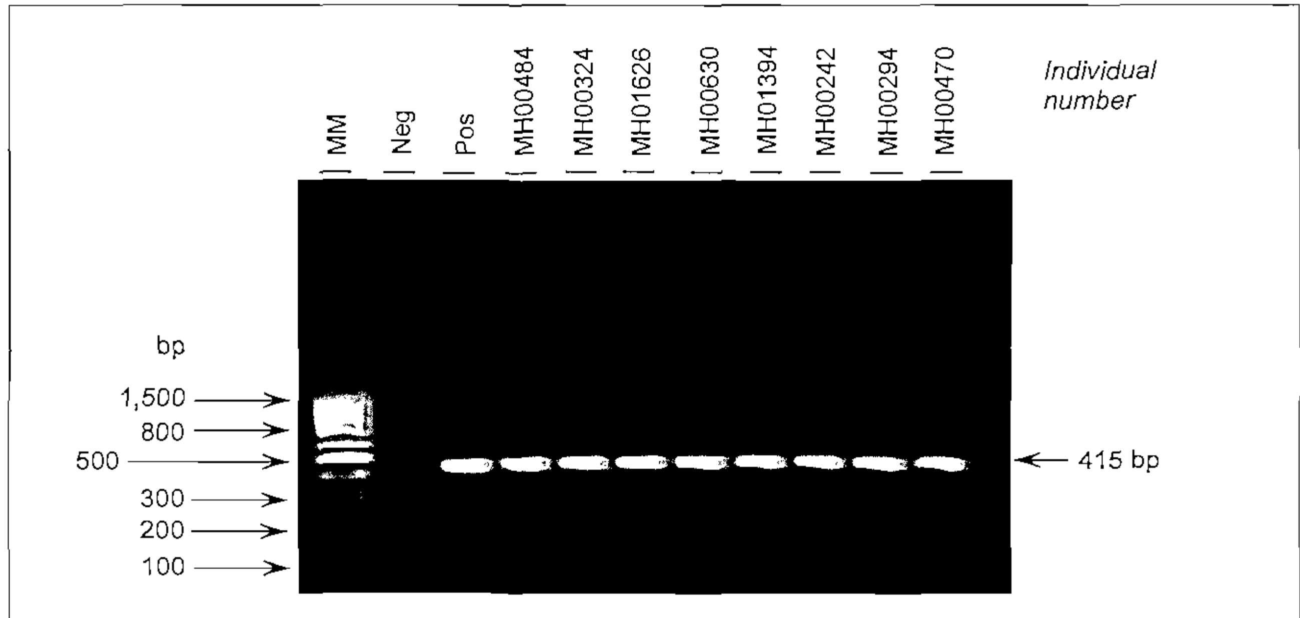
observed recently in a single MHS individual from North America (Sambuughin *et al.*, 2005). However, it is currently unknown whether these alterations are specific to those families or if they occur more frequently. The Val4849Ile alteration was recently reported in four unrelated MH pedigrees from the UK (Halsall and Robinson, 2004) and the frequency of this alteration in the UK population was determined to be 0.92. Therefore the Val4849Ile alteration does not represent a family-specific mutation. As the alteration has been reported in several families from the UK, it is likely that it may play a role in the development of the MH phenotype in the South African population. However, to date South African families harbouring this alteration have not been observed.

The Arg4861His alteration was first reported by Monnier *et al.* (2001) and was detected in three unrelated CCD pedigrees. It is observed in a highly conserved region of the RYR1 gene. The Arg4861His alteration was also detected in a single CCD pedigree, presenting with complete segregation in all 27 affected individuals investigated, but was absent in unaffected individuals. Three members of the family were also subsequently diagnosed as MHS via an IVCT. However, none of the individuals experienced a fulminant MH reaction under anaesthesia (Davis *et al.*, 2003). An association between MH and CCD was first reported by Denborough *et al.* (1973), as discussed in Section 2.4 (page 11). The alteration was subsequently reported in one MH family from the UK (Halsall and Robinson, 2004) and is currently being used for the genetic diagnosis of MH susceptibility. Although the alteration was not detected in any South African MH probands analysed, several independent genetic factors may predispose an individual to MH and/or CCD, as discussed in Section 5.2.2 (page 399). This observation could explain why the alteration was detected in individuals with both disorders. All individuals had a mild form of MH during anaesthetic procedures, as discussed in Section 2.2 (page 6). Analysis of all these factors may identify genetic determinants involved in susceptibility to MH in the South African population.

4.9.12 Exon 102 of the RYR1 gene

Thus far, one alteration resulting in the MH phenotype has been reported in exon 102. Ibarra *et al.* (2006) observed the Ala4894Thr alteration in one MH family due to a G14680A nucleotide transition. Five alterations have been observed in this exon in probands diagnosed with CCD, as discussed in Section 3.7.72 (page 153), which resides in hotspot three of the RYR1 gene. Genomic DNA was amplified via PCR as discussed in Section 4.2 (page 159) and the results are presented in Figure 4.91.

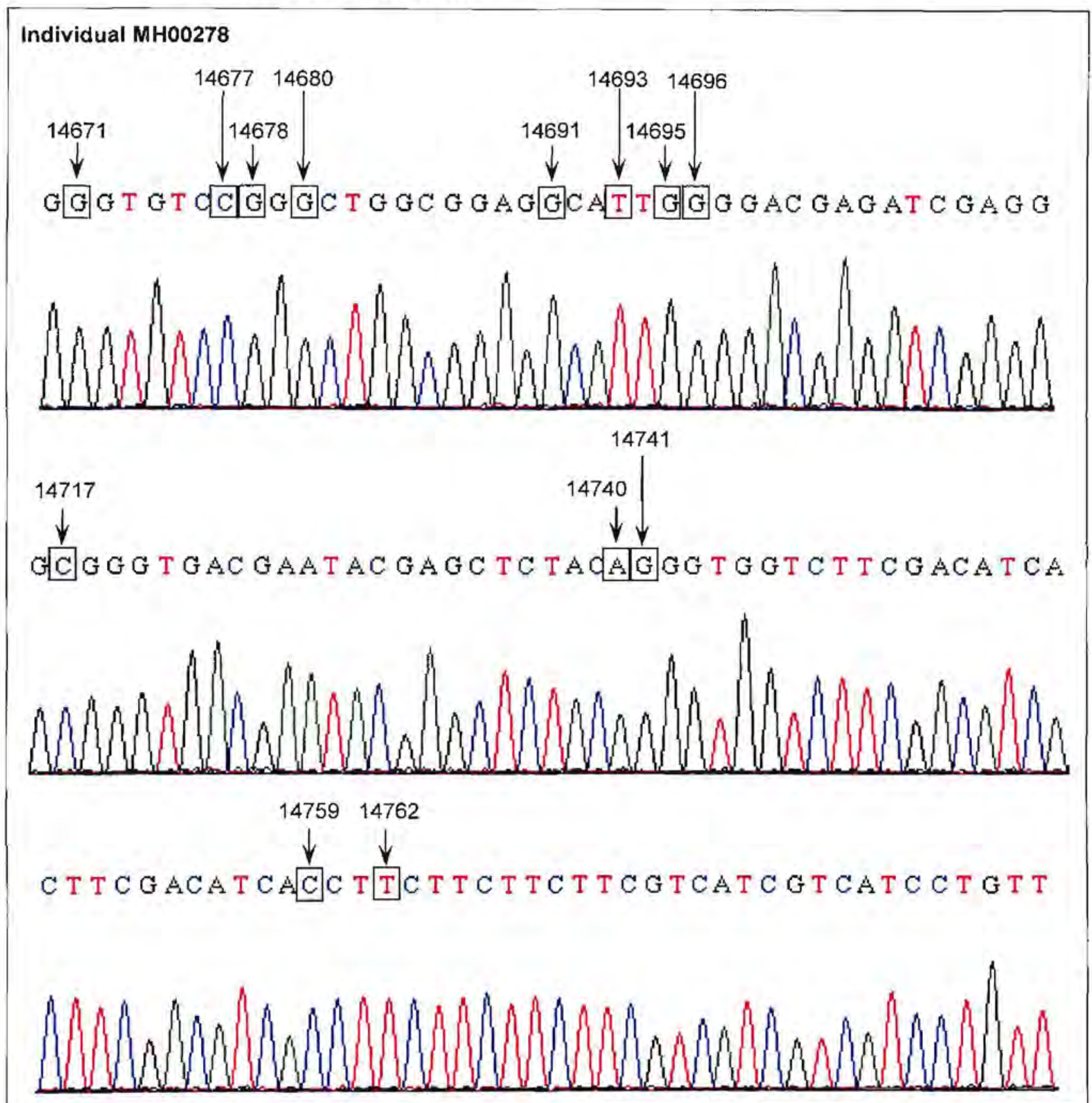
Figure 4.91: Photographic representation of amplified PCR products encompassing exon 102



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Fragment distortion, MM overloading and non-specific amplification were observed, as discussed in Sections 4.2 and 4.3.

Figure 4.92 indicates the sequence generated for individual MH00278. Alterations that have thus far been reported to occur in this region of the RYR1 gene are indicated. Sequencing was conducted using the reverse primer (RYRex102R) and for this reason sequences are depicted as the reverse complement. The Ala4894Thr alteration was not observed in any of the MH probands analysed in Phase 3. As the mutation has only been observed in a single family from Japan (Ibarra *et al.*, 2006), this may indicate that it is family-specific.

Figure 4.92: Representative electropherogram of exon 102 indicating the nucleotide positions of the Gly4891Arg, Arg4893Trp, Arg4893Gln, Arg4893Pro, Ala4894Thr, Gly4897Val, Ile4898Thr, Gly4899Arg, Gly4899Glu, Ala4906Val, Arg4914Gly, Arg4914Thr, Thr4920Asn and Phe4921Ser alterations



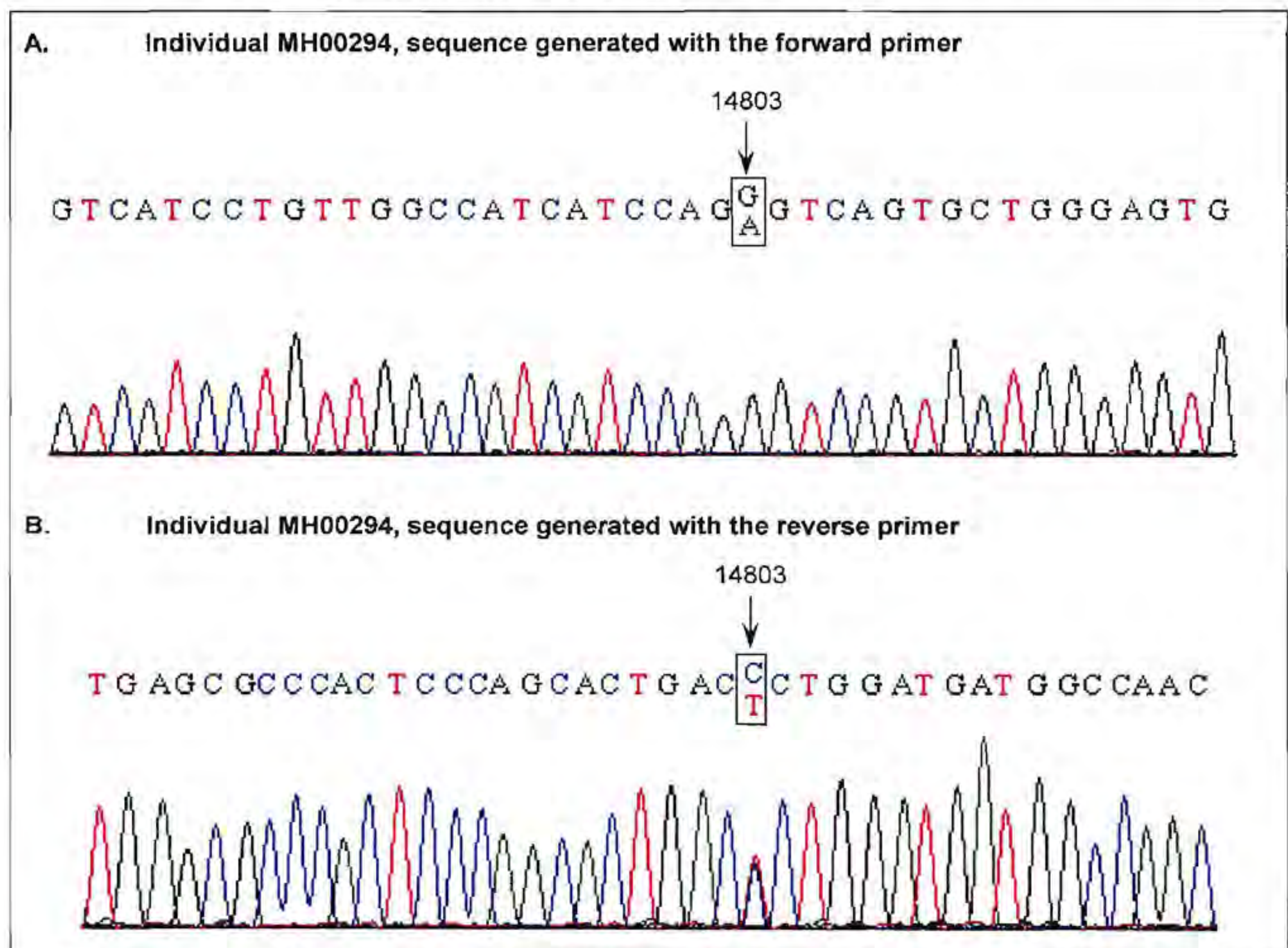
A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Gly4891Arg at nucleotide 14671; Arg4893Trp and Arg4893Gln at nucleotide position 14677; Arg4893Pro at nucleotide 14678; Ala4894Thr at nucleotide 14680; Gly4897Val at nucleotide 14691; Ile4898Thr at nucleotide 14693; Gly4899Arg at nucleotide 14695; Gly4899Glu at nucleotide 14696; Ala4906Val at nucleotide 14717; Arg4914Gly at nucleotide 14740; Arg4914Thr at nucleotide 14741; Thr4920Asn at nucleotide 14759 and Phe4921Ser at nucleotide 14762.

4.9.12.1 The Gly4935Ser alteration in the RYR1 gene

In one individual (MH00294), a single novel alteration was observed in exon 102 of the RYR1 gene. The Gly4935Ser alteration is due to a G14803A nucleotide transition and has

never been reported in any other population studied worldwide. The different bases encode two different amino acids, namely Gly and Ser. The sequence depicted on the electropherogram of Figure 4.93A indicates the alteration observed using the forward primer and similarly the sequence depicted in the electropherogram of Figure 4.93B indicates the alteration observed using the reverse primer. Sequencing in both the forward and reverse directions confirmed the presence of the Gly3935Ser alteration.

Figure 4.93: Representative electropherograms indicating the Gly4935Ser alteration observed in exon 102



A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Gly4935Ser at nucleotide 14803.

Table 4.11 depicts the partial amino acid sequence of exon 102 of the RyR1 protein. Multiple sequence alignments encompassing the nucleotide position of the Gly4935Ser alteration were retrieved from GenBank® (P21817, Q92736, NP_001027, P16960 and P11716). This region of the RyR1 protein is highly conserved and harbours several alterations associated with CCD. The region also exhibits homology to the $InsP_3R$ and is highly conserved through evolution and among different RyR1 species.

Table 4.11: Conserved amino acids obtained from different RyR isoforms and species surrounding the novel and reported mutations in exon 102 of the RyR1 protein

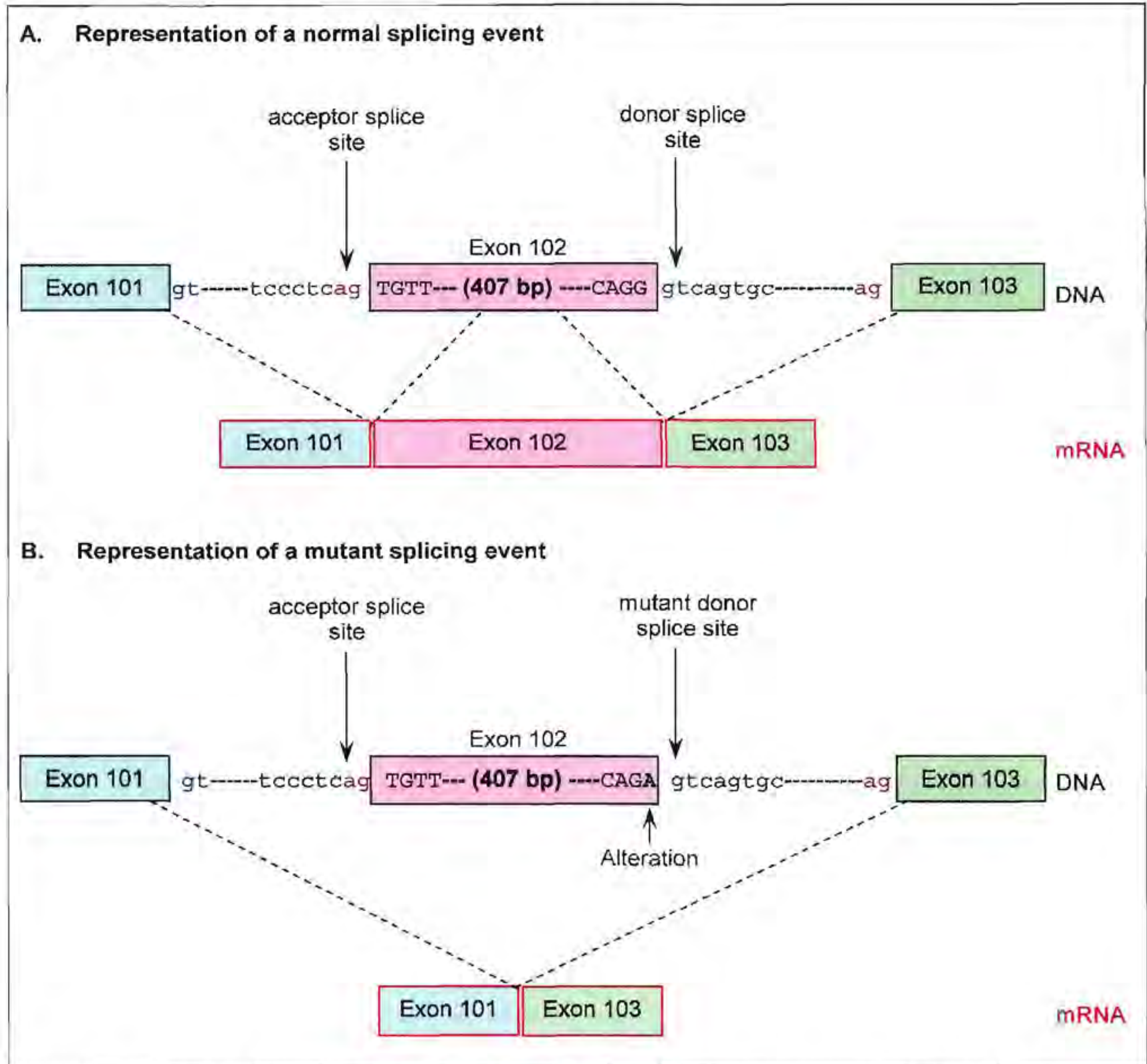
Isoform	Species	Alignment of RyR protein sequences
RyR1	Human	EIEDPAGDEY ELYRVVFDIT FFFFVIVILL AIIQGLIIDA FGELRDQQEQ VKEDMET
RyR2	Human	EIEDPAGDEY EIYRIIFDIT FFFFVIVILL AIIQGLIIDA FGELRDQQEQ VKEDMET
RyR3	Human	EIEDPAGDPY EMYRIVFDIT FFFFVIVILL AIIQGLIIDA FGELRDQQEQ VREDMET
*ryr*1	Pig	EIEDPAGDEY ELYRVVFDIT FFFFVIVILL AIIQGLIIDA FGELRDQQEQ VREDMET
*ryr*1	Rabbit	EIEDPAGDEY ELYRVVFDIT FFFFVIVILL AIIQGLIIDA FGELRDQQEQ VKEDMET

RyR1 = RyR expressed in human skeletal muscle; RyR2 = RyR expressed in human cardiac muscle; RyR3 = RyR expressed in human brain; *ryr1* = RyR1 protein expressed in animals. Amino acid residues that are not conserved among different isoforms and species are highlighted in grey. The site of the conserved reported Gly amino acid at nucleotide 14803 which was observed in the study presented here is indicated in blue. The sites at which previously reported alterations that occur in the depicted region are indicated: Ala4906Val = red; Arg4914Gly or Arg4914Thr = green; Thr4920Asn = pink and Phe4921Ser = orange. The accession numbers are as follows: RyR1 human = P21817; RyR2 human = Q92736; RyR3 human = NP_001027; *ryr*1 pig = P16960 and *ryr*1 rabbit = P11716.

The Gly4935Ser alteration observed in exon 102 occurs adjacent to the 5' donor splice site (`gt`). Studies have indicated that the exon sequence plays an important role during splice site selection. Sadusky *et al.* (2004) indicated that the intron splice site sequence is flanked by a partially conserved exon sequence. Reed and Maniatis (1986) identified deletions and substitutions in exon sequences that could alter the pattern of splice-site selection, which resulted in the splice site adjacent to the altered exon not being used. The alteration of a G-to-A nucleotide could result in the elimination of critical G:C base pairing between exon 102 and the uridine-rich small nuclear RNA (snRNA) which binds to the 5' splice site. The weakening of the interaction between the exon and the snRNA will result in a substantial loss of the splicing signal. However, as the splicing signal is not completely destroyed this will result in a mixture of both full-length and shortened mRNA species from the primary transcript. A schematic representation of the proposed abnormal splicing at the intron-exon boundary of exon 102 is illustrated in Figure 4.94.

A similar occurrence has been reported by Huang *et al.* (1993) in the human glycophorin A gene. The authors described a single G-to-A transition in the terminal position of exon III at position -1 that resulted in partial inactivation of the adjacent 5' splice site, which caused skipping of various exons and the alternative use of other constitutive splice sites. Their study demonstrated that constitutive splicing could be converted into alternative splicing in the presence of a single splice site mutation.

Figure 4.94: A schematic representation of the normal and abnormal splicing at the intron-exon boundary of exon 102



Adapted from Monnier *et al.* (2003). The nucleotide that is altered is indicated in green and may result in the loss of a donor splice site.

Thus far, a mutation in the splice site in an exon of the RYR1 gene has not been reported to be associated with the MH phenotype in any other population. However, Monnier *et al.* (2003) identified a 14646+2.99 kb A-to-G mutation associated with the classical form of MmD with ophthalmoplegia within the intron sequence between exons 101 and 102. The alteration created a cryptic splice site and resulted in the insertion of a 119 bp out-of-frame intronic fragment in the cDNA. The homozygous proband harboured both mutated and normal transcripts, which could be due to competition between the normal and cryptic splice sites. The authors suggested that the expression of the cryptic splicing mutation is tissue-specific, because of the varying nature of the specific splicing machinery present in a given cell type. In addition, Rueffert *et al.* (2000) identified a novel polymorphism in the

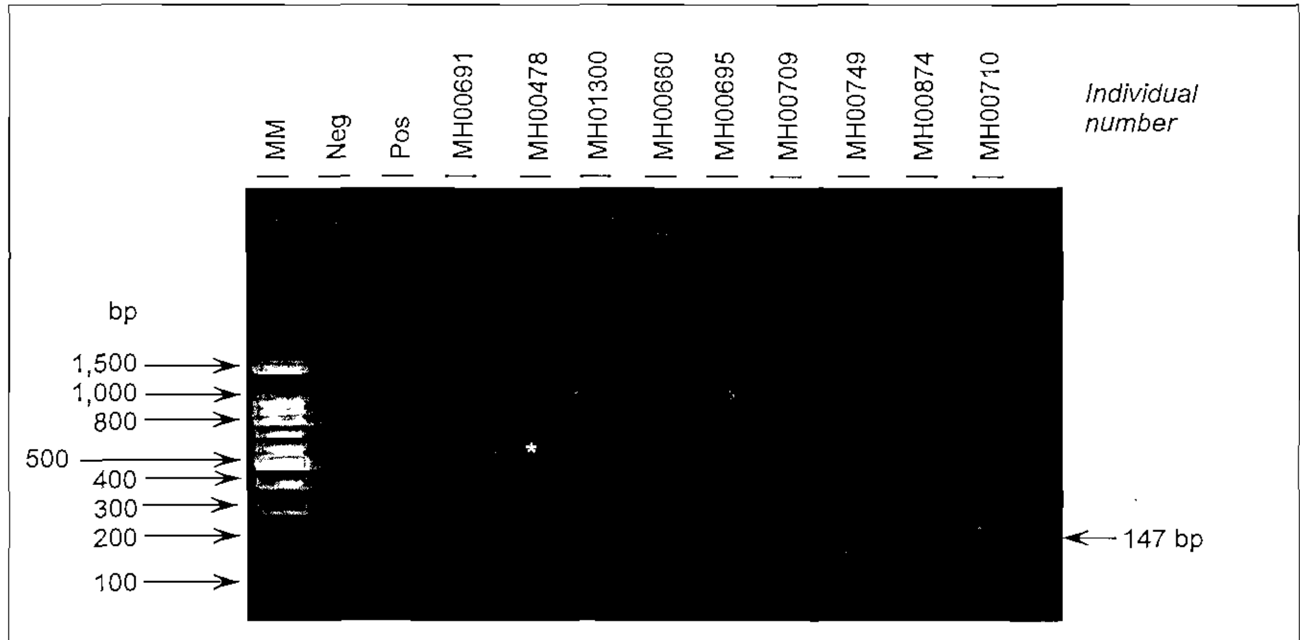
splice site of intron 45 of the RYR1 gene. However, the authors indicated that the transversion in the splice donor site did not result in MHS, as the base change was observed in 14 MHS probands and 16 out of 120 unaffected chromosomes. Statistical analysis indicated that there was no difference in frequency between the MHS and MHN group.

The Gly4935Ser alteration was, however, not observed in any other family members of this pedigree, which suggests that it arose as a spontaneous mutation in individual MH00294 and was not inherited. These results suggest that a second alteration may play a role in the development of this disorder in pedigree MH104. Since the Gly4935Ser alteration was not observed in any other family members, DNA from MH00294 was re-amplified and sequenced to verify the result. The alteration was again observed in the re-amplified sequence, confirming the original result. As the daughter of individual MH00294 died during dental surgery when she was two years old from MH, it is unknown whether she inherited the alteration, as DNA was not obtained from this individual. Although the alteration did not segregate with the MH phenotype, it may still play a role in the development of MH. Causative alterations associated with MH have been reported previously that do not segregate with the phenotype. Adeokun *et al.* (1997) reported that the Gly341Arg alteration was not causative of MH, as it did not exhibit complete co-segregation with the MH phenotype in one family. However, further analysis by Tong *et al.* (1997) confirmed the causative status of this alteration via functional analysis. Therefore, it is possible that a causative alteration may not co-segregate with MHS in all families that harbour a mutation of the RYR1 gene. The causative status of this alteration remains undetermined and should be confirmed via functional analysis and screening of unaffected chromosomes.

4.9.13 Exon 103 of the RYR1 gene

A region of 147 bp of exon 103 was sequenced in order to detect four alterations, known as Ile4938Met, Asp4939Glu (Halsall and Robinson, 2004), Ala4940Thr (Sambuughin *et al.*, 2005) and Gly4942Val (Galli *et al.*, 2002), as well as to identify any novel alterations that may occur in this region. The PCR conditions for exon 103 were optimised as discussed in Section 4.2 (page 159). PCR amplification was successful for 38 MH probands in Phase 2. An additional three MHS probands that have not previously been amplified were analysed for alterations in this exon in Phase 3. Figure 4.95 is a photographic representation of the amplicon encompassing exon 103.

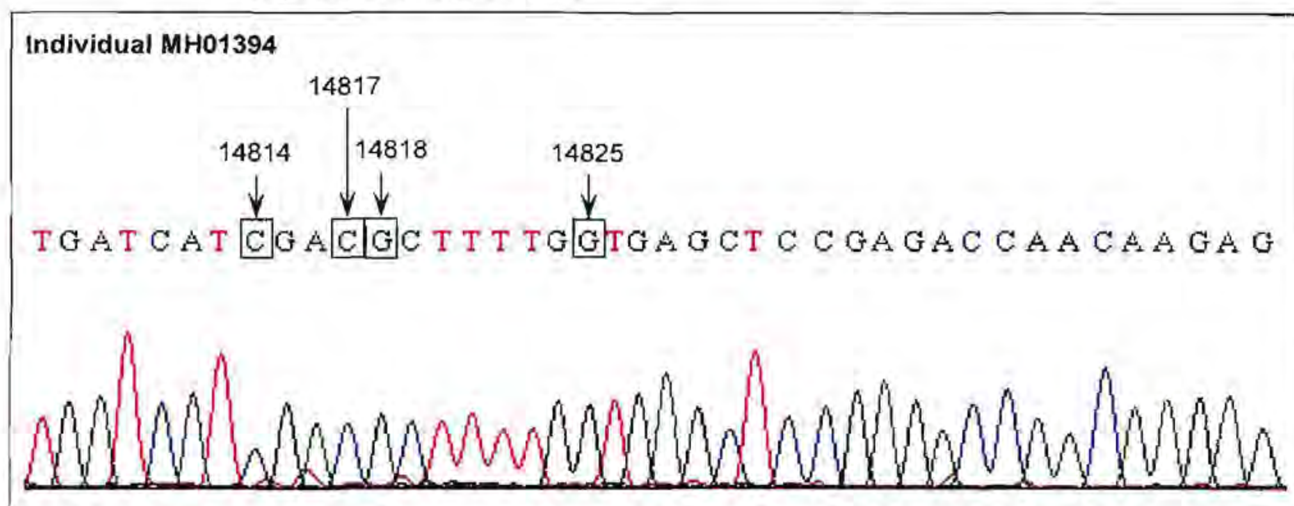
Figure 4.95 Photographic representation of amplified PCR products encompassing exon 103



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. An artefact in the gel matrix was detected, as indicated by the white asterisk (*), as discussed in Section 4.3. In addition, variation in exonic PCR efficiency was observed, as discussed in Section 4.2.

The amplified region was subsequently sequenced in order to investigate the presence of reported and novel alterations in this region of the RYR1 gene. Sequencing was conducted using the reverse primer (RYREx103R). The reverse primer was used, as two of the mutations are situated in close proximity to the location of the forward primer. Sequences are therefore illustrated as the reverse complement. All 41 samples analysed in Phase 2 and Phase 3 were amplified and sequenced successfully. A representative electropherogram obtained for individual MH01394, illustrating the nucleotide positions of previously reported alterations that occur in the amplified region of exon 103, is indicated in Figure 4.96.

Figure 4.96: Representative electropherogram of exon 103 indicating the nucleotide positions of the Ile4938Met, Asp4939Glu, Ala4940Thr and Gly4942Val alterations



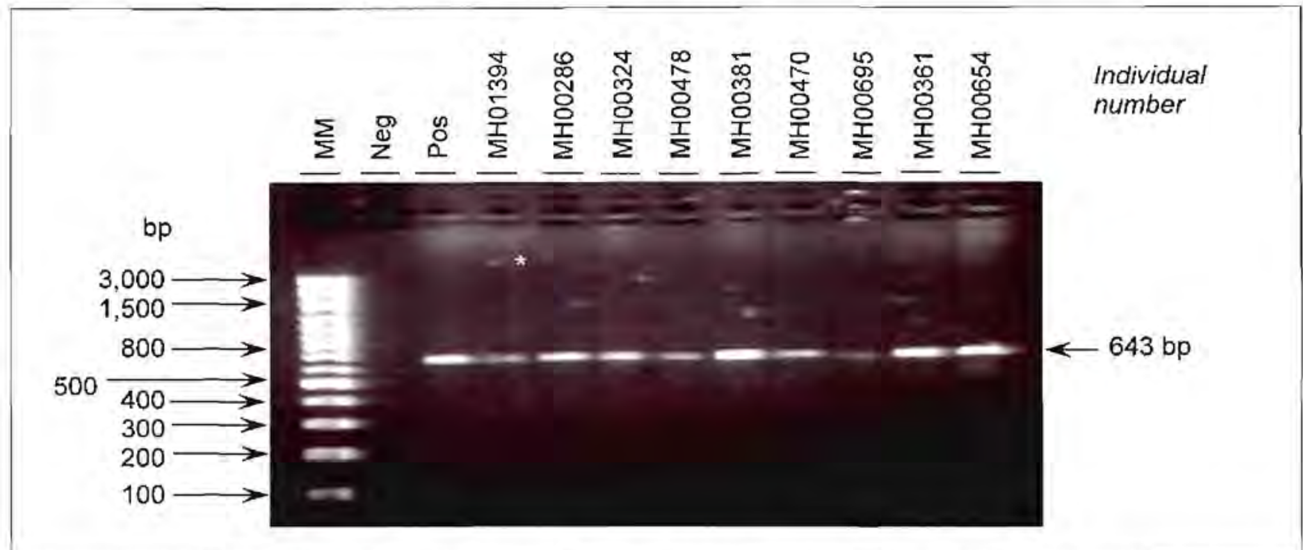
A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Ile4938Met at nucleotide 14814; Asp4939Glu at nucleotide 14817; Ala4940Thr at nucleotide 14818 and Gly4942Val at nucleotide 14825.

The Ile4938Met and Asp4939Glu alterations were reported in single UK pedigrees, respectively (Halsall and Robinson, 2004). In addition, the Ala4940Thr and Gly4942Val alterations have also only been reported in single MHS individuals. As the alterations have only been observed recently, the frequency of these alterations in other populations has not yet been determined, and could represent mutations that are private to those specific families. None of the 41 South African MH individuals that were sequenced in Phase 2 and Phase 3 harboured the Ile4938Met, Asp4939Glu, Ala4940Thr, Gly4942Val alterations or harboured any novel alterations in this region.

4.9.14 Exon 104 of the RYR1 gene

Exons 104 and 105 were simultaneously amplified and the PCR reaction was optimised as discussed in Section 4.2 (page 159). Thereafter, the PCR products were purified and sequenced. Figure 4.97 depicts the amplified products of exons 104 and 105. However, as exon 105 resides outside hotspot three, it is discussed in Section 4.10.46 (page 383). Thus far, two alterations have been reported in exon 104 that may be associated with the MH phenotype. Monnier *et al.* (2002) identified a Pro4973Leu alteration in a single MH pedigree from France. Ibarra *et al.* (2006) reported a Phe4960Tyr alteration in one MH family from Japan.

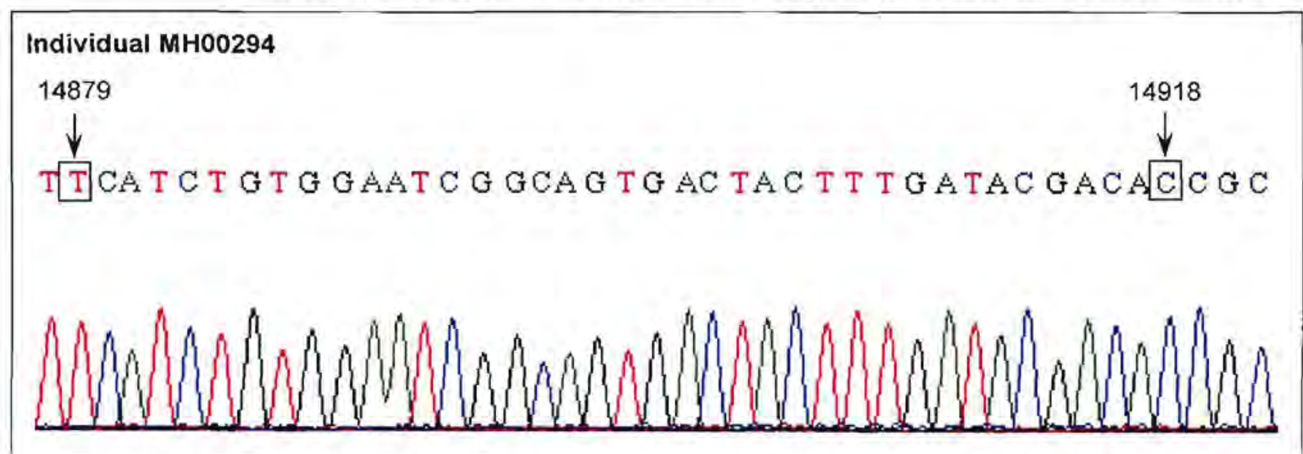
Figure 4.97: Photographic representation of amplified PCR products encompassing exons 104 and 105



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Variation in amplification efficiency, an artefact in the gel matrix, as indicated by the white asterisk (*) and overloaded MM were noted, as discussed in Sections 4.2 and 4.3.

Fifteen individuals were successfully screened for nucleotide substitutions in exon 104. A representative result generated via automated sequencing is presented in Figure 4.98, which illustrates the positions of the Phe4960Tyr and Pro4973Leu reported to occur in exon 104.

Figure 4.98: Representative electropherogram of exon 104 indicating the nucleotide positions of the Phe4960Tyr and Pro4973Leu alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Phe4960Tyr at nucleotide 14879 and Pro4973Leu at nucleotide 14918.

None of the 15 South African MH individuals harboured the Pro4973Leu or Phe4960Tyr alterations, or presented with any novel alterations. Both the Pro4973Leu and Phe4960Tyr alterations were reported in single pedigrees with MH (Monnier *et al.*, 2002; Ibarra *et al.*, 2006). Therefore, both alterations may be private to those specific families.

4.10 EXONS LOCATED OUTSIDE THE HOTSPOTS OF THE RYR1 GENE

Comprehensive screening for mutations in all exons of the RYR1 gene has led to a higher detection rate of mutations in both MH and CCD probands (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006). Thus far, an additional 38 alterations associated with either MH or CCD have been reported to occur outside the mutational hotspots, as discussed in Section 2.11.3.3.4 (page 62). Phase 3 therefore represents the first study in which all exons that reside outside the mutational hotspots were screened in order to identify alterations that may result in MHS in the South African population. These regions of the RYR1 gene were screened in order to estimate the distribution of RYR1 mutations in the South African MH population.

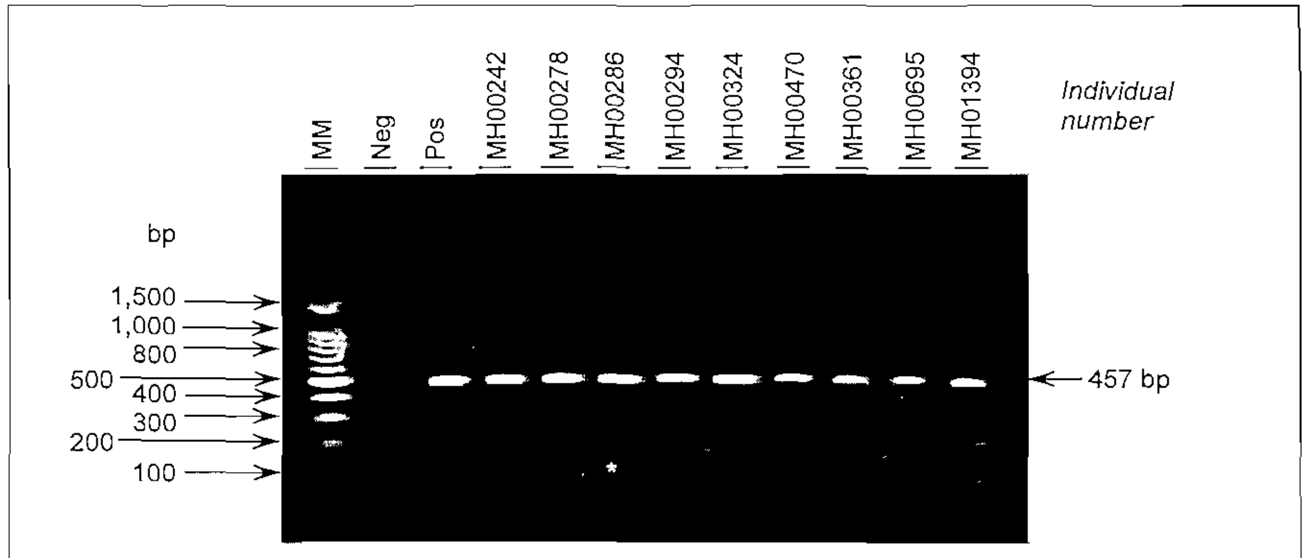
As functional domains outside the three hotspots have been described, it is likely that other regions of the RYR1 gene, if mutated, may result in MHS (Dulhunty and Pouliquin, 2003). As discussed in Section 2.9 (page 22), amino acids 1 - 1680 of the RyR1, which are encoded by exons 1 - 34, hold critical determinants for E-C coupling (Perez *et al.*, 2003b). In addition, Proenza *et al.* (2002) indicated that a region encompassing residues 1837 - 2168, which are encoded by exons 35 to 39, interact with the II-III loop of the DHPR. Nakai *et al.* (1998) reported that amino acids 1681 - 3770, which are encoded by exons 34 to 79, contain critical components of the RyR1 protein that are required to restore bi-directional signalling between the RyR1 and DHPR. Exon 35 encodes residues that form the low affinity Ca^{2+} binding site and exon 74 encodes the residues that harbour a CaM binding site (Menegazzi *et al.*, 1994). In the study presented here, an additional three alterations that reside outside the three hotspots were observed in six South African MH probands. The alterations occurred in exons 34, 38 and 73. The remaining analysed exons did not harbour any alterations associated with MH. However, several SNPs were detected in these regions of the RYR1 gene, as listed in Appendices B and C (page 447 and 451). A summary of mutations obtained for MH individuals analysed in Phase 3 is listed in Table 4.17 (page 387). The results obtained for each exon region outside the mutational hotspots of the RYR1 gene that was screened are described and discussed separately in the subsequent sections of this chapter.

4.10.1 Exon 1 of the RYR1 gene

A 457 bp region was amplified using conditions listed in Table 4.1 (page 161). The results of the amplified PCR product encompassing exon 1 are presented in Figure 4.99.

Successful amplification was achieved in all 15 MH samples obtained from South African probands.

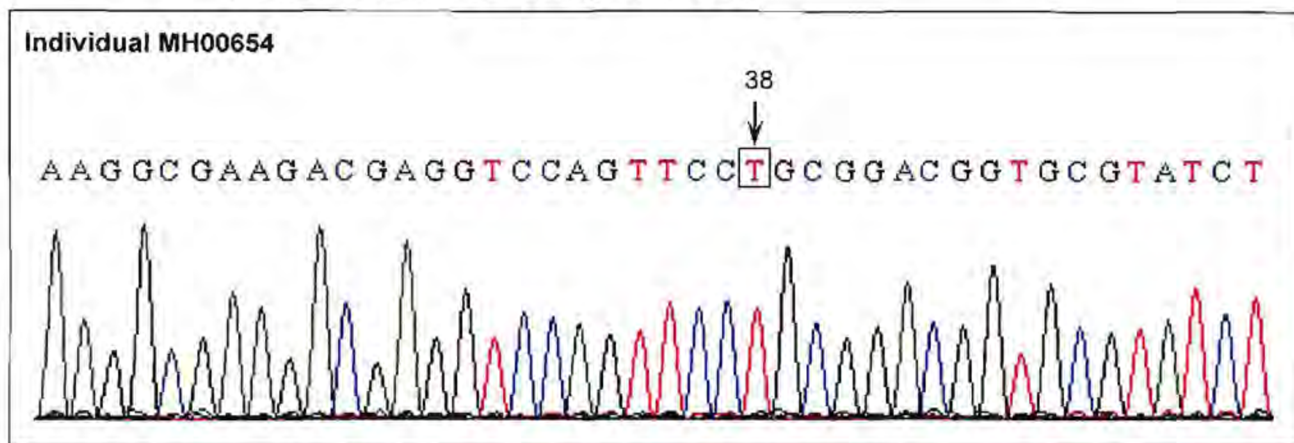
Figure 4.99: Photographic representation of amplified PCR products encompassing exon 1



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure and appears overloaded, as discussed in Section 4.2; Neg = negative control; Pos = positive control. An artefact in the gel matrix, as indicated by the white asterisk (*), non-specific amplification, fragment smear, variation in PCR efficiency, fragment and MM distortion were observed, as discussed in Sections 4.2 and 4.3.

Exon 1 has been reported to harbour one alteration that has previously been identified in one individual diagnosed with MH from Japan, namely the Leu13Arg alteration (Ibarra *et al.*, 2006). This exon resides adjacent to the first mutational hotspot. Due to the identification of a single alteration and the possibility of additional unidentified mutations that may be associated with MH in exon 1, the entire exon was amplified and subsequently sequenced. A representative result obtained for individual MH00654 generated via automated sequencing, illustrating the nucleotide position of the reported Leu13Arg alteration, is presented in Figure 4.100. Sequencing was conducted using the forward primer (RYRex1F).

Figure 4.100: Representative electropherogram of exon 1 indicating the nucleotide position of the Leu13Arg alteration



A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Leu13Arg at nucleotide 38.

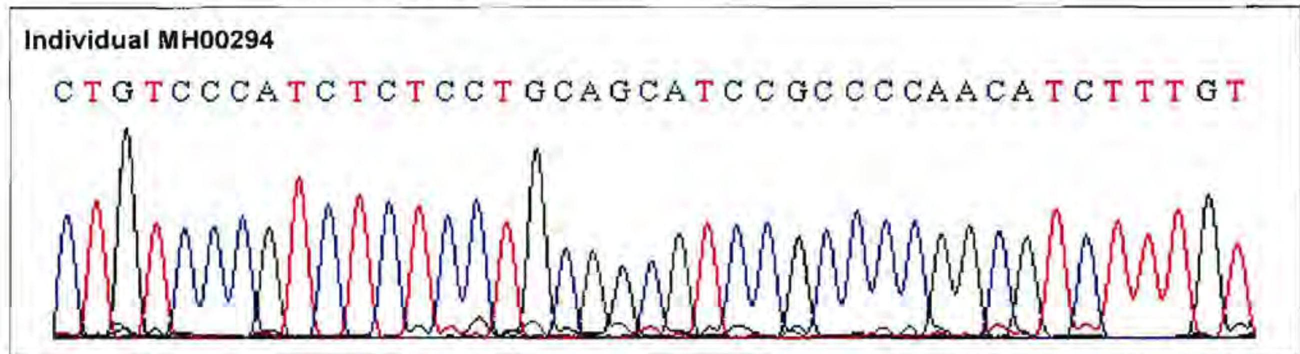
None of the 15 South African MH probands harboured any novel or reported alterations in exon 1 of the RYR1 gene. The Leu13Arg alteration that was previously identified in this exon has only been reported in a single family and may represent a family-specific alteration. However, exons outside the RYR1 hotspots have only recently been screened for alterations that may result in the MH phenotype (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006), as discussed in Section 4.10 (page 273). Therefore, the frequency of the Leu13Arg alteration in various populations is currently unknown. The causative status of the Leu13Arg alteration has not been determined, as discussed in Section 5.6 (page 413). Functional characterisation of this alteration may indicate that it plays a role in the development of MHS. In addition, as exon 1 encodes amino acids that play a role in E-C coupling (Perez *et al.*, 2003b), it may harbour additional alterations that could be responsible for or contribute to the MH phenotype.

4.10.2 Exon 18 of the RYR1 gene

Exon 18 does not currently harbour any reported alterations associated with MHS and is observed adjacent to hotspot one. Exons 17 and 18 were simultaneously amplified via PCR, as discussed in Section 4.7.10 (page 204) and the results of the amplified PCR product encompassing exons 17 and 18 are depicted in Figure 4.43 (page 205). The amplified region for all 15 MH probands was subsequently sequenced in order to investigate the presence of novel alterations in this region. Sequencing was conducted using the standard protocol and the forward primer (RYRex17F) was used in the

sequencing reaction. A representative electropherogram obtained for individual MH00294 illustrating a portion of the amplified region for exon 18, is depicted in Figure 4.101.

Figure 4.101: Representative electropherogram illustrating a portion of the amplified region of exon 18



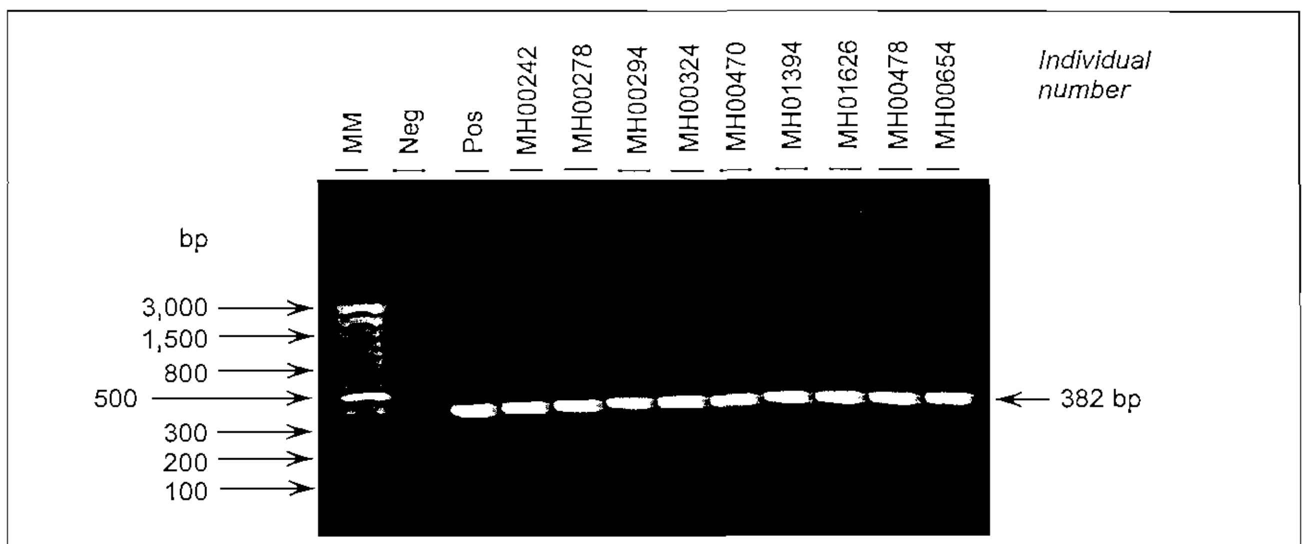
A = adenine; C = cytosine; G = guanine; T = thymine. Low-level background peaks were observed, as discussed in Section 4.5.

Thus far, screening of all 106 exons in MH probands from North America, Italy, France and Japan (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006) has not identified alterations associated with the MH phenotype in exon 18 of the RYR1 gene. In addition, novel alterations associated with MH were not observed in any of the South African probands analysed in the study presented here. Therefore, this exon does not play a role in the development of MH in the population cohorts investigated. However, exon 18 resides in a region that supports E-C coupling (Perez *et al.*, 2003b), therefore alterations in this exon may have a functional effect on the RyR1 protein. In addition, only a limited number of individuals from certain populations have been screened for alterations that may reside in exon 18. Sambuughin *et al.* (2005) analysed a cohort of 30 North American MH probands for alterations in the RYR1 gene. Monnier *et al.* (2005) screened 133 families from either France or Italy and Galli *et al.* (2006) analysed the RYR1 gene in 50 Italian MHS individuals. In addition, Wu *et al.* (2006) screened 27 Japanese CCD probands and Ibarra *et al.* (2006) analysed 56 Japanese MHS probands for alterations that may occur in the RYR1 gene. As discussed in Section 4.8.2 (page 215), the absence of reported alterations in exon 18 may be due to the small number of individuals thus far screened worldwide for alterations that may result in the MH phenotype. In addition, due to the population-specific nature of the majority of alterations associated with MH, alterations that may result in the MH phenotype may not have been detected in exon 18, as only a limited number of populations have thus far been screened.

4.10.3 Exon 19 of the RYR1 gene

The PCR reaction was optimised as discussed in Section 4.2 (page 159) and the results are presented in Figure 4.102. Thus far, exon 19 does not harbour any reported alterations associated with MH susceptibility. However, a single Asn759Asp alteration has been reported in exon 19 of the RYR1 gene in one family diagnosed with CCD (Kossugue *et al.*, 2005). Sequencing was conducted using the reverse primer (RYRex19R) and sequences are illustrated as the reverse complement. A representative electropherogram obtained for individual MH00630, depicting the nucleotide position of the reported Asn759Asp alteration, is illustrated in Figure 4.103.

Figure 4.102: Photographic representation of amplified PCR products encompassing exon 19



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. As discussed in Sections 4.2 and 4.3, the fragments and MM appear distorted and a barrier in the agarose gel resulted in non-linear migration of the fragments.

None of the 15 MHS probands harboured any novel alterations in exon 19 of the RYR1 gene. In addition, as none of the South African probands were diagnosed with CCD, it would be expected that they did not harbour the Asn759Asp alteration. As discussed in Section 4.10.2 (page 275), analysis of this region of the RYR1 gene in a larger group of individuals from various populations would have to be conducted in order to determine whether alterations associated with the MH phenotype reside in exon 19.

Figure 4.103: Representative electropherogram of exon 19 indicating the nucleotide position of the Asn759Asp alteration

A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Asn759Asp at nucleotide 2274.

4.10.3.1 Synonymous substitutions in the amplified region of exon 19 of the RYR1 gene

Two SNPs were identified in the amplified region. The first SNP (T25990G) was observed in the intron sequence between exon 18 and 19 and the second SNP (C26165T) was identified in exon 19. Both synonymous substitutions have been reported as SNPs of the RYR1 gene in GenBank® and have been described to be in LD (International Human Genome Sequencing Consortium, 2004; with accession numbers rs4802474 and rs3745847, respectively). Thus far, LD between the T25990G and C26165T SNPs has been reported in populations from Europe, America, West Africa and Asia (International Human Genome Sequencing Consortium, 2004; with accession numbers rs4802474, and rs3745847, respectively). As discussed in Section 4.7.9.1 (page 202), the presence of LD between the two SNPs is based on the small physical distance between the SNPs. As discussed in Section 4.7.4.1 (page 185), SNPs observed in the RYR1 gene may have an impact on gene expression. In this manner, SNPs observed in the RYR1 gene may not be directly associated with MH, however, via epistasis a SNP may contribute a portion of the effect and may do so additively as well as interactively.

4.10.3.1.1 SNP T25990G

In the study presented here, the heterozygous T25990G SNP was detected in seven South African MH probands. In addition, the homozygous T25990G SNP was detected in four probands and the remaining four probands did not harbour the SNP, as listed in Appendix C (page 451). Figure 4.104 depicts the sequences generated for the

heterozygous and homozygous T25990G SNP, respectively. The frequency of the T25990G SNP has been determined in three different populations (International Human Genome Sequencing Consortium, 2004; with accession number rs4802474). In the European and Asian populations the frequency of the G/G genotype has been identified as 0.58 and 0.46 respectively, whereas in the African American group the frequency of the G/T genotype has been identified as 0.61 (International Human Genome Sequencing Consortium, 2004; with accession number rs4802474).

Figure 4.104: Representative electropherograms indicating the T25990G SNP observed in the intron sequence between exons 18 and 19 of the RYR1 gene

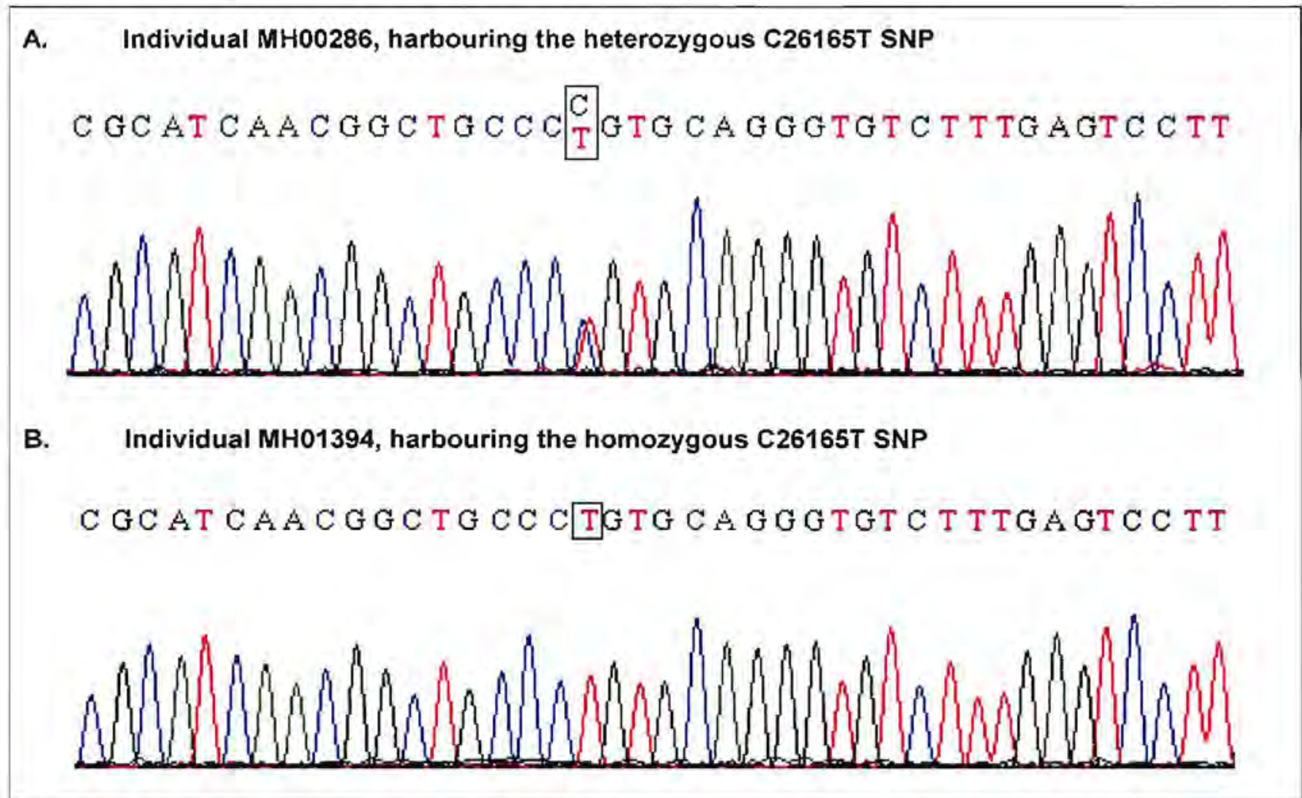
A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.3.1.2 SNP C26165T

The heterozygous C26165T SNP was observed in seven South African MH probands and the homozygous SNP was detected in four probands, in the study presented here, as listed in Appendix B (page 447). Figure 4.105 depicts the sequences generated for the heterozygous and homozygous C26165T SNP, respectively. The genotype frequencies for this alteration have been determined in three different populations (International Human Genome Sequencing Consortium, 2004; with accession number rs3745847). In the European, Asian and African American groups, the C/C genotype occurs least often with frequencies of 0.04, 0.17 and 0.22, respectively. In the African American population the

C/T genotype is the most common, with a frequency of 0.61 and in the European and Asian populations the T/T genotype is the most common and has the following genotype frequencies: 0.58 and 0.48, respectively (International Human Genome Sequencing Consortium, 2004; with accession number rs3745847).

Figure 4.105: Representative electropherograms indicating the C26165T SNP observed in exon 19 of the RYR1 gene

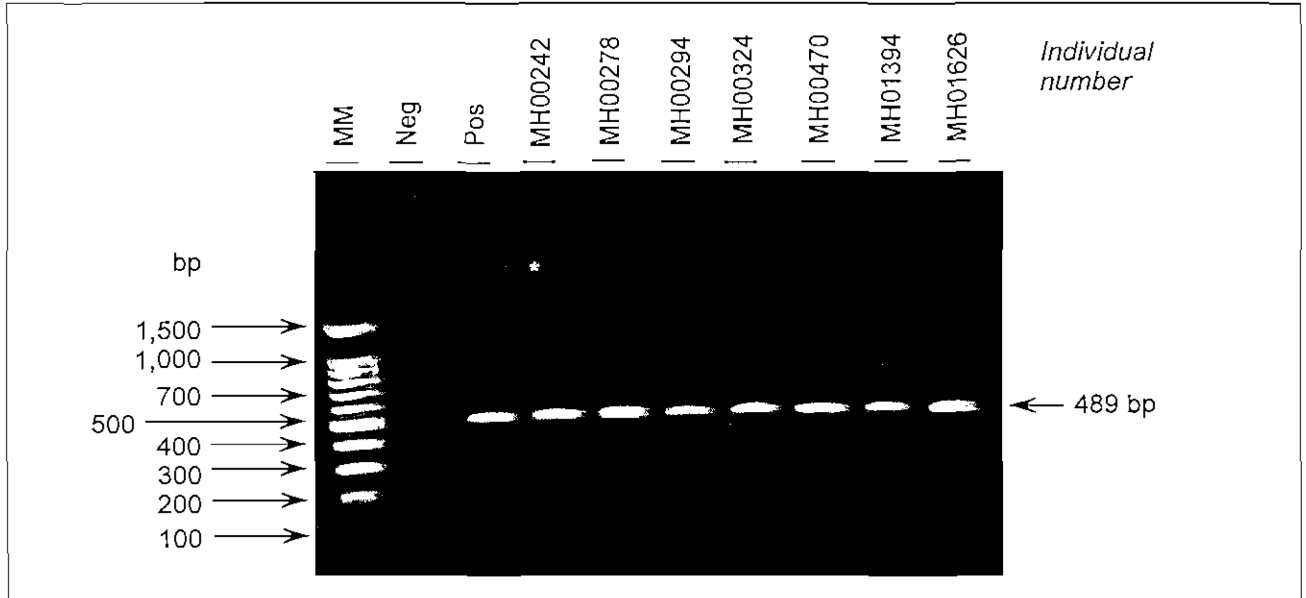


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.4 Exon 20 of the RYR1 gene

Thus far, exon 20 has not been reported to harbour any alterations that result in the MH phenotype and only a single SNP has been reported in GenBank® (International Human Genome Sequencing Consortium, 2004; with accession number rs2304147) to occur in this region of the RYR1 gene. Exon 20 resides outside the mutation hotspots in close proximity to hotspot one. In order to identify novel alterations that may occur in exon 20, a 489 bp region encompassing this exon was amplified, as discussed in Section 4.2 (page 159). Thereafter, the PCR product was purified (Figure 4.106) and sequenced according to the standard sequencing protocol. A representative result generated via automated sequencing, illustrating a portion of the amplified region of exon 20, is presented in Figure 4.107.

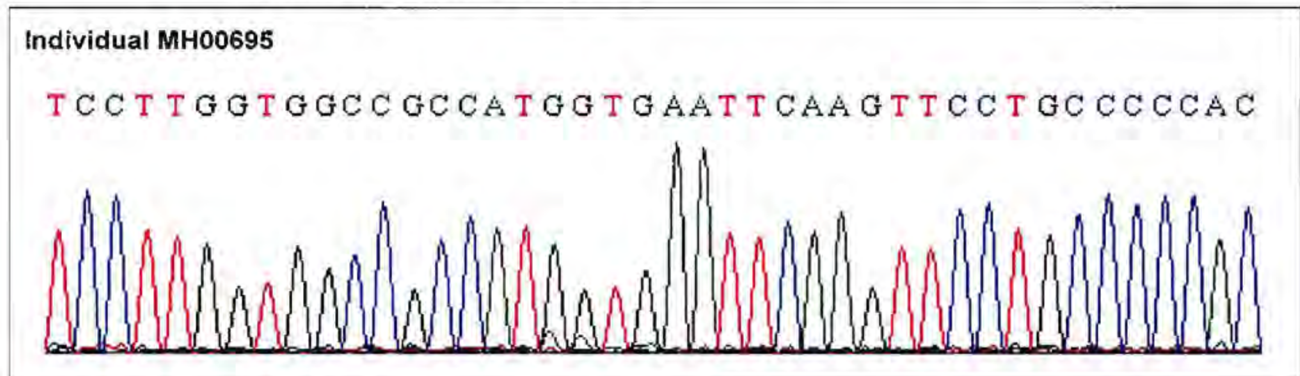
Figure 4.106: Photographic representation of amplified PCR products encompassing exon 20



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure and appears overloaded (Section 4.6); Neg = negative control; Pos = positive control. An artefact in the gel matrix, as indicated by the white asterisk (*), non-specific amplification, fragment distortion, overloaded MM and variation in amplification efficiency were observed, which may be attributed to reasons outlined in Sections 4.2 and 4.3.

Sequencing was conducted using the forward primer (RYRex20F). None of the 15 MHS probands analysed in the study presented here harboured any novel alterations in this region. As alterations associated with MH have not been reported in this exon in any population analysed thus far, it may indicate that this exon does not harbour mutations associated with MH. However, exons outside the RYR1 hotspots have only recently been screened for alterations that may result in the MH phenotype (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006), as discussed in Section 4.10 (page 273). It is only via further analysis that the exact role of this exon will be clarified in the context of the MH phenotype, as discussed in Section 4.10.2 (page 275).

Figure 4.107: Representative electropherogram illustrating a portion of the amplified region of exon 20



A = adenine; C = cytosine; G = guanine; T = thymine.

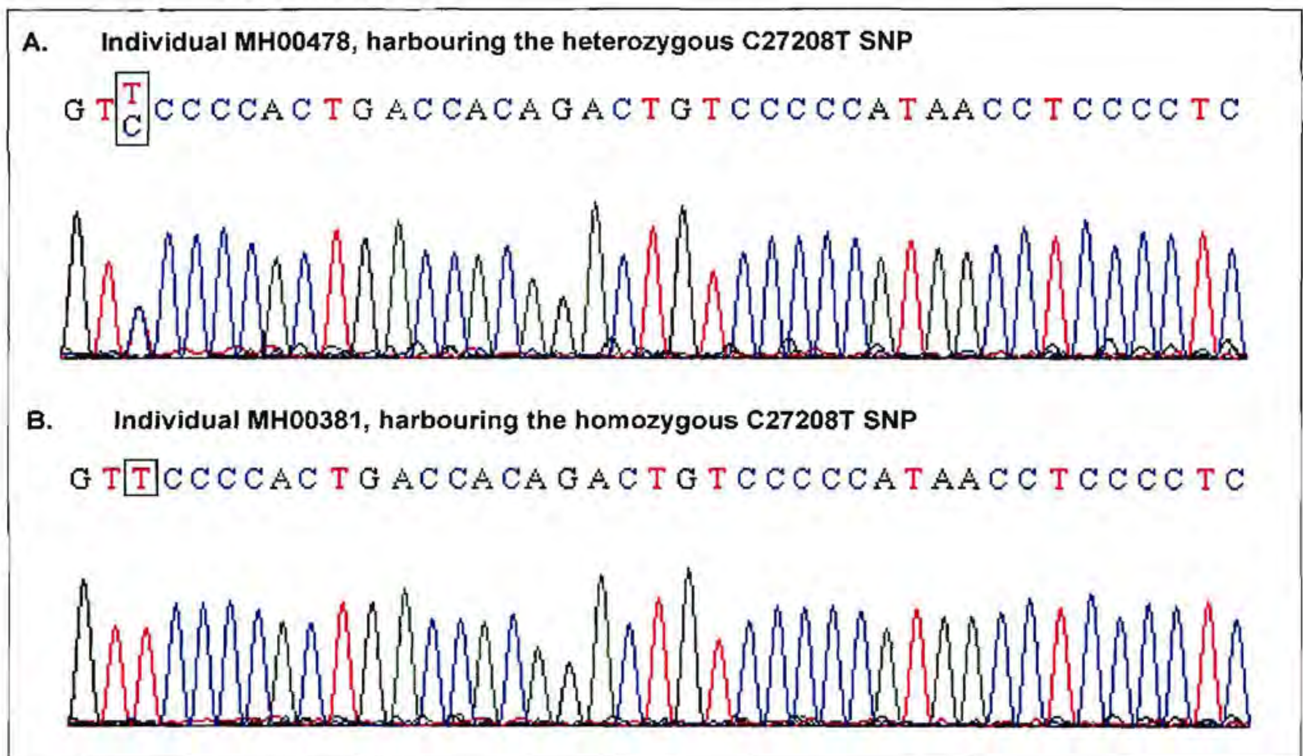
4.10.4.1 Synonymous substitution in the amplified region of exon 20 of the RYR1 gene

In seven South African MH probands, a heterozygous C27208T SNP was observed, as listed in Appendix C (page 451). All seven individuals harboured two peaks representing two different nucleotides i.e. T and C. In addition, four individuals were homozygous (T/T) for the SNP and the remaining four individuals did not harbour the SNP. As discussed in Section 4.7.4.1 (page 185), SNPs observed in the RYR1 gene may play a role in the development of MH, via epistasis.

4.10.4.1.1 SNP C27208T

The sequences presented in Figure 4.108 are representative electropherograms illustrating the heterozygous and homozygous C27208T SNP, respectively. The synonymous substitution was identified in the intron sequence of the RYR1 gene between exons 19 and 20 and is indicated as a SNP in GenBank® (International Human Genome Sequencing Consortium, 2004; with accession number rs2304147). The genotype frequency of this SNP has been determined in the European, Asian and Sub-Saharan African populations. In all populations analysed, the C/T and T/T genotypes are the most common and their frequencies vary between 0.32 and 0.63. The C/C genotype was the least likely to be observed in all populations and the genotype frequencies vary between 0.02 and 0.16 (International Human Genome Sequencing Consortium, 2004; with accession number rs2304147).

Figure 4.108: Representative electropherograms indicating the C27208T SNP observed in the intron sequence between exons 19 and 20 of the RYR1 gene

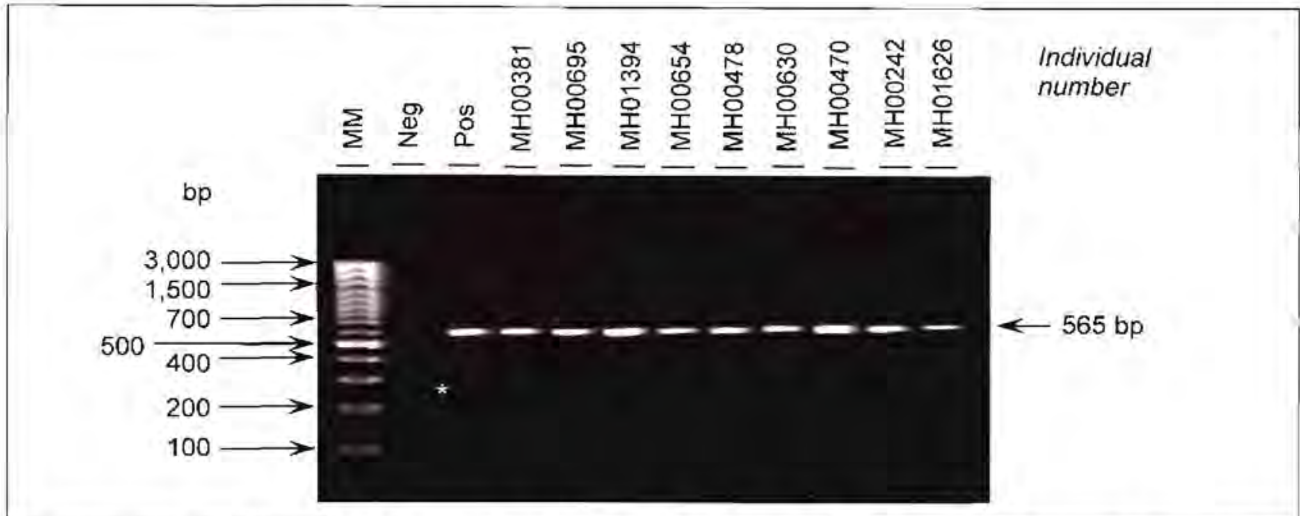


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.5 Exons 21 and 22 of the RYR1 gene

The standard PCR protocol was used to amplify a region of 565 bp encompassing both exons 21 and 22 of the RYR1 gene, as listed in Table 4.1 (page 161). PCR amplification was successful for all 15 samples analysed and the results of PCR amplification are depicted in Figure 4.109.

Figure 4.109: Photographic representation of amplified PCR products encompassing exons 21 and 22



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. As discussed in Sections 4.2 and 4.3, an artefact in the gel matrix, as indicated by the white asterisk (*), variation in amplification efficiency, fragment distortion and overloaded MM were observed.

Fifteen MH probands amplified via PCR were successfully sequenced using the standard protocol. A representative electropherogram obtained for individual MH01626, illustrating a portion of the amplified region of exon 21, is indicated in Figure 4.110.

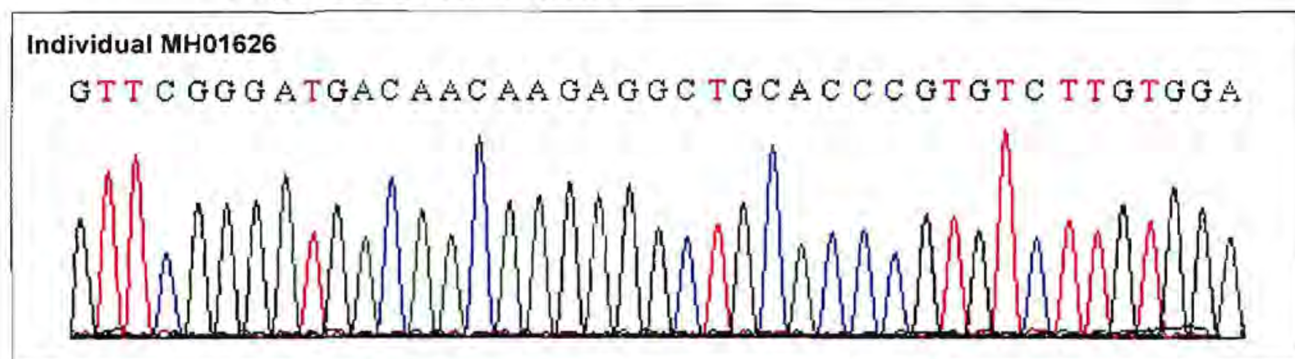
Figure 4.110: Representative electropherogram illustrating a portion of the amplified region of exon 21

A = adenine; C = cytosine; G = guanine; T = thymine.

Alterations associated with MH have thus far not been reported in either exons 21 or 22 (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006). A representative electropherogram obtained for individual MH01626, illustrating a portion of the amplified region of exon 22, is depicted in Figure 4.111. In the study presented here, alterations associated with MH were not observed in any of the South African MH probands analysed. As discussed in Section 2.9 (page 22), this region of the RyR1 protein holds critical determinants for E-C coupling (Perez *et al.*, 2003b).

Therefore, alterations observed in this region of the RYR1 gene may have functional consequences and result in MH susceptibility. As discussed in Section 4.10.2 (page 275), analysis of these exons would have to be conducted in various populations of adequate size, in order to determine if these two exons harbour alterations that contribute to the phenotype responsible for MHS.

Figure 4.111: Representative electropherogram illustrating a portion of the amplified region of exon 22

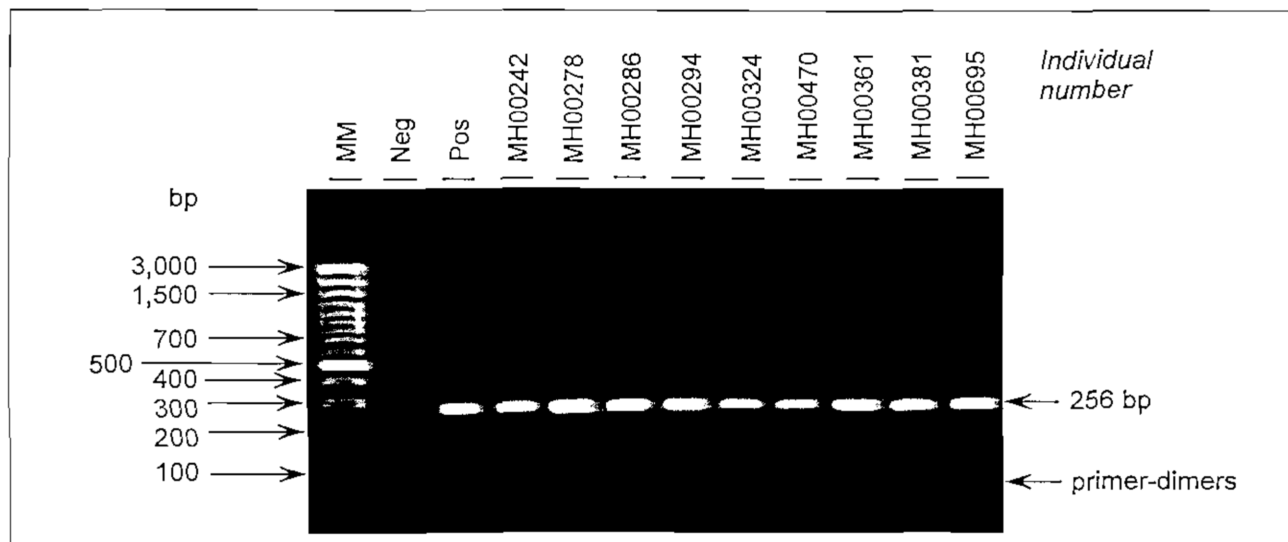


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.6 Exon 23 of the RYR1 gene

Exon 23 is located outside the mutational hotspot regions of the RYR1 gene. However, as discussed in Section 2.9 (page 22), this exon resides in a region of the gene that supports E-C coupling. Due to the role that this region of the RyR1 protein plays in E-C coupling, the amplified region of exon 23 was sequenced and analysed for novel alterations. Thus far, alterations associated with the MH phenotype have not been reported in this region of the gene (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006). A 256 bp region encompassing this exon was amplified as discussed in Section 4.2 (page 159). Figure 4.112 is a photographic representation of the amplicon encompassing exon 23.

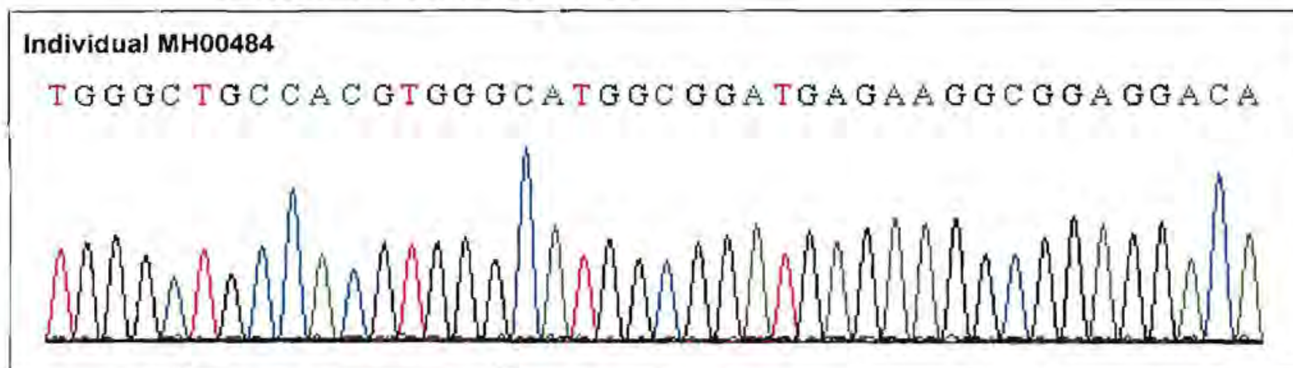
Figure 4.112: Photographic representation of amplified PCR products encompassing exon 23



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Variation in amplification efficiency between samples, primer-dimers and MM distortion were observed, as discussed in Sections 4.2 and 4.3.

A representative result generated via automated sequencing obtained for individual MH00484 illustrating a portion of the amplified region is presented in Figure 4.113. Sequencing was conducted using the reverse primer (RYRex23R). For this reason, sequences are depicted as the reverse complement. None of the 15 MHS probands analysed in the study presented here harboured any novel alterations in this region. Due to the absence of alterations in exon 23 in the South African population included in this investigation, it was concluded that this exon does not harbour alterations that add to the list of MH mutations in this cohort. However, as this exon has only recently been screened for MH mutations (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006), alterations associated with MH may be observed in this exon in MHS individuals that have thus far not been screened, as discussed in Section 4.10.2 (page 275).

Figure 4.113: Representative electropherogram illustrating a portion of the amplified region of exon 23

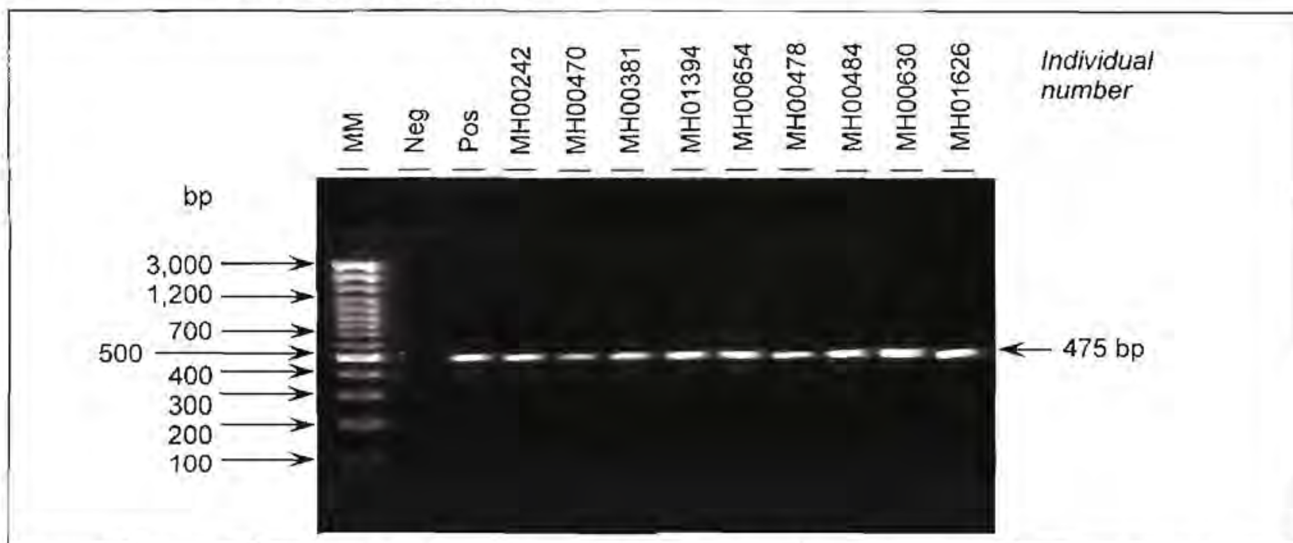


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.7 Exon 24 of the RYR1 gene

Alterations associated with MH have never been reported to occur in exon 24. Fifteen MH probands that have not previously been analysed for novel alterations in this region of the RYR1 gene were screened during the Phase 3 study. The PCR conditions were optimised as discussed in Section 4.2 (page 159). The amplified product was subsequently electrophoresed, and the results of the amplified products are illustrated in Figure 4.114.

Figure 4.114: Photographic representation of amplified PCR products encompassing exon 24

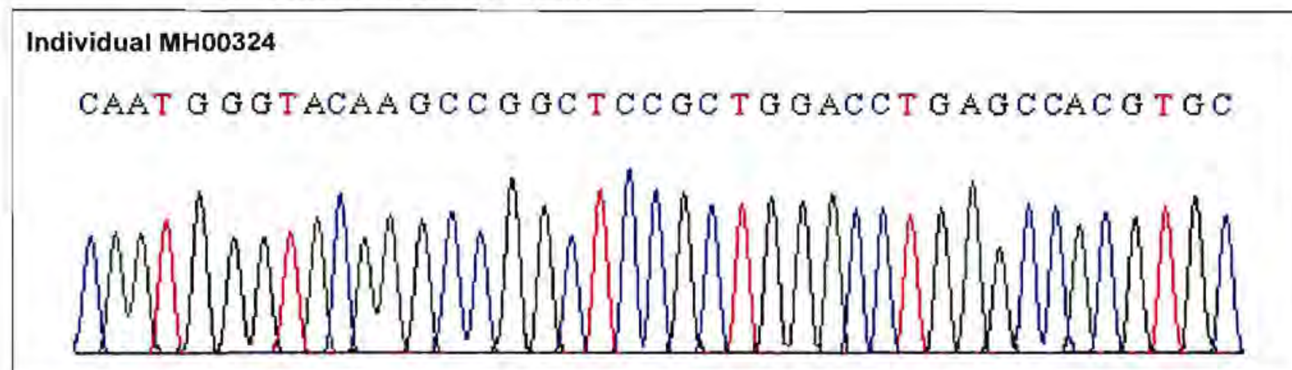


Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. The agarose gel exhibited MM overloading, fragment distortion and variation in amplification efficiency, as discussed in Sections 4.2 and 4.3.

Of the 15 samples amplified, 15 were successfully sequenced according to the standard protocol. Sequencing was conducted using the forward primer (RYRex24F), and a representative result illustrating a portion of the amplified region of exon 24, generated via

automated sequencing, is presented in Figure 4.115. Alterations associated with MH were not observed in the amplified region of exon 24. However, two SNPs were detected in this region of the RYR1 gene, as discussed in Section 4.10.7.1 (page 288). Mutation screening in various populations of sufficient size may be required in order to determine if this exon harbours alterations that play a role in the development of the MH phenotype, as discussed in Section 4.10.2 (page 275).

Figure 4.115: Representative electropherogram illustrating a portion of the amplified region of exon 24



A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.7.1 Synonymous substitutions in the amplified region of exon 24 of the RYR1 gene

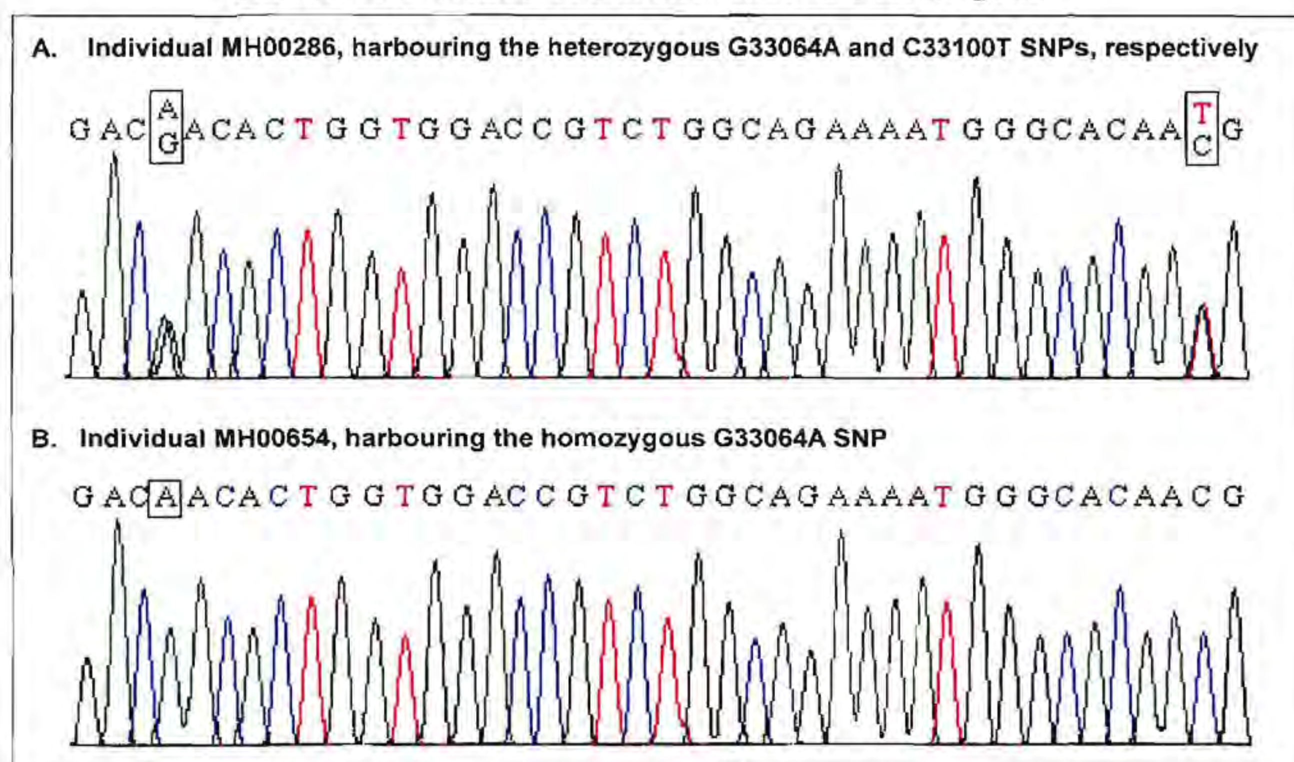
In eight South African MH probands, a heterozygous G33064A SNP was identified. In addition, five individuals harboured the homozygous G33064A SNP, as listed in Appendix B (page 447). The remaining individuals included in the study presented here, did not exhibit the SNP. In addition, the C33100T SNP was identified in six South African probands analysed in the study presented here, as listed in Appendix B (page 447). All six individuals harboured the heterozygous SNP, but the homozygous SNP was not detected in any individuals analysed. The remaining individuals included in the study presented here did not exhibit the SNP. Both SNPs may not directly result in the MH phenotype in these individuals, however, the detected SNPs may contribute to the MH phenotype via epistasis, as discussed in Section 4.7.4.1 (page 185).

4.10.7.1.1 SNP G33064A

The G33064A SNP is a synonymous substitution, as it occurs at the third codon position and does not result in an alteration in the amino acid, Thr. The synonymous substitution was identified in the coding region of the RYR1 gene and is indicated as a SNP of the

RYR1 gene in GenBank® (International Human Genome Sequencing Consortium, 2004; with accession number rs2228069). The sequences depicted in Figure 4.116 are representative electropherograms illustrating the heterozygous and homozygous G33064A SNP, respectively. The genotype frequency of the G33064A SNP has been determined in cohorts from the European, African American and Asian populations. In the European and African American populations, the A/G genotype occurs more frequently and the frequencies have been identified as 0.46 and 0.55, respectively. The Asian population had the following genotype frequencies: 0.46 for A/A, 0.33 for the A/G genotype and 0.21 for G/G (International Human Genome Sequencing Consortium, 2004; with accession number rs2228069).

Figure 4.116: Representative electropherograms indicating the G33064A and C33100T SNPs observed in exon 24 of the RYR1 gene



A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.7.1.2 SNP C33100T

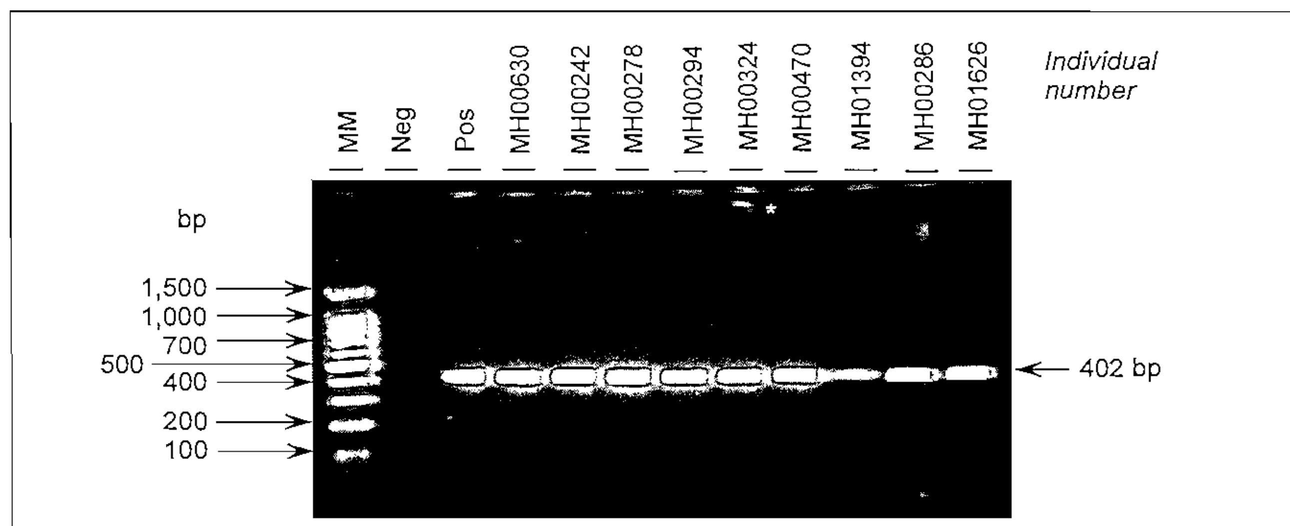
The C33100T synonymous substitution was identified in the coding region of the RYR1 gene and is indicated as a SNP of the RYR1 gene in GenBank® (International Human Genome Sequencing Consortium, 2004; with accession number rs2228070). The C33100T SNP retains the Asn amino acid. The sequences depicted in Figure 4.116A are a representative electropherogram illustrating the heterozygous C33100T SNP. The

genotype frequencies have been determined in the North American and African American populations (International Human Genome Sequencing Consortium, 2004; with accession number rs2228070), where the C/C genotype occurs more frequently, and has a frequency of 0.61, followed by the C/T genotype, with a frequency of 0.33 and the T/T genotype with a frequency of 0.06.

4.10.8 Exon 25 of the RYR1 gene

PCR was used to amplify a region of 402 bp of exon 25 from the RYR1 gene. This region of the RYR1 has not been reported to harbour mutations responsible for the MH phenotype and does not contain any reported SNPs. Exon 25 resides outside the three mutational hotspots, between hotspots one and two. The PCR reaction was optimised, as discussed in Section 4.2 (page 159), and the results of the amplified PCR product encompassing exon 25 are presented in Figure 4.117.

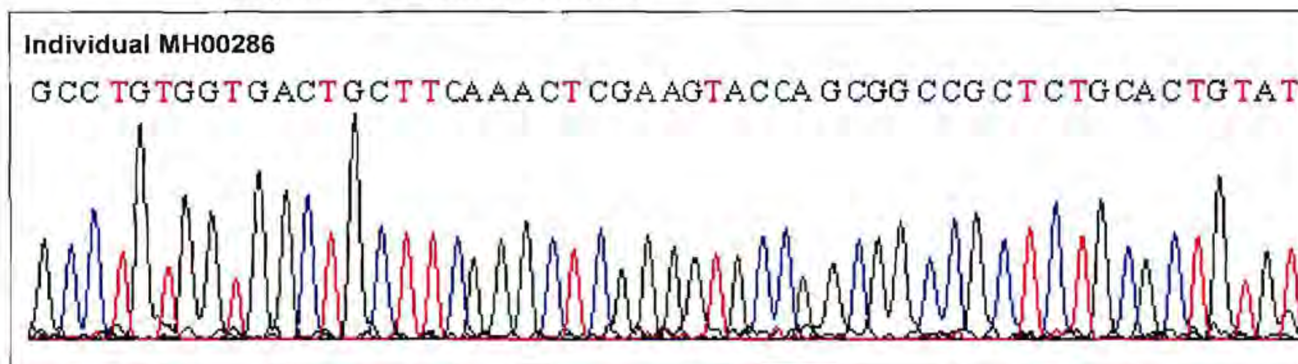
Figure 4.117: Photographic representation of amplified PCR products encompassing exon 25



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. An artefact in the gel matrix, as indicated by the white asterisk (*) was detected, as discussed in Section 4.3. Non-specific secondary amplification and background smears were observed, as discussed in Section 4.2. A variation in amplification efficiency of the samples was noted, as discussed in Section 4.2.

Amplification was considered successful for all 15 samples obtained from the South African probands. The amplified region was sequenced, in order to investigate the presence of novel mutations that may occur in this region of the RYR1 gene. Sequencing was conducted using the reverse primer (RYRex25R), thus sequences are depicted as the reverse complement. A representative electropherogram obtained for individual MH00286, illustrating a portion of the amplified region for exon 25, is indicated in Figure 4.118.

Figure 4.118: Representative electropherogram illustrating a portion of the amplified region of exon 25



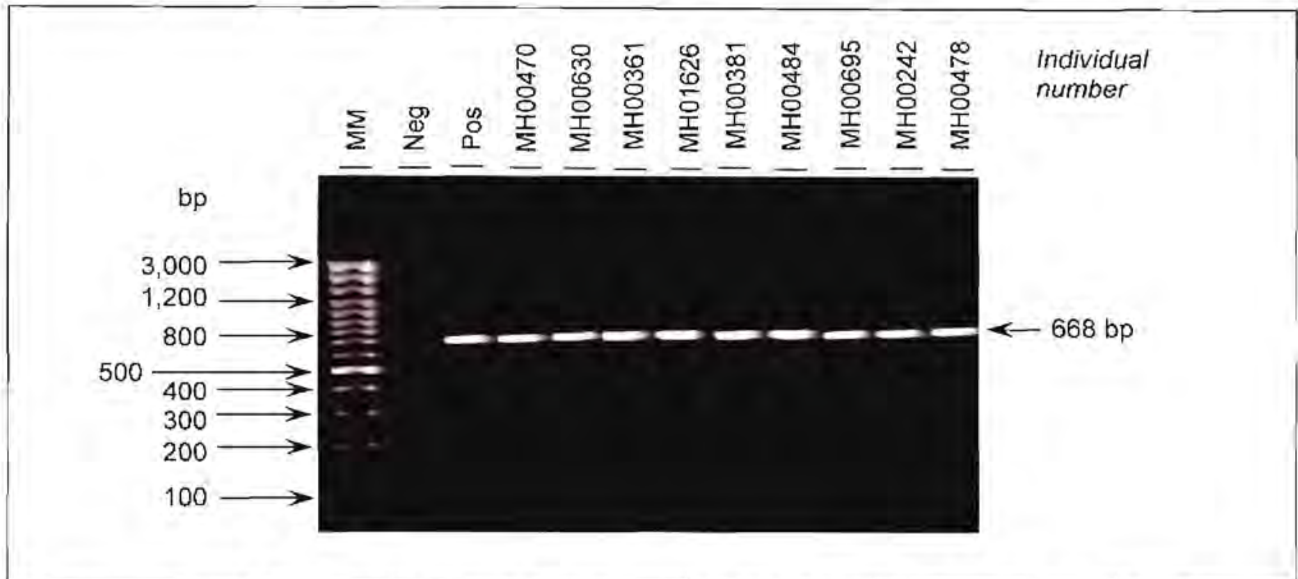
A = adenine; C = cytosine; G = guanine; T = thymine. As discussed in Section 4.5, background peaks were observed.

All 15 individuals included in the study presented here were analysed for alterations in exon 25 of the RYR1 gene. None of the individuals analysed harboured any novel alterations in exon 25. As exon 25 has only recently been analysed in a limited number of individuals (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006), as discussed in Section 4.10.2 (page 275), further analysis of this exon may yield alterations associated with MH.

4.10.9 Exons 26 and 27 of the RYR1 gene

In order to identify novel alterations that may occur in exons 26 or 27, a region of 668 bp was amplified using PCR conditions listed in Table 4.1 (page 161). The amplified product was subsequently electrophoresed and results obtained for PCR amplification of exons 26 and 27 are depicted in Figure 4.119.

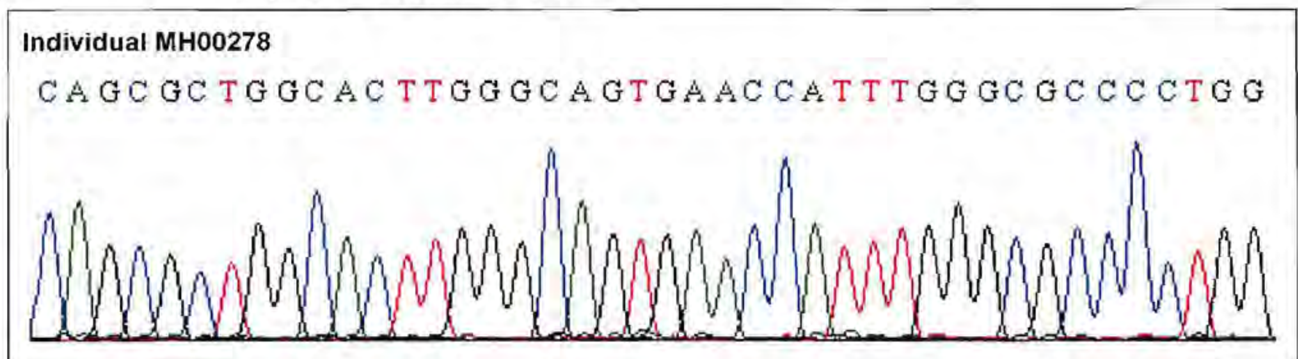
Figure 4.119: Photographic representation of amplified PCR products encompassing exons 26 and 27



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. As discussed in Sections 4.2 and 4.3, slanted fragments, background smears and MM distortion were observed.

The 15 successfully amplified samples obtained via PCR were purified and sequenced according to the standard protocol. A representative electropherogram obtained for individual MH00278, illustrating a portion of the amplified region for exon 26, is presented in Figure 4.120.

Figure 4.120: Representative electropherogram illustrating a portion of the amplified region of exon 26



A = adenine; C = cytosine; G = guanine; T = thymine.

Sequencing was conducted using the reverse primer (RYRex26R) and sequences are depicted as the reverse complement. Sequences were subsequently analysed in order to investigate for the presence of novel alterations that may occur in exons 26 and 27. A representative electropherogram obtained for individual MH00278, illustrating a portion of the amplified region for exon 27, is presented in Figure 4.121. None of the 15 individuals that were sequenced in the study presented here harboured any novel alterations. As

discussed in Section 4.10.2 (page 275), since both these exons 26 and 27 have only recently been screened (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006), analysis in a larger group of MHS individuals from a variety of populations would verify if alterations that may be associated with the disorder occur in either exons 26 or 27.

Figure 4.121: Representative electropherogram illustrating a portion of the amplified region of exon 27

A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.9.1 Synonymous substitution in the amplified region of exons 26 and 27 of the RYR1 gene

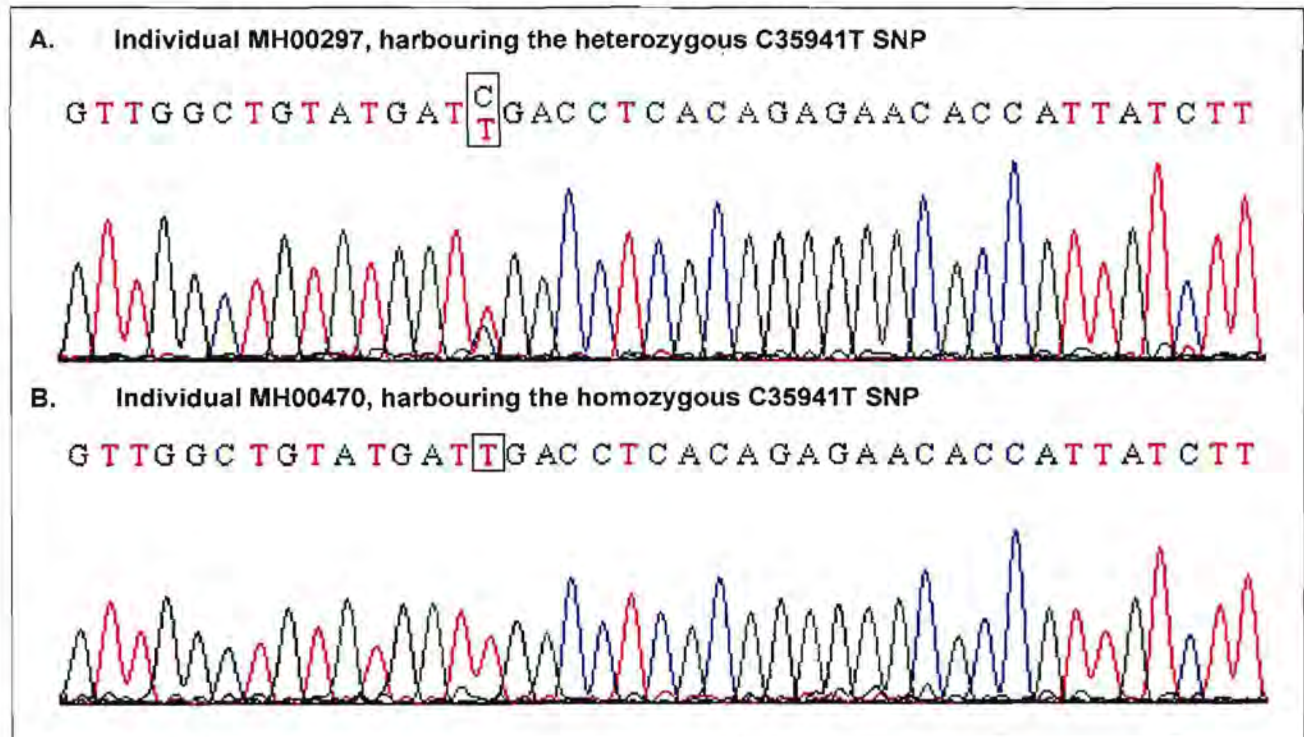
Analysis of exon 26 identified a C35941T SNP in the coding region of the gene. In eight South African MH probands, two peaks representing two different nucleotides i.e. C and T, were identified. In addition, one individual (MH00470) harboured the homozygous C35941T SNP. The remaining individuals included in the study presented here did not harbour the SNP, as listed in Appendix B (page 447). As discussed in Section 4.7.4.1 (page 185), the alterations may contribute to the MH phenotype via epistasis.

4.10.9.1.1 SNP C35941T

The C35941T SNP is a synonymous substitution and retains the amino acid Ile. The synonymous substitution has previously been identified as a SNP of the RYR1 gene in GenBank® (International Human Genome Sequencing Consortium, 2004; with accession number rs11083462). The sequences depicted in Figure 4.122 are representative electropherograms illustrating the heterozygous and homozygous C35941T SNP, respectively. The genotypes of this SNP have been determined in the European, African American, Sub-Saharan African and Asian populations (International Human Genome Sequencing Consortium, 2004; with accession number rs11083462). In the European and

African American populations the C/T genotype occurs more frequently and the frequencies have been identified as 0.46 and 0.52, respectively. In the Asian and Sub-Saharan African population the frequencies of the C/C genotype have been identified as 0.56 and 0.44, respectively, whereas the C/T genotype has a frequency of 0.39 for the Asian population and 0.42 for the Sub-Saharan African population.

Figure 4.122: Representative electropherograms indicating the C35941T SNP observed in exon 26 of the RYR1 gene

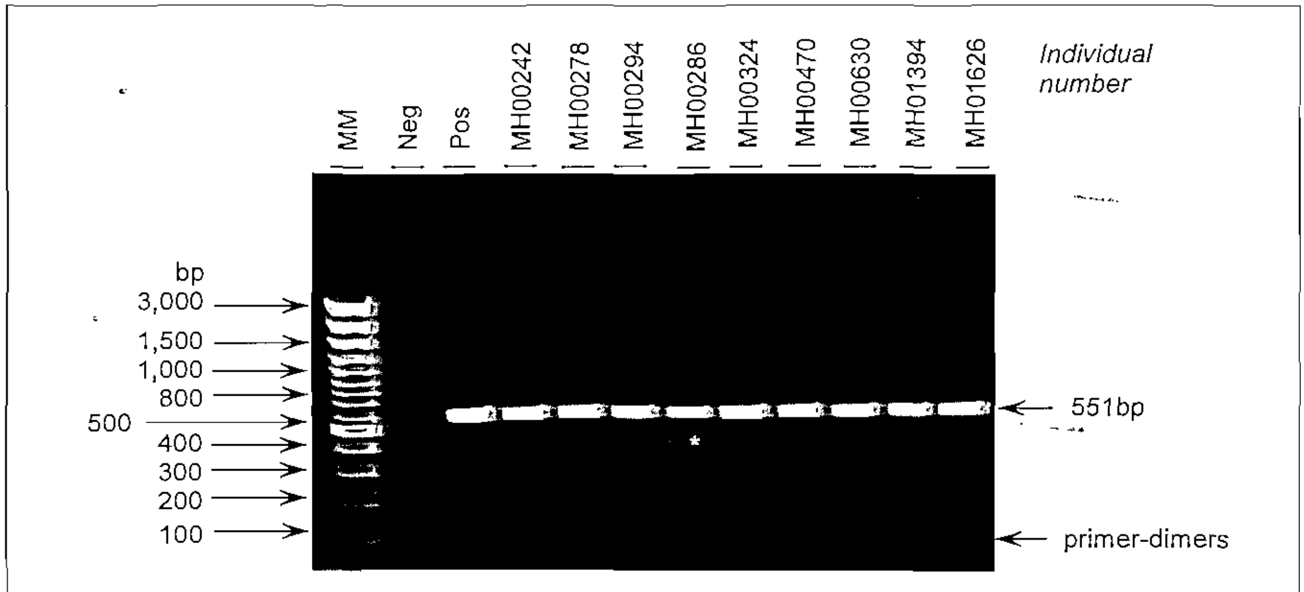


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.10 Exon 28 of the RYR1 gene

PCR was conducted in order to amplify a 551 bp region of exon 28 of the RYR1 gene. Exon 28 does not currently harbour any reported alterations associated with the MH phenotype. PCR was optimised as discussed in Section 4.2 (page 159). Amplification results obtained for the PCR region encompassing exon 28 are depicted in Figure 4.123.

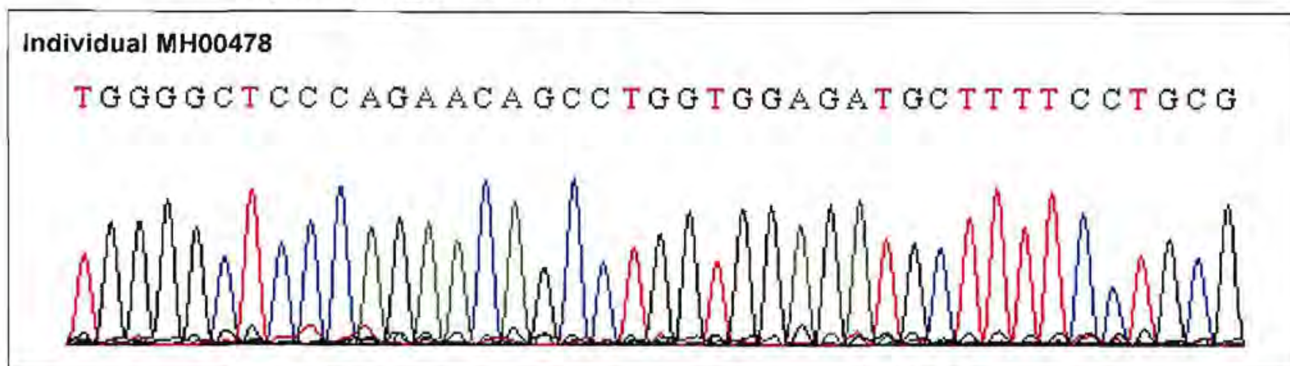
Figure 4.123: Photographic representation of amplified PCR products encompassing exon 28



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Primer-dimers, an artefact in the gel matrix, as indicated by the white asterisk (*), MM and fragment distortion were observed, as discussed in Sections 4.2 and 4.3.

A representative result generated via automated sequencing is presented in Figure 4.124. Sequencing was conducted using the reverse primer (RYRex28R) and the sequences illustrated in the electropherogram are depicted as the reverse complement. Novel alterations in exon 28 were not observed in any of the probands analysed in the study presented here. As discussed in Section 4.10 (page 273), additional alterations outside the mutational hotspots have only recently been observed in populations from France, Italy, North America and Japan (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006). Therefore, analysis of this exon in various populations, which includes a sufficient number of individuals, as discussed in Section 4.10.2 (page 275), may identify alterations in exon 28 that result in susceptibility to MH.

Figure 4.124: Representative electropherogram illustrating a portion of the amplified region of exon 28

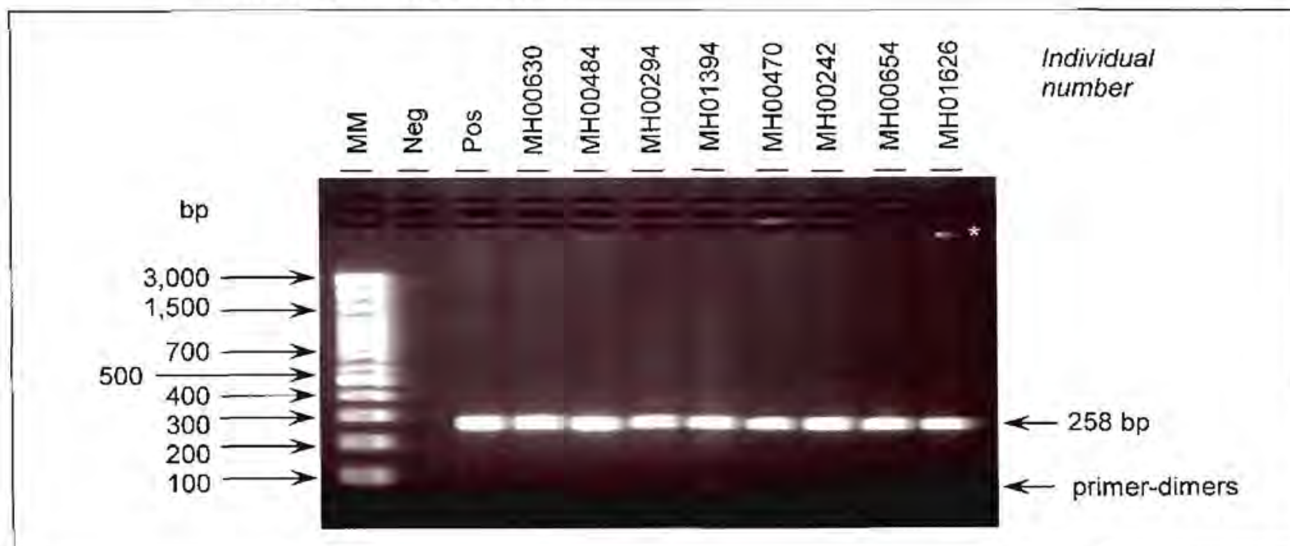


A = adenine; C = cytosine; G = guanine; T = thymine. The sequence exhibited background peaks, as discussed in Section 4.5.

4.10.11 Exon 29 of the RYR1 gene

To date alterations have not been reported to occur within exon 29 of the RYR1 gene. A region of 258 bp was amplified, as discussed in Section 4.2 (page 159) and sequenced in order to detect any novel alterations that may occur in this region of the RYR1 gene. Figure 4.125 is a photographic representation of the amplicon encompassing exon 29.

Figure 4.125: Photographic representation of amplified PCR products encompassing exon 29

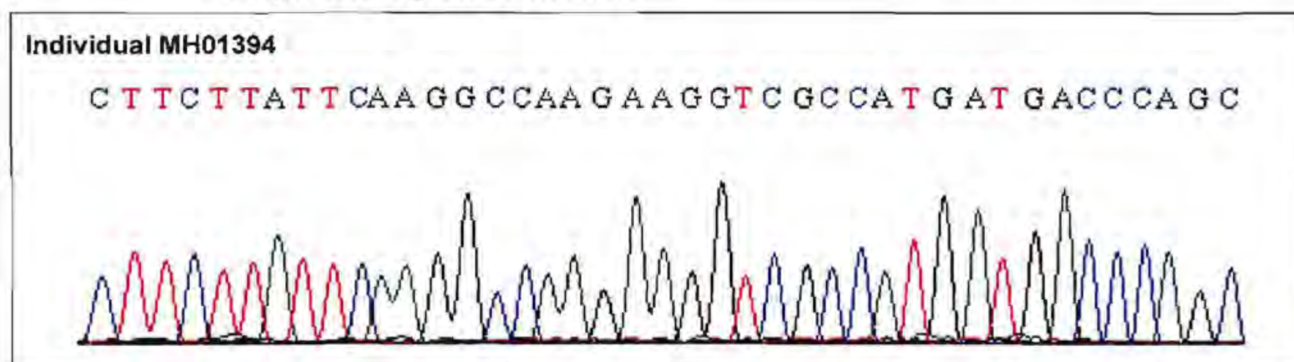


Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. An artefact in the gel matrix, as indicated by the white asterisk (*) was detected, as discussed in Section 4.3. In addition, non-specific secondary amplification and primer-dimers were observed, as well as MM distortion, as discussed in Sections 4.2 and 4.3.

The samples of the 15 MH probands analysed in the study presented here were all amplified and sequenced successfully using the optimised protocols. Sequencing was conducted using the forward primer (RYRex29F). A representative sequence electropherogram obtained for individual MH01394, depicting a portion of the amplified

region of exon 29, is illustrated in Figure 4.126. Novel alterations in exon 29 were not observed in any of the South African probands analysed in the study presented here. As this exon has only recently been analysed in a limited number of individuals from Italy, France, North America, Japan (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006) and South Africa, further analysis of this exon would have to be conducted to determine if alterations associated with the MH phenotype occur in exon 29 of the RYR1 gene, as discussed in Section 4.10.2 (page 275).

Figure 4.126: Representative electropherogram illustrating a portion of the amplified region of exon 29

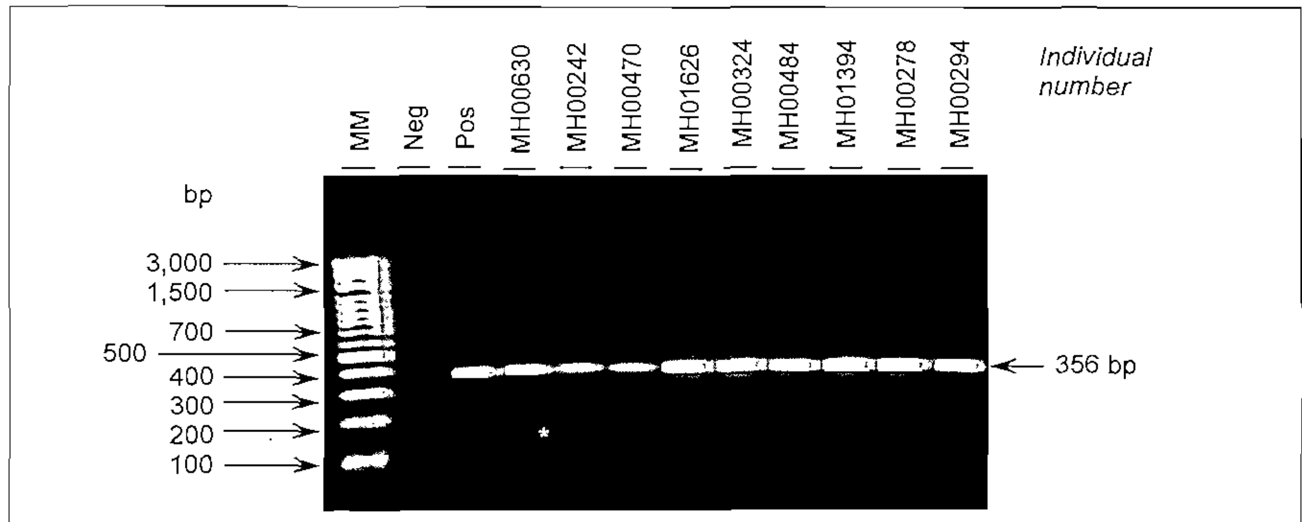


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.12 Exon 30 of the RYR1 gene

A 356 bp product encompassing exon 30 was amplified via PCR, as listed in Table 4.1 (page 161). The region currently does not harbour any reported alterations associated with the MH phenotype. The product was electrophoresed on a 2% (w/v) mini agarose gel and Figure 4.127 is a photographic representation of the amplicon encompassing the amplified exon.

Figure 4.127: Photographic representation of amplified PCR products encompassing exon 30



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. An artefact in the gel matrix, as indicated by the white asterisk (*) was detected. In addition, the amplification efficiencies varied between samples, non-specific amplification and MM overloading were observed, as discussed in Sections 4.2 and 4.3.

The amplified region was sequenced in order to investigate the presence of novel alterations in the amplified region of exon 30. Sequencing was conducted using the standard protocol. The forward primer (RYRex30F), was used in the sequencing reaction. A representative sequence electropherogram obtained for individual MH00630 depicting a portion of the amplified region of exon 30, is illustrated in Figure 4.128. Exon 30 was screened for the first time in the South African MH population and novel alterations were not detected in any of the 15 MH probands analysed in Phase 3. As this region of the RyR1 is involved in E-C coupling (Perez *et al.*, 2003b) alterations observed in exon 30 may have a functional effect on the protein. The absence of alterations associated with MHS may be due to the relatively few individuals and populations that have been analysed thus far for alterations in exon 30 (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006), as discussed in Section 4.10.2 (page 275).

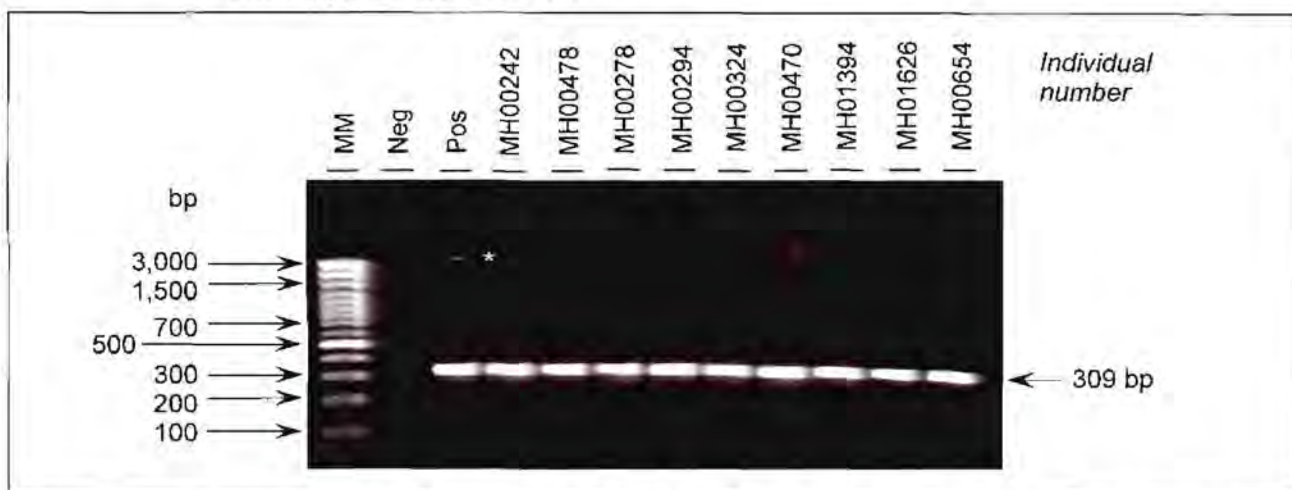
Figure 4.128: Representative electropherogram illustrating a portion of the amplified region of exon 30

A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.13 Exon 31 of the RYR1 gene

Amplification of a region of the RYR1 gene encompassing exon 31 was conducted and the reaction was optimised, as described in Section 4.2 (page 159). The 309 bp product was electrophoresed and the results are presented in Figure 4.129. Thus far, exon 31 does not harbour any reported alterations associated with MH susceptibility.

Figure 4.129: Photographic representation of amplified PCR products encompassing exon 31

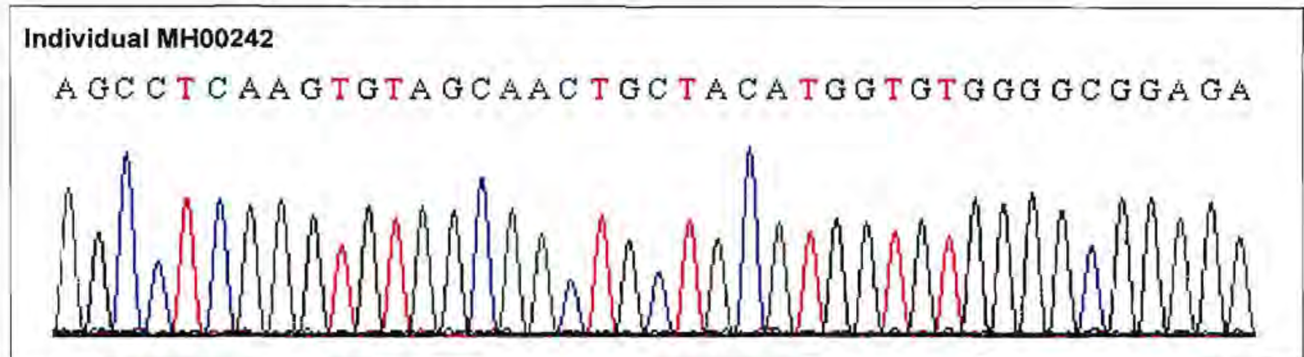


Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. As discussed in Sections 4.2 and 4.3, an artefact in the gel matrix, as indicated by the white asterisk (*), background smears, slanted fragments and a barrier in the gel were observed. In addition, the MM and sample fragments appear distorted.

A representative result generated via automated sequencing, indicating a portion of the amplified region of exon 31, is presented in Figure 4.130. Sequencing was conducted using the reverse primer (RYRex31R) and sequences are thus depicted as the reverse complement. None of the 15 MHS probands analysed in the study presented here harboured any novel alterations in this region. Exon 31 has only recently been screened

for MH mutations worldwide (Monnier *et al*., 2005; Sambuughin *et al*., 2005; Galli *et al*., 2006; Ibarra *et al*., 2006; Wu *et al*., 2006) and in South Africa, therefore alterations that contribute to the phenotype responsible for MHS may be observed in this exon in MH individuals that have thus far not been screened, as discussed in Section 4.10.2 (page 275).

Figure 4.130: Representative electropherogram illustrating a portion of the amplified region of exon 31

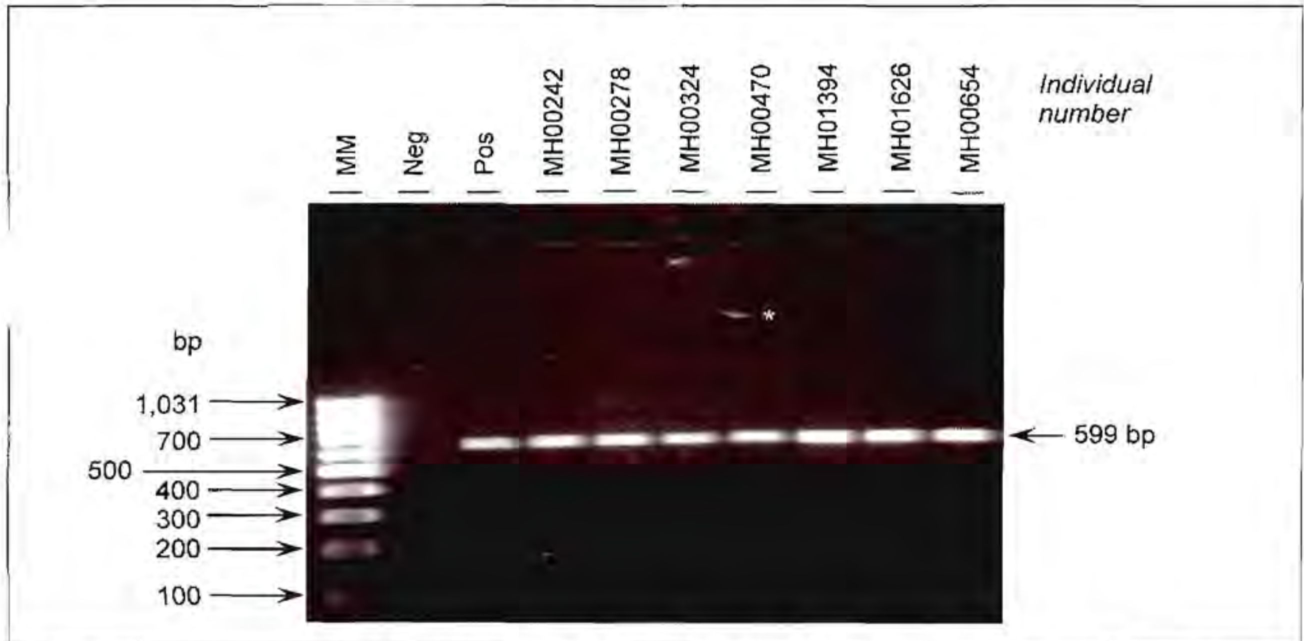


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.14 Exons 32 and 33 of the RYR1 gene

In order to identify novel or reported alterations that may be associated with MHS in exons 32 and 33 simultaneously, a 599 bp region was amplified, as discussed in Section 4.2 (page 159) and subsequently sequenced. The results of the amplified PCR product encompassing exons 32 and 33 are presented in Figure 4.131. Amplification of this region of the RYR1 gene was obtained for all 15 samples from South African probands.

Figure 4.131: Photographic representation of amplified PCR products encompassing exons 32 and 33



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. An artefact in the gel matrix, as indicated by the white asterisk (*) was observed, as discussed in Section 4.3. Variations in amplification efficiency of the samples and MM distortion were observed, as discussed in Sections 4.2 and 4.3.

The amplified region was subsequently sequenced, using the reverse primer (RYRex32R). Therefore, sequences are depicted as the reverse complement. A representative electropherogram obtained for individual MH00630, illustrating a portion of the amplified region for exon 32, is depicted in Figure 4.132.

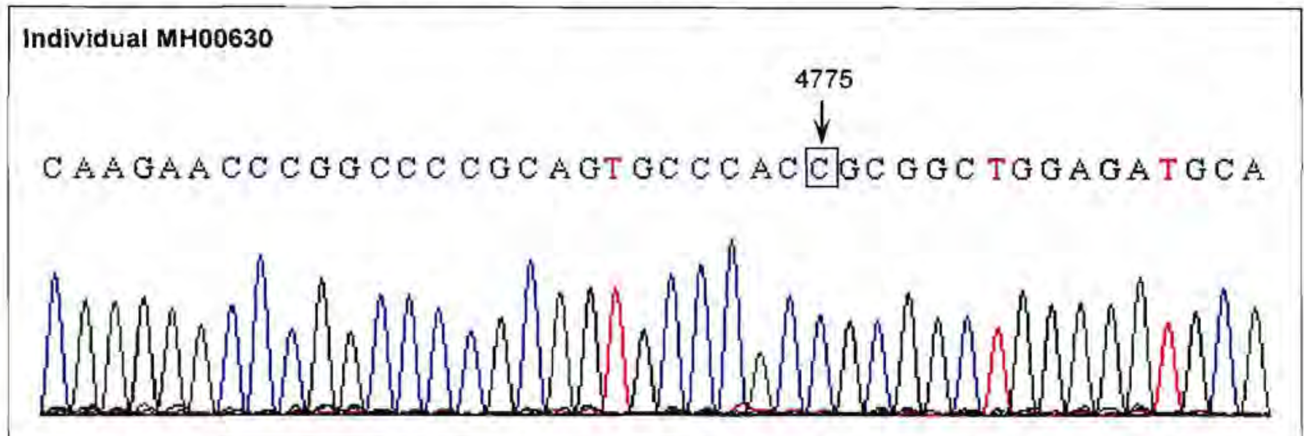
Figure 4.132: Representative electropherogram illustrating a portion of the amplified region of exon 32

A = adenine; C = cytosine; G = guanine; T = thymine.

To date, alterations associated with MH have not been reported to occur in exon 32 of the RYR1 gene. However, Ibarra *et al.* (2006) recently reported a single alteration, Pro1592Leu, in exon 33 of the RYR1 gene, even though this exon resides outside the RYR1 mutational hotspots. The alteration is due to a C4775T substitution and was

observed in one MHS family from Japan. A representative electropherogram obtained for individual MH00630, illustrating the nucleotide position of the reported Pro1592Leu alteration, is depicted in Figure 4.133.

Figure 4.133: Representative electropherogram of exon 33 indicating the nucleotide position of the Pro1592Leu alteration



A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Pro1592Leu at nucleotide 4775.

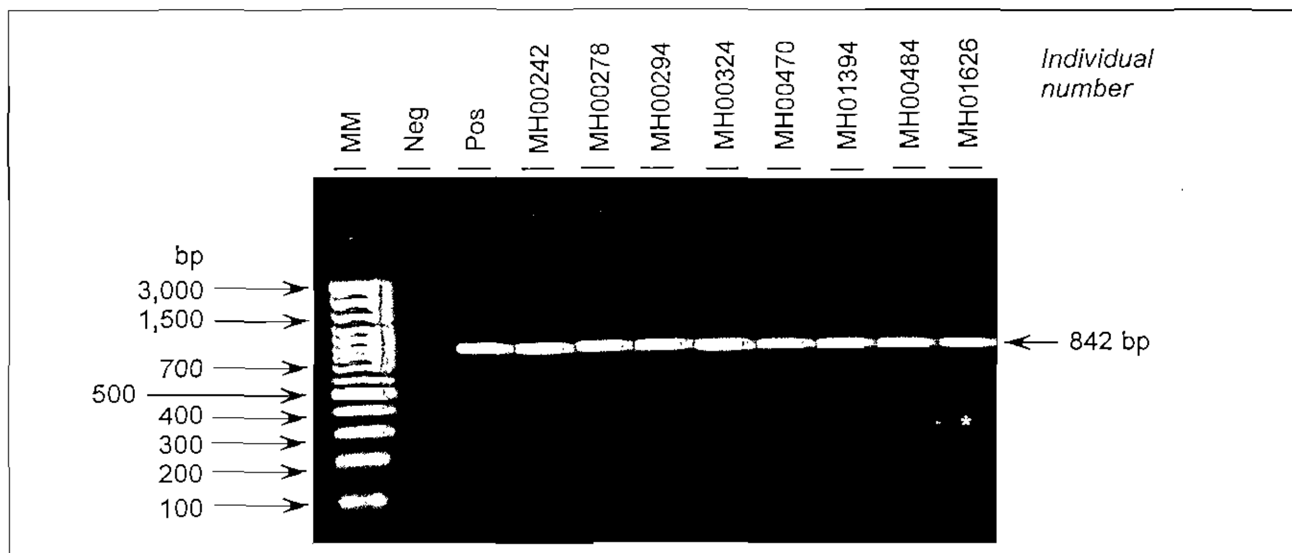
All 15 individuals included in the study presented here were analysed for alterations in exons 32 and 33 of the RYR1 gene. The Pro1592Leu alteration was not detected in any of the 15 South African probands analysed and in addition, novel alterations were not detected in either exons 32 or 33. The fact that the Pro1592Leu alteration has thus far been observed in only one family suggests that it may be unique to the family described by Ibarra *et al.* (2006). In addition, the alteration may be population-specific, and could be detected only in individuals of Asian origin. However, as the alteration has only been described recently (Ibarra *et al.*, 2006), the frequency of the alteration would have to be determined in other populations. Recent identification of a novel alteration that may be associated with MH in this region of the RYR1 gene may indicate that this exon could play a role in the development of the MH phenotype. Analysis of these exons in larger groups of MH individuals from various populations may identify additional alterations in exons 32 and 33 that are associated with MH, as discussed in Section 4.10.2 (page 275).

4.10.15 Exon 34 of the RYR1 gene

Exon 34 harbours several alterations associated with MH, even though it resides outside the mutational hotspots, between hotspots one and two. The Ser1728Phe alteration was detected in one individual from North America (Sambuughin *et al.*, 2005). Two different alterations that occur in exon 34 have been reported in MHS individuals from Japan

(Ibarra *et al.*, 2006). The Arg1667Cys alteration (Ibarra *et al.*, 2006) was observed in three families and the Pro1773Ser alteration (Ibarra *et al.*, 2006) was observed in one family. In addition, Gillard *et al.* (1992) identified a Leu1786Pro alteration and a Pro1787Leu alteration in single families. The PCR conditions were optimised listed in Table 4.1 (page 161). The amplified product was subsequently electrophoresed on a 2% (w/v) mini agarose gel as illustrated in Figure 4.134.

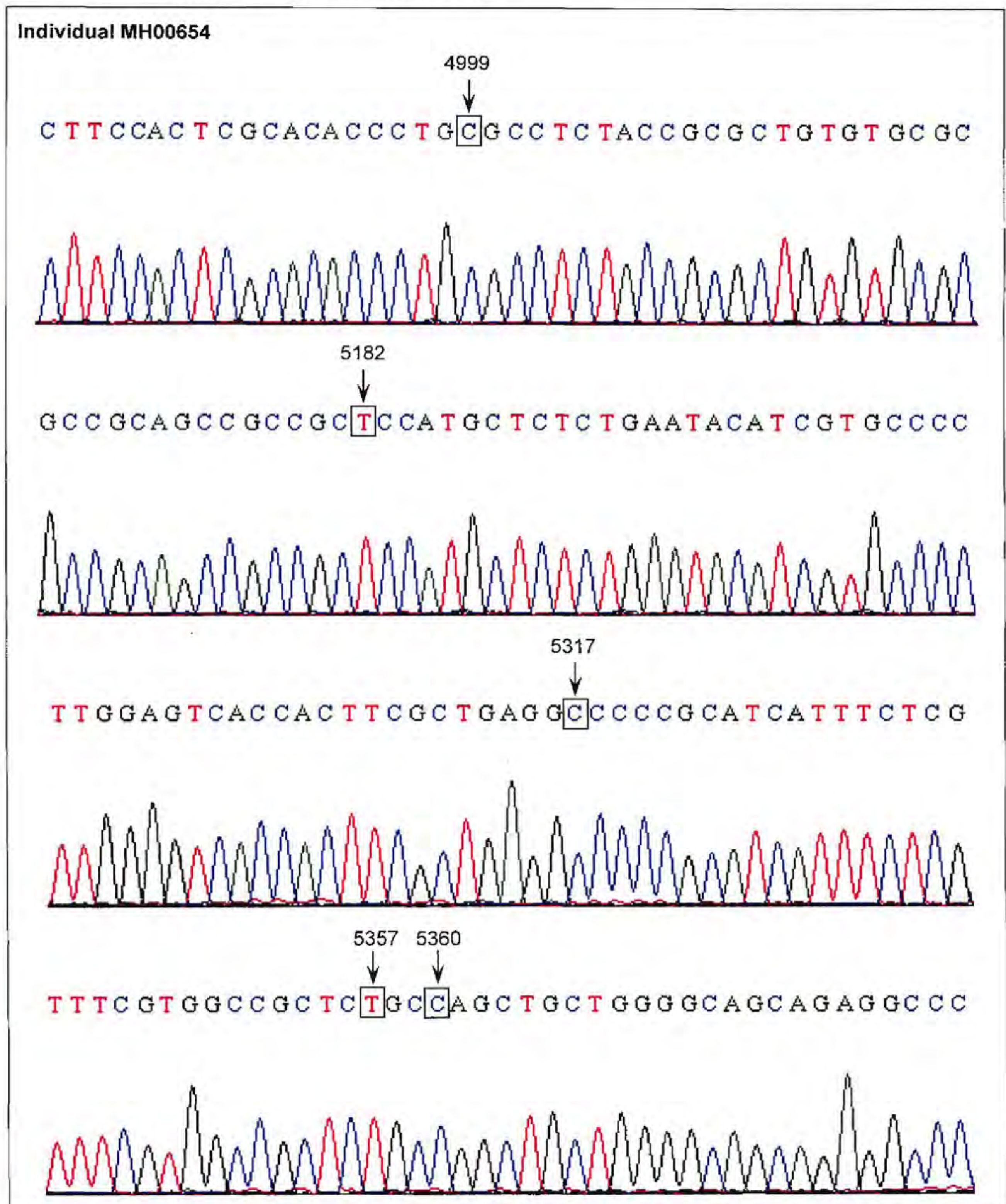
Figure 4.134: Photographic representation of amplified PCR products encompassing exon 34



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. An artefact in the gel matrix, as indicated by the white asterisk (*) was detected, as discussed in Section 4.7. In addition, non-secondary amplification, variation in the PCR efficiency of the amplified products and MM distortion were observed, as discussed in Sections 4.2 and 4.3.

A representative result generated via automated sequencing is presented in Figure 4.135, indicating the nucleotide positions of the reported alterations. Sequencing was conducted using the forward primer (RYRex34F).

Figure 4.135: Representative electropherogram of exon 34 indicating the nucleotide positions of the Arg1667Cys, Ser1728Phe, Pro1773Ser, Leu1786Pro and Pro1787Leu alterations



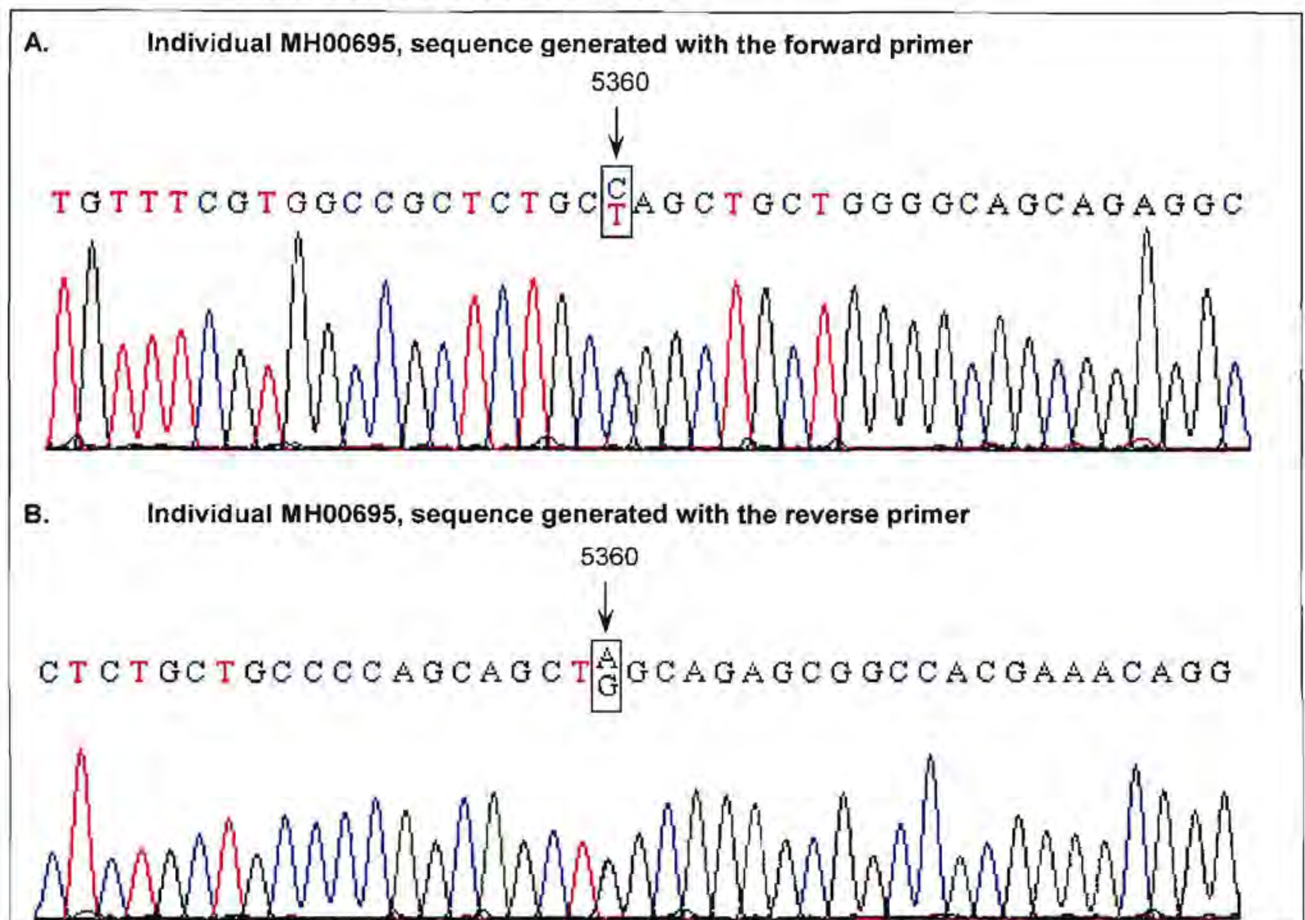
A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Arg1667Cys at nucleotide 4999; Ser1728Phe at nucleotide 5182; Pro1773Ser at nucleotide 5317; Leu1786Pro at nucleotide 5357 and Pro1787Leu at nucleotide 5360.

A single alteration, Pro1787Leu, was identified in one MH proband from South Africa, as discussed in Section 4.10.15.1 (page 305). The remaining individuals analysed did not harbour the alteration. In addition, the Ser1728Phe, Arg1667Cys, Leu1786Pro and Pro1773Ser alterations were not detected. Three of the alterations, namely Ser1728Phe (Sambuughin *et al.*, 2005), Leu1786Pro (Gillard *et al.*, 1992) and Pro1773Ser (Ibarra *et al.*, 2006), were detected in single families from North America, Europe and Japan, respectively. Therefore, they may be unique to those specific families. The Arg1667Cys alteration was observed in three families from Japan (Ibarra *et al.*, 2006), therefore the alteration is not family-specific. As it has only been reported in one population, the Arg1667Cys alteration may exhibit regional differences that have been associated with MH (Halsall and Robinson, 2004). However, as discussed in Section 4.10.2 (page 275), screening of all the exons of the RYR1 gene has only been conducted in certain populations (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006). Therefore, analysis of exon 34 in a larger group of individuals from various populations would determine if the alterations are family- or population-specific.

4.10.15.1 The Pro1787Leu alteration in the RYR1 gene

A single alteration, Pro1787Leu, was identified in one South African MH proband, MH00695 (MH114-1). At nucleotide position 5360, two peaks were observed that indicate the presence of two different bases, namely C and T. The different bases result in the expression of two amino acids, namely Pro and Leu, respectively. The sequences depicted for the electropherograms illustrated in Figure 4.136 indicate the Pro1787Leu alteration observed in individual MH00695.

Figure 4.136: Representative electropherograms indicating the Pro1787Leu alteration observed in exon 34



A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Pro1787Leu at nucleotide 5360.

Table 4.12 depicts the partial amino acid sequence of exon 34 of the RyR1 protein. Multiple sequence alignments encompassing the position of the Pro1787Leu alteration were retrieved from GenBank® (P21817, Q92736, NP_001027, P16960 and P11716). This region of the RyR1 protein is highly conserved among RyR1 species, but is not conserved among RyR isoforms. However, alterations that result in disease do not always reside in highly conserved regions. In contrast, alterations that are in highly conserved regions may not lead to a disorder (Jurkat-Rott and Lehmann-Horn, 2005). The authors indicated that two disease-causing mutations, i.e. Phe413Cys and Gln552Arg observed in the homodimeric chloride channel, which is encoded by CLCN1 gene, occur in regions that are not highly conserved. Dulhunty *et al.* (2005) reported that alterations in unconserved residues in a region of the DHPR II-III had functional consequences. The authors reported alterations that were critical for the functional interaction between RyR1 and DHPR. However, the presence of the alterations did not alter the structure of the DHPR. Therefore, in order to determine the causative status of an alteration, all criteria listed by the EMHG, as discussed in Section 5.6 (page 413) should be considered and

interpreted for a given novel alteration before being identified as causative or as a polymorphism. In addition, animal models or gene-expression profiling may clarify these aspects in the future.

Table 4.12: Conserved amino acids from different RyR isoforms and species surrounding the reported mutations in exon 34 of the RyR1 protein

Isoform	Species	Alignment of RyR protein sequences
RyR1	Human	GLPGVGVTTS LRPPHHFSPP CFVAALPAAG AAEAPARLSP AIPLEALRDK ALRMLG
RyR2	Human	GLPGIGLSTS LRPRMQFSSP SFVSI----- -SNECYQYSP EFPLDILKSK TIQMLT
RyR3	Human	GLPGVGMSTC LRPELHFSHT CFVSTISEL- -----YQHSP YIPLDILKTK AINMLT
ryr1	Pig	GLPGVGVTTS LRPPHHFSAP CFVAALPAVG AAEAPARLSP SIPLEALRDK ALRMLG
ryr1	Rabbit	GLPGVGVTTS LRPPHHFSPP CFVAALPAAG AAEAPARLSP AIPLEALRDK ALRMLG

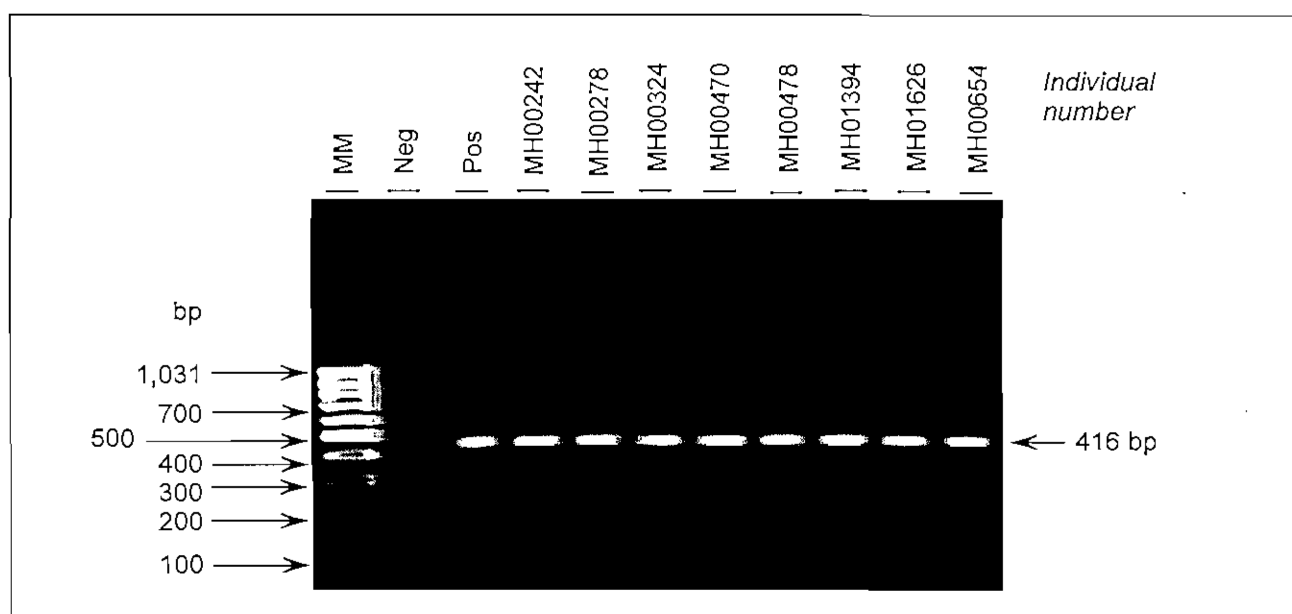
RyR1 = RyR expressed in human skeletal muscle; RyR2 = RyR expressed in human cardiac muscle; RyR3 = RyR expressed in human brain; *ryr1* = RyR1 protein expressed in animals. Amino acid residues that are not conserved among different isoforms and species are highlighted in grey. The site of the reported Pro amino acid at nucleotide 5360 which was observed in the study presented here is indicated in red. The site at which the previously reported alteration that occurs in the depicted region is indicated as follows: Leu1786Pro = blue. The accession numbers are as follows: RyR1 human = P21817; RyR2 human = Q92736; RyR3 human = NP_001027; *ryr1* pig = P16960 and *ryr1* rabbit = P11716.

This investigation represents the first report of this alteration in the South African population. The Pro1787Leu alteration has previously been identified in two MH families (Gillard *et al.*, 1992). In one family, the alteration was observed in the proband and the father of the proband, however, it was not identified in the brother who was diagnosed via the IVCT as being MHS. In the second family the substitution was present in the MHN father of the proband, however, it was not identified in either of the MHS children. Therefore, the authors indicated that the alteration was a polymorphism and not a candidate mutation. In the study presented here, the alteration was observed in the proband (MH114-1) and in the sister of the proband (MH114-7), who was diagnosed as MHN. The alteration was not observed in any other members of the family, including MH114-5 and MH114-6, both of whom were diagnosed as MHS. As the alteration has not been identified in a conserved region of the RyR1 protein and is observed in both MHN and MHS individuals, it is unlikely that the alteration is causative of the MH phenotype in pedigree MH114. The Pro1787Leu alteration may play a secondary role in the development of the disorder. As discussed in Section 5.1 (page 393), the alteration may have a minor phenotypic effect, in which additional alterations and/or polymorphisms and environmental factors contribute to the phenotype. However, it is likely that a second unidentified alteration may result in the disorder in family MH114.

4.10.16 Exon 35 of the RYR1 gene

Exon 35 has not been reported to harbour any reported alterations that result in the MH phenotype. A 416 bp product was successfully amplified in 15 South African MH probands and the PCR was optimised as described in Section 4.2 (page 159). The results of the amplified region encompassing exon 35 are illustrated in Figure 4.137. All 15 samples obtained via PCR were successfully sequenced according to the standard protocol. The forward primer (RYRex35F) was used for sequencing. A representative electropherogram obtained for individual MH00381, depicting a portion of the amplified region of exon 35, is indicated in Figure 4.138.

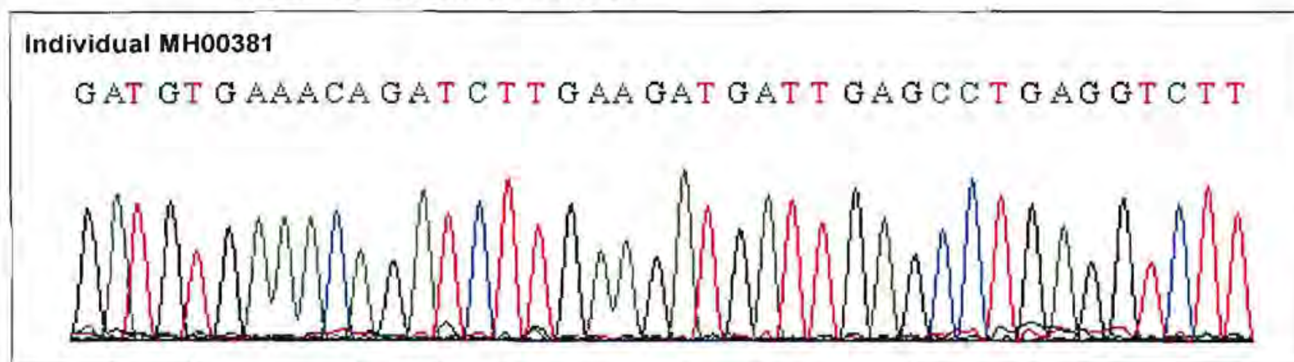
Figure 4.137: Photographic representation of amplified PCR products encompassing exon 35



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. As discussed in Section 4.3, the MM appears distorted. Variation in amplification efficiency was observed between exonic regions, as discussed in Section 4.2.

Alterations associated with MH have not been reported in exon 35. In the study presented here, alterations associated with MH were not observed in any of the South African MH probands. However, Zorzato *et al.* (1990) indicated that a low affinity Ca^{2+} binding site occurred at residues 1873 - 1923 (encoded by exon 35), indicating that this region of the RYR1 gene plays a functional role and if mutated could result in disease. Screening of various populations of adequate sample size would therefore have to be conducted in order to clarify the role of this exon in the development of MHS, as discussed in Section 4.10.2 (page 275).

Figure 4.138: Representative electropherogram illustrating a portion of the amplified region of exon 35

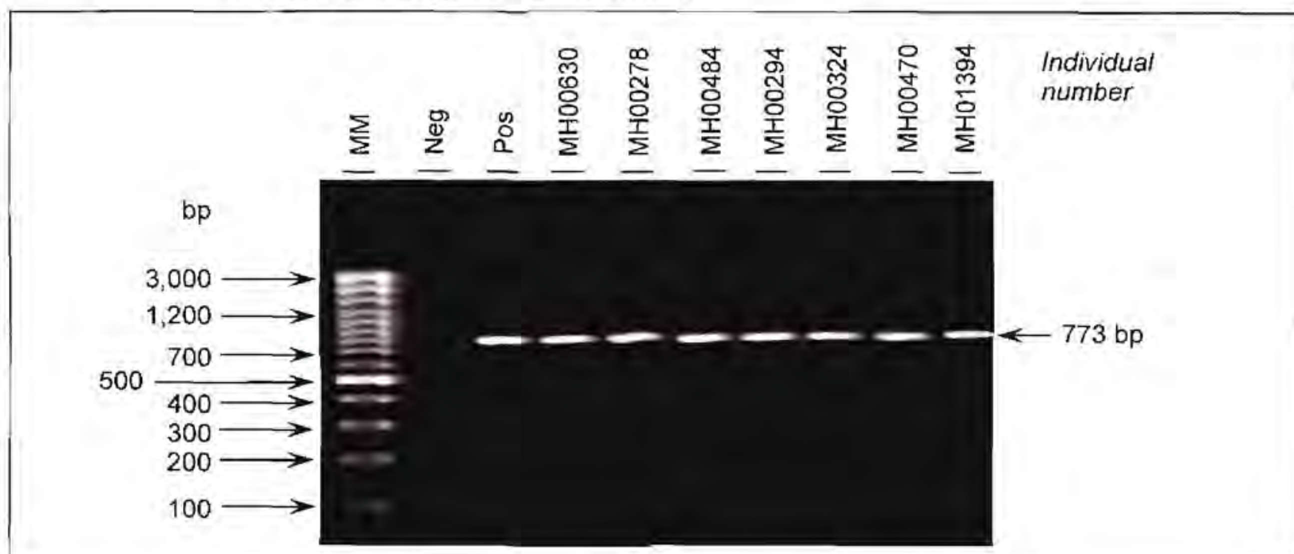


A = adenine; C = cytosine; G = guanine; T = thymine. As discussed in Section 4.5, background peaks were observed.

4.10.17 Exons 36 and 37 of the RYR1 gene

Alterations associated with MH have never been reported to occur in either exons 36 or 37. Using optimised PCR conditions listed in Table 4.1 (page 161) resulted in efficient amplification of the 773 bp product. The amplified product was subsequently electrophoresed on a 2% (w/v) mini agarose gel as depicted in Figure 4.139.

Figure 4.139: Photographic representation of amplified PCR products encompassing exons 36 and 37

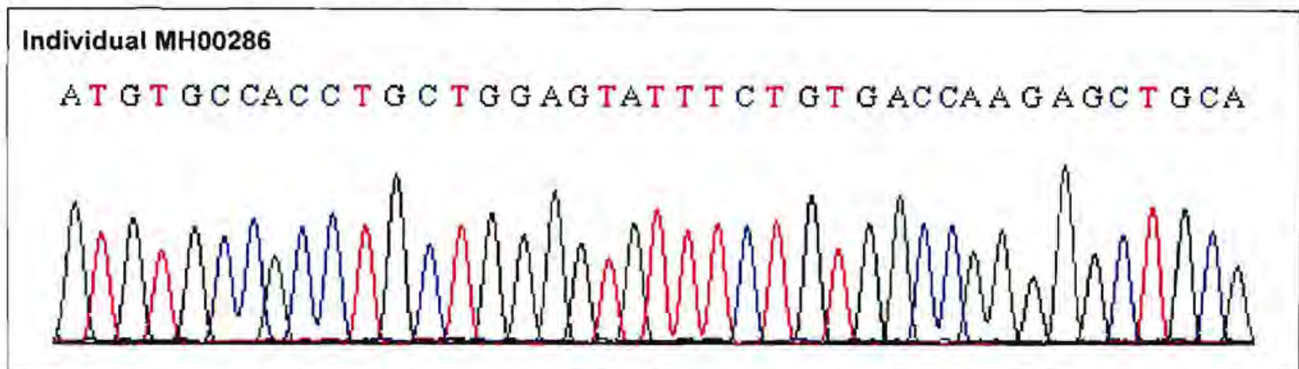


Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. As discussed in Sections 4.2 and 4.3, the MM and amplified products appeared distorted.

Fifteen MH probands that have previously not been analysed for novel alterations in this region of the RYR1 gene were screened during the Phase 3 study. A representative result generated via automated sequencing is presented in Figure 4.140 for exon 36 and is depicted in Figure 4.141 for exon 37. The forward primer (RYRex36F) was used and all 15

samples analysed in this molecular investigation were amplified and sequenced successfully.

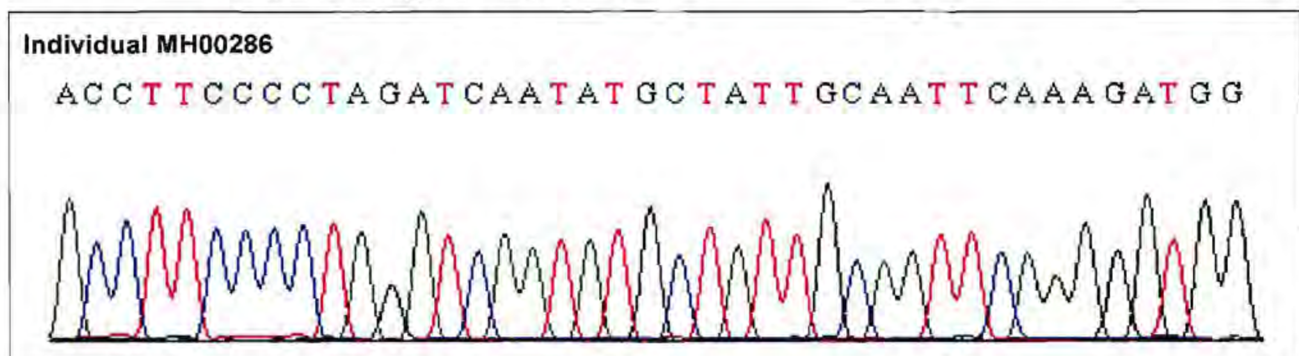
Figure 4.140: Representative electropherogram illustrating a portion of the amplified region of exon 36



A = adenine; C = cytosine; G = guanine; T = thymine.

Novel alterations were not identified in any of the 15 South African MH probands analysed in the study presented here. As exons 36 and 37 have only recently been analysed worldwide (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006), the frequency of alterations in different populations has not been determined, as discussed in Section 4.10.2 (page 275).

Figure 4.141: Representative electropherogram illustrating a portion of the amplified region of exon 37



A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.17.1 Synonymous substitutions in the amplified region of exon 36 and 37 of the RYR1 gene

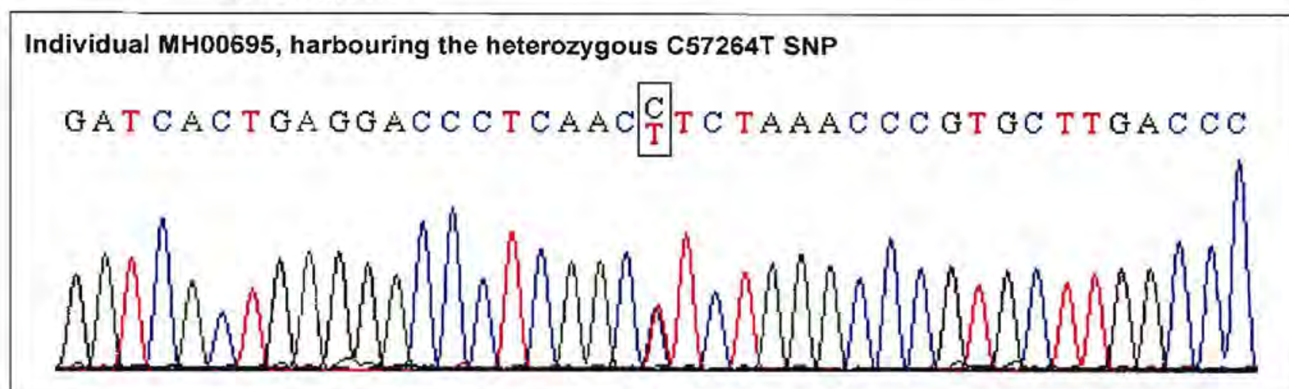
Two SNPs were identified within the analysed region of exons 36 and 37. The C57264T SNP was detected in the intron sequence between exons 36 and 37 and the A57545G SNP was identified in exon 37 of the RYR1 gene. Both synonymous substitutions are indicated as SNPs of the RYR1 gene in Genbank® and have been reported to be in LD

(International Human Genome Sequencing Consortium, 2004; with accession numbers rs17708009 and rs2228068, respectively). LD between the C57264T and A57545G SNPs has been identified in populations from Europe, Asia, North America and Africa. As discussed in Section 4.7.9.1 (page 202), LD between the two SNPs occurred due to their physical distance. As discussed in Section 4.7.4.1 (page 185), synonymous SNPs observed in this region may have functional consequences. Susceptibility to MH may be due to epistasis, in which several different mutations and SNPs lead to a threshold being reached. The contribution of all these factors would have to be analysed further in order to resolve each of their effects on the MH phenotype.

4.10.17.1.1 SNP C57264T

Analysis of 15 MHS probands from South Africa identified four individuals that were heterozygous for the C57264T SNP, as listed in Appendix C (page 451). The remaining individuals did not harbour the SNP. The genotype and allele frequencies worldwide for the C57264T SNP have been determined and vary between different populations (International Human Genome Sequencing Consortium, 2004; with accession number rs17708009). In the majority of cases, the C/C genotype is the most common and the T/T genotype has never been observed in any populations analysed. The European population had the following genotype frequencies: 0.79 for C/C and 0.21 for C/T. In the Asian population the genotype frequencies were as follows: 0.98 for C/C and 0.02 for C/T. Lastly, the population from Sub-Saharan Africa had the following genotype frequencies: 0.97 for C/C and 0.03 for C/T (International Human Genome Sequencing Consortium, 2004; with accession number rs17708009). Figure 4.142 depicts the sequences generated for an individual heterozygous for the C57264T SNP.

Figure 4.142: Representative electropherogram indicating the C57264T SNP observed in the intron sequence between exons 36 and 37 of the RYR1 gene

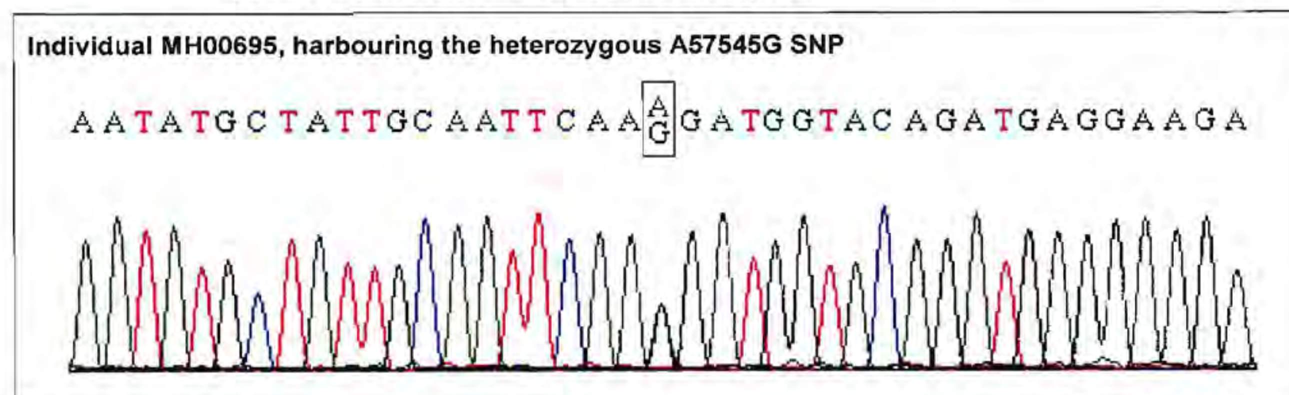


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.17.1.2 SNP A57545G

Analysis of 15 MHS probands from South Africa identified four individuals that were heterozygous for the A57545G SNP. The remaining individuals did not harbour the SNP, as listed in Appendix B (page 447). The worldwide genotype frequencies per population have also been determined for the A57545G SNP and the most common genotype is A/A. The G/G genotype has never been observed in any population analysed, worldwide (International Human Genome Sequencing Consortium, 2004; with accession number rs2228068). The average frequencies of the SNP in the three populations (European, Asian and Sub-Saharan Africa) are as follows: 0.88 for A/A and 0.12 for A/G (International Human Genome Sequencing Consortium, 2004; with accession number rs2228068). Figure 4.143 depicts the sequence generated for an individual heterozygous for the A57545G SNP.

Figure 4.143: Representative electropherogram indicating the A57545G SNP observed in exon 37 of the RYR1 gene

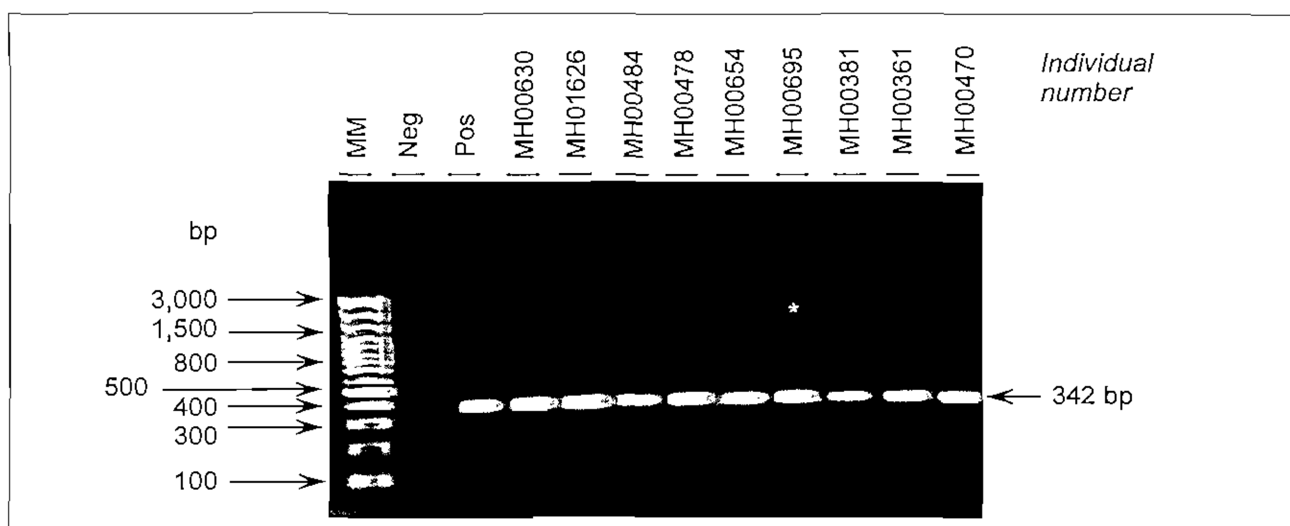


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.18 Exon 38 of the RYR1 gene

Exon 38 harbours a single alteration, Gly2060Cys that is due to a G6178T transition. The alteration has previously been identified in one individual diagnosed as MHS via the IVCT (Gillard *et al.*, 1992). In the study presented here, 15 South African MH probands were analysed for the first time for reported or novel alterations that may occur in exon 38 of the RYR1 gene, using optimised PCR conditions listed in Table 4.1 (page 161). Figure 4.144 is a photographic representation of the amplicon encompassing exon 38 of the RYR1 gene.

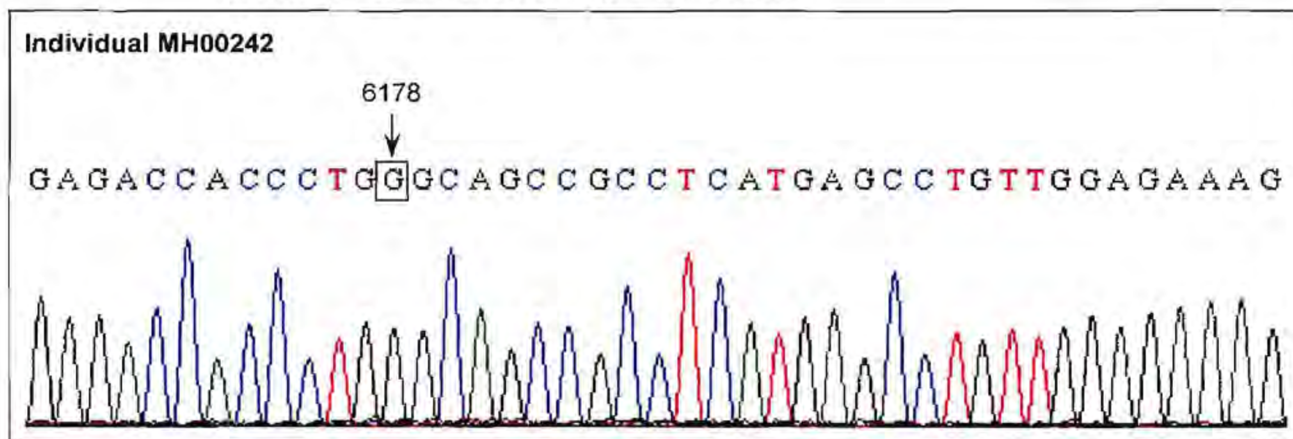
Figure 4.144: Photographic representation of amplified PCR products encompassing exon 38



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. An artefact in the gel matrix, as indicated by white asterisk (*), variation in amplification efficiency of the amplicons, a barrier in the gel and distortion of the MM were observed, as discussed in Sections 4.2 and 4.3.

A total of 15 MH probands were successfully amplified and sequenced according to the standard protocol. Four individuals, namely MH00294, MH00695, MH00470 and MH00478, harboured the Gly2060Cys alteration in exon 38, as discussed in Section 4.10.18.1 (page 314). The remaining 11 South African MH probands analysed in the study presented here did not harbour this alteration. A representative electropherogram obtained for individual MH00242, illustrating the nucleotide position of the previously reported Gly2060Cys alteration, is presented in Figure 4.145.

Figure 4.145: Representative electropherogram of exon 38 indicating the nucleotide position of the Gly2060Cys alteration

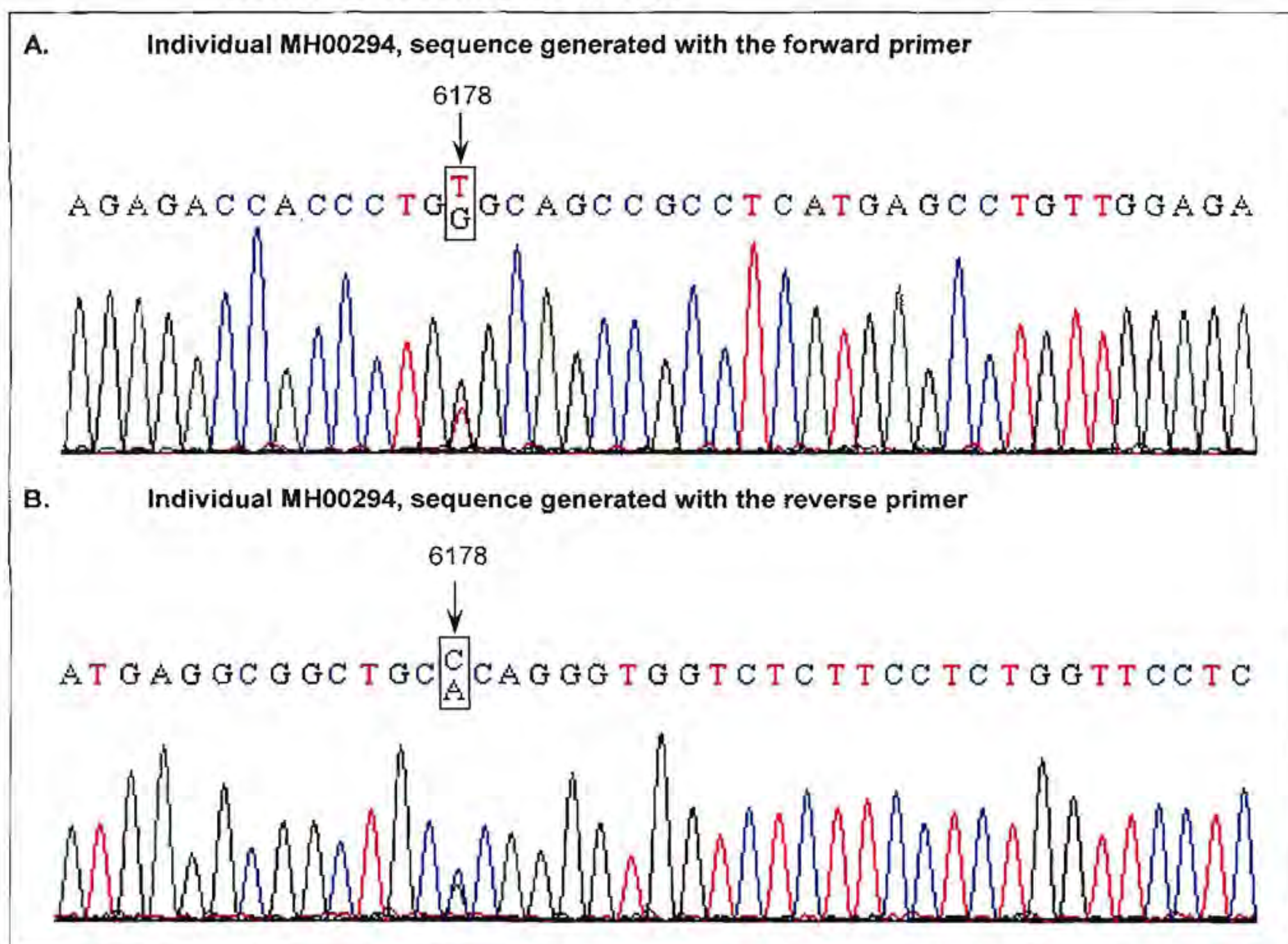


A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Gly2060Cys at nucleotide 6178.

4.10.18.1 The Gly2060Cys alteration in the RYR1 gene

At nucleotide position 6178, two peaks were observed that had similar intensities. The result indicates the presence of two bases, G and T, which results in the expression of two different amino acids, namely Gly and Cys. The sequence depicted on the electropherogram of Figure 4.146A indicates the alteration observed using the forward primer and similarly the sequence depicted on the electropherogram of Figure 4.146B indicates the alteration observed using the reverse primer. Sequencing of exon 38 in the forward and reverse directions confirmed the presence of the Gly2060Cys alteration.

Figure 4.146: Representative electropherograms indicating the Gly2060Cys alteration observed in exon 38



A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Gly2060Cys at nucleotide 6178.

The variant affects an amino acid that is conserved among RyR1 species, but is not conserved among RyR isoforms. Table 4.13 depicts the partial amino acid sequences of exon 38 of the RyR1 protein obtained from RyR isoforms and other vertebrate *ryr1* proteins. Multiple alignments encompassing the position of the alteration were obtained from GenBank® (P21817, Q92736, NP_001027, P16960 and P11716), and the position of the Gly2060Cys alteration detected in this study is indicated in blue. As discussed in Section 4.10.15.1 (page 305), alterations that result in disease may not reside in highly conserved regions.

Table 4.13: Conserved amino acids from different RyR isoforms and species surrounding the novel and reported mutation in exon 38 of the RyR1 protein

Isoform	Species	Alignment of RyR protein sequences
RyR1	Human	CPLPEEIRQD LLDFHQDLLA HCGIQLDGEE EEPEEETTLG SRLMSLLEKVR
RyR2	Human	CPCPEEIRDQ LLDFHEDLMT HCGIELDEDG SDGNSDLTIR GRLLSLVEKVK
RyR3	Human	CPCPEEIREE YLDFHEDLLL HCGVPLE-EE EEEEEEDTSW GKLCALVYKIK
ryr1	Pig	CPLPDEIRQD LLEFHQDLLT HCGIQLEGEE EEPEEEATLG SRLMSLLEKVR
ryr1	Rabbit	CPLPEEVRQD LLEFHQDLLA HCGIQLEGEE EEPEEEATLG SRLMSLLEKVR

RyR1 = RyR expressed in human skeletal muscle; RyR2 = RyR expressed in human cardiac muscle; RyR3 = RyR expressed in human brain; *ryr1* = RyR1 protein expressed in animals. Amino acid residues that are not conserved among different isoforms and species are highlighted in grey. The site of the reported Gly amino acid at nucleotide 6187 is indicated in blue. The accession numbers are as follows: RyR1 human = P21817; RyR2 human = Q92736; RyR3 human = NP_001027; *ryr1* pig = P16960 and *ryr1* rabbit = P11716.

This Gly2060Cys alteration represents the first mutation in exon 38 associated with MHS in the South African population. However, the Gly2060Cys alteration in the study conducted by Gillard *et al.* (1992) failed to segregate with MH in the family tested, which may indicate that the alteration is not causative of MH. In addition, functional analysis of the Gly2060Cys alteration has been conducted, as listed in Appendix A (page 443). Zhou *et al.* (2006a) indicated that the alteration did not result in channel hypersensitivity. However, the authors indicated that low levels of RyR1 were detected, indicating that the Gly2060Cys alteration may play a role in the development of the MH phenotype.

In the study presented here, the alteration segregated with the MH phenotype in one South African family (MH108). The alteration was observed in the proband, MH00470 (MH108-1) and the three children of the proband, MH108-3, MH108-4 and MH108-5, who were all diagnosed as MHS. The alteration was absent in the MHN wife of the proband (MH108-2). The Gly2060Cys alteration was the second RYR1 alteration observed in this family. An Arg2336His alteration was identified in exon 43 of the RYR1 in this family, as discussed in Section 4.8.4.1 (page 222). The presence of two alterations in a single proband has been reported previously. Ibarra *et al.* (2006) reported six probands that harboured potentially causative compound heterozygous sequence variations and indicated that the individuals exhibited CICR enhancement. Therefore, although the Gly2060Cys alteration may not be causative of MH in this family, it may contribute to the MH phenotype via epistasis, as discussed in Section 5.4 (page 406). Functional analysis would have to be conducted in order to determine if the presence of both alterations affects the MH phenotype and results in a fulminant MH episode.

In family MH122, the Gly2060Cys alteration was observed in the proband MH00478 (MH122-1) and was absent in the child of the proband (MH122-2). The MH status of

MH122-2 is currently unknown. Thus far, analysis of the coding region of the RYR1 gene identified only the Gly2060Cys alteration in this family. This may indicate either that the alteration plays a role in the development of the disorder or that a second unidentified alteration plays a primary role in the development of MH in family MH122. Zhou *et al.* (2006a) reported low levels of the RyR1 protein in HEK293 cells that harboured the Gly2060Cys alteration, as listed in Appendix A (page 443). The consequence of low levels of RyR1 protein is currently unknown. In addition, as discussed in Section 5.2.3 (page 402), as multiple RYR1 isoforms exist in a variety of tissues, alterations observed in the skeletal muscle RYR1 may also exist in other tissues. Therefore, a single alteration may affect a wider variety of organelles than previously thought.

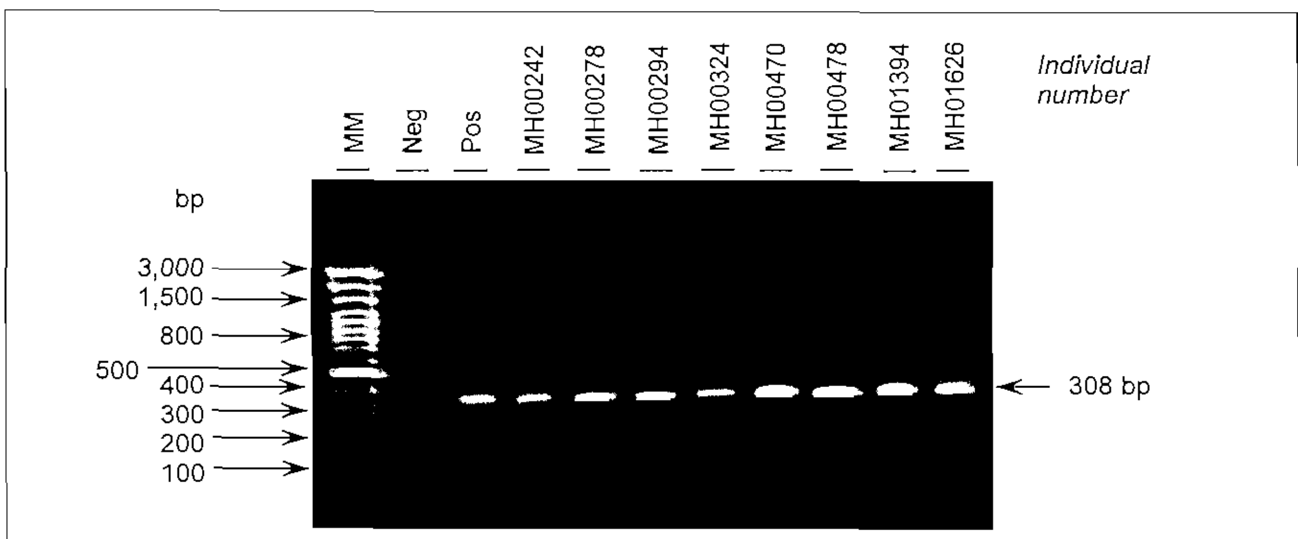
In the study presented here, two South African families exhibited discordance between the Gly2060Cys alteration and the IVCT phenotype. The Gly2060Cys alteration was observed in proband MH00294 (MH104-26) and was subsequently identified in the mother of the proband (MH104-19), who had never been tested for MH. The alteration was not identified in the brother of the proband (MH104-24), who has been tested as MH positive, or in his wife (MH104-25) who had been diagnosed as MHN. In family MH114, the proband MH00695 (MH114-1) harboured the alteration and the alteration was also observed in MH114-7 who was diagnosed as MHN. The alteration was absent in the remaining members of the family, including two MHS individuals, namely MH114-5 and MH114-6. The Gly2060Cys alteration represents the second alteration observed in both families MH104 and MH114. The proband (MH114-1) was also observed to harbour the Pro1787Leu alteration, as discussed in Section 4.10.15.1 (page 305). The proband (MH104-26) was identified to harbour the Gly4935Ser alteration, as discussed in Section 4.9.12.1 (page 265). Both the Gly2060Cys and the Pro1787Leu alterations were observed in the proband (MH114-1) and the MHN sister of the proband (MH114-7). In family MH104, the Gly2060Cys alteration was observed in the mother of the proband who was not tested and was absent in an individual diagnosed as MHS. In addition, the Gly4935Ser alteration was only observed in the proband. This may indicate that the discordant IVCT results obtained from families MH114 and MH104 are either false positive or false negative diagnoses. However, as all three alterations may represent polymorphisms, the exact role that they play in the development of the disorder is currently unknown. The presence of two alterations that are not causative of MH in an MH proband has been reported previously. Zhou *et al.* (2006a) described a proband that had inherited the Ala1577Thr alteration from one parent and the Gly2060Cys alteration from the other parent. However, neither of the substitutions affected the functional properties of the RyR1

channel. Therefore, all three alterations may contribute to the MH phenotype via epistasis, as discussed in Section 5.4 (page 406).

4.10.19 Exon 47 of the RYR1 gene

Exon 47 occurs outside the mutational hotspots, adjacent to the third mutational hotspot. The 308 bp product encompassing the alteration was successfully amplified using an optimised PCR protocol, as described in Section 4.2 (page 159), and is depicted in Figure 4.147.

Figure 4.147: Photographic representation of amplified PCR products encompassing exon 47



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. As discussed in Sections 4.2 and 4.3, variation in amplification efficiency, MM and amplified fragment distortion were observed.

Recently, an Arg2508His alteration was detected in exon 47 in one MH family from Italy (Galli *et al.*, 2006). In addition, four alterations have been reported in probands diagnosed with CCD, as listed in Table 4.14.

Table 4.14: Reported alterations in exon 47 of the RYR1 gene

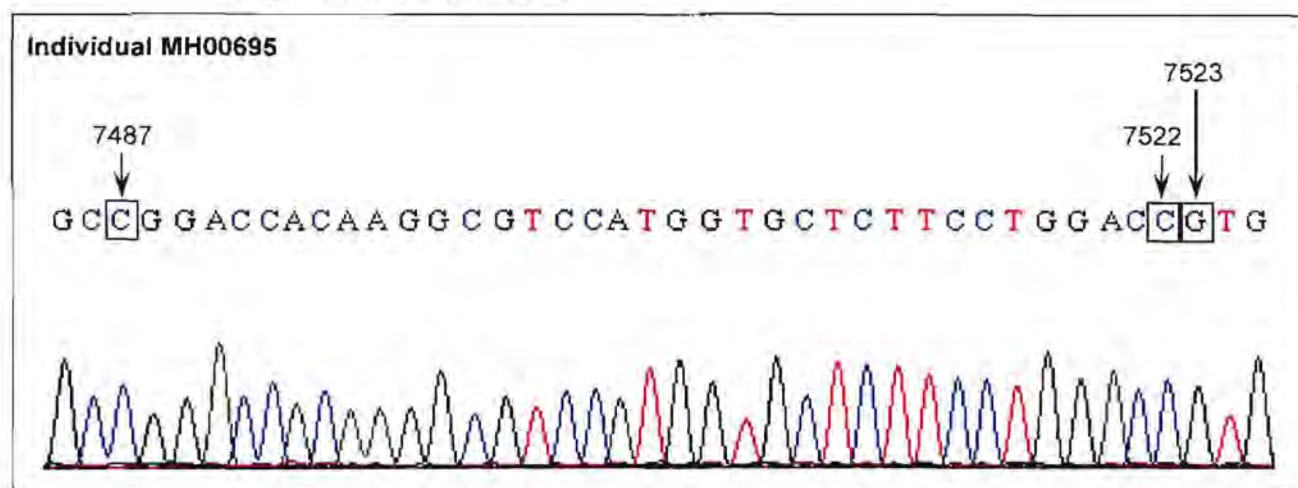
Amino acid change	Nucleotide change	Reference	Amino acid change	Nucleotide change	Reference
Pro2496Leu[1]	C7487T	Ibarra *et al.*, 2006	Arg2508Gly[1]	C7522G	Ibarra *et al.* 2006
Arg2508Cys[1]	C7522T	Wu *et al.*, 2006	Arg2508His	G7523A	Galli *et al.*, 2006

[1] = indicates alterations observed in patients clinically diagnosed with CCD.

All 15 MH probands that were amplified via PCR were subsequently sequenced using the standard protocol. A representative electropherogram obtained for individual MH00695

illustrating the nucleotide positions of the previously reported alterations observed in exon 47 is indicated in Figure 4.148. None of the 15 South African MH individuals that were sequenced harboured the Pro2946Leu, Arg2508Cys, Arg2508Gly and Arg2508His alteration or any novel alterations. It would be expected that the alterations previously observed in probands clinically diagnosed with CCD would not be observed in any of the South African probands analysed in the study presented here, as the South African probands included in this investigation have not been diagnosed with CCD. As this Arg2508His alteration has only been observed recently (Galli *et al.*, 2006), the frequency of the alteration has not been determined in other populations. Masumiya *et al.* (2003) reported, using the yeast two hybrid technique, that amino acids 2497 - 2520 (coded by exon 47) consist of residues that interact with FKBP12. The interaction between the FKBP12 and RyR1 is responsible for stabilising the full conductance state, channel gating and modulating the sensitivity of Ca^{2+} activation of the RyR1. Therefore, although South African MH probands analysed in the study presented here did not harbour any alterations associated with MH, further analysis in a larger group of individuals should be conducted for exon 47, due to its functional importance, as discussed in Section 4.10.2 (page 275).

Figure 4.148: Representative electropherogram of exon 47 indicating the nucleotide positions of the Pro2946Leu, Arg2508Cys, Arg2508Gly and Arg2508His alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Pro2496Leu at nucleotide 7487; Arg2508Cys and Arg2508Gly at nucleotide 7522 and Arg2508His at nucleotide 7523.

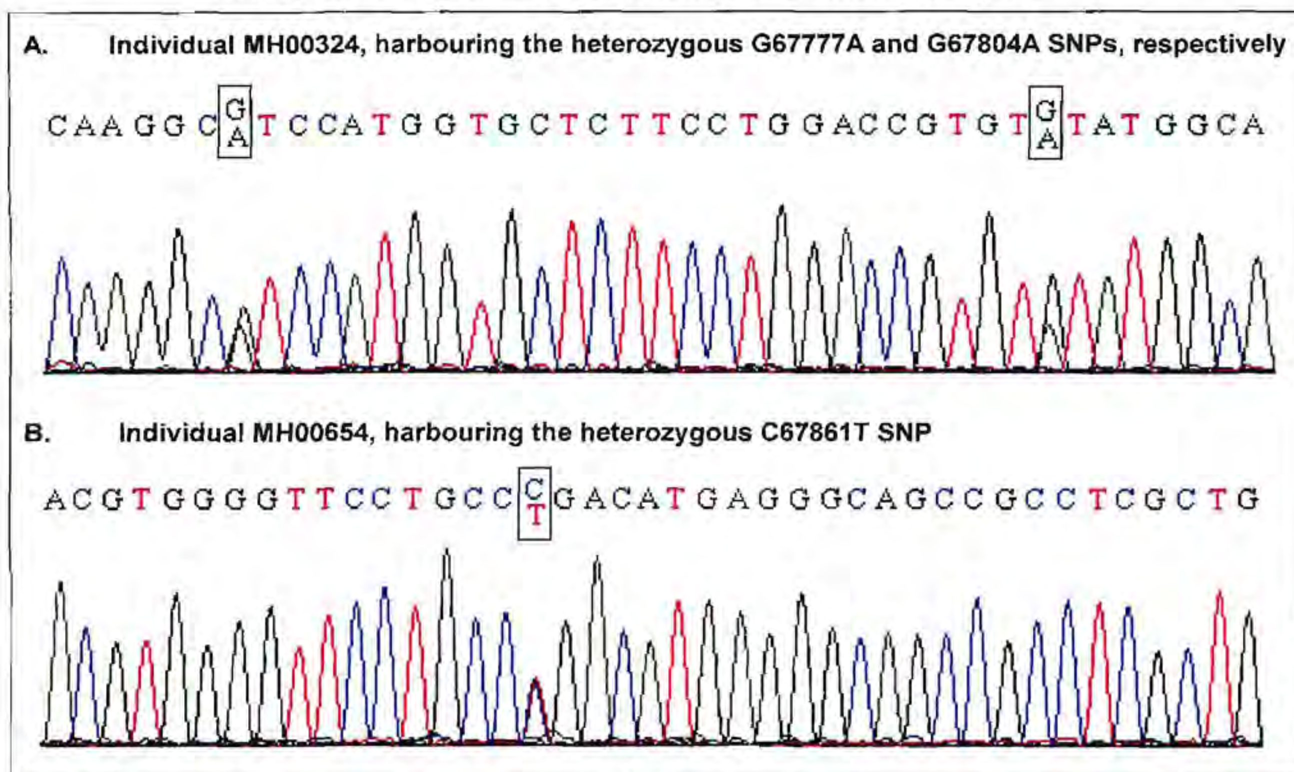
4.10.19.1 Synonymous substitutions in the amplified region of exon 47 of the RYR1 gene

Within the amplified region of exon 47, four SNPs were identified. Three SNPs (G67777A, G67804A and C67861T) occurred in exon 47 and the fourth SNP (C67901G) was observed in the intron sequence between exons 47 and 48, as listed in Appendices B and C (pages 447 and 451). As discussed in Section 4.7.4.1 (page 185), the SNPs may affect gene expression and function of the RYR1 gene. In this regard, SNPs observed in the RYR1 gene may not be directly associated with MH, however, via epistasis each SNP, in combination may affect the phenotype of a specific individual, which could not be predicted from their separate effects.

4.10.19.1.1 SNP G67777A

The heterozygous G67777A SNP was observed in three South African MH probands. All three individuals harboured two peaks representing two different nucleotides, namely G and A. The sequence depicted in Figure 4.149A is a representative electropherogram illustrating the heterozygous G67777A SNP. The G67777A synonymous substitution retains the Ala amino acid and is indicated as a SNP of the RYR1 gene in Genbank® (International Human Genome Sequencing Consortium, 2004; with accession number rs2228072). The genotype frequencies for the SNP have been reported in a variety of populations, including North American, African American, Asian and European (International Human Genome Sequencing Consortium, 2004; with accession number rs2228072). The G/G genotype is the most common and has been identified to have a frequency of 0.86, whereas the A/G and A/A genotypes are less likely to occur and have frequencies of 0.09 and 0.05, respectively.

Figure 4.149: Representative electropherograms indicating the G67777A and G67804A SNPs observed in exon 47 of the RYR1 gene



A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.19.1.2 SNP G67804A

The heterozygous G67804A SNP was detected in two South African MH probands. Figure 4.149A is a representative electropherogram illustrating the heterozygous G67804A SNP. The G67804A synonymous substitution retains the Val amino acid and has been reported as a SNP of the RYR1 gene in Genbank® (International Human Genome Sequencing Consortium, 2004; with accession number rs2071088). The genotype frequencies have been reported in a variety of populations, including North American, African American, Asian and European (International Human Genome Sequencing Consortium, 2004; with accession number rs2071088). For the G67804A SNP the G/G genotype is the most common (frequency between 0.83 and 0.86), whereas the A/G genotype is less likely to occur (frequency between 0.15 and 0.14). However, the A/A genotype has never been identified worldwide for the G67804A SNP (International Human Genome Sequencing Consortium, 2004; with accession number rs2071088).

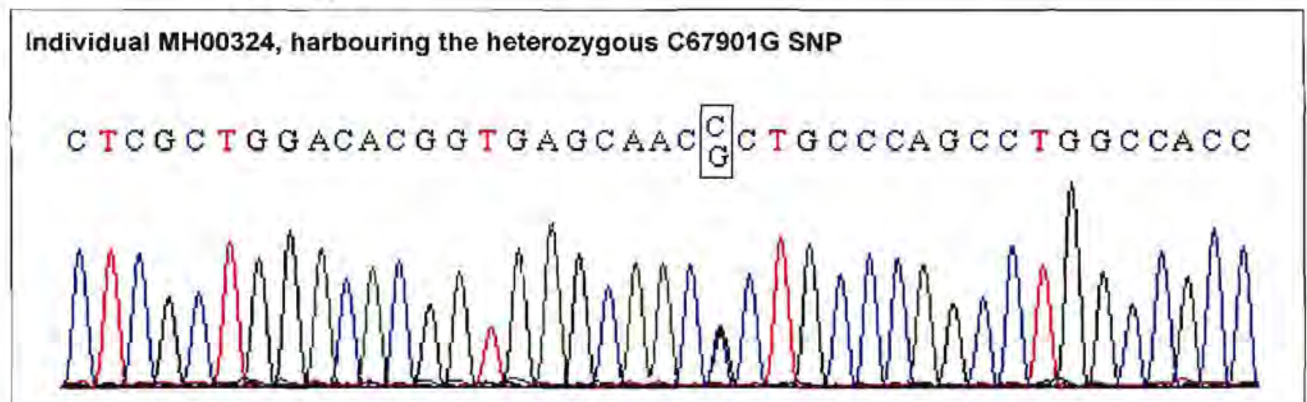
4.10.19.1.3 SNP C67861T

Two South African MH probands analysed in the study presented here harboured the heterozygous C67861T SNP. The SNP occurs in the coding region of exon 47 and retains the Pro amino acid. The synonymous substitution has been reported as a SNP in Genbank® (International Human Genome Sequencing Consortium, 2004; with accession number rs1465698). However, the genotype and allele frequencies for this alteration have not been reported, worldwide. The sequence illustrated in Figure 4.149B is a representative electropherogram illustrating the heterozygous C67861T SNP.

4.10.19.1.4 SNP C67901G

In addition to SNPs in the coding region of exon 47, a single SNP was identified in the intron sequence between exons 47 and 48. The heterozygous C67901G SNP was observed in four South African MH probands. The C67901G synonymous substitution has been reported as a SNP in Genbank® (International Human Genome Sequencing Consortium, 2004; with accession number rs2960323) and the genotype frequencies for this alteration have not been determined, worldwide. The sequence illustrated in Figure 4.150 is a representative electropherogram illustrating the heterozygous C67901G SNP.

Figure 4.150: Representative electropherogram indicating the C67901G SNP observed in the intron sequence between exons 47 and 48 of the RYR1 gene



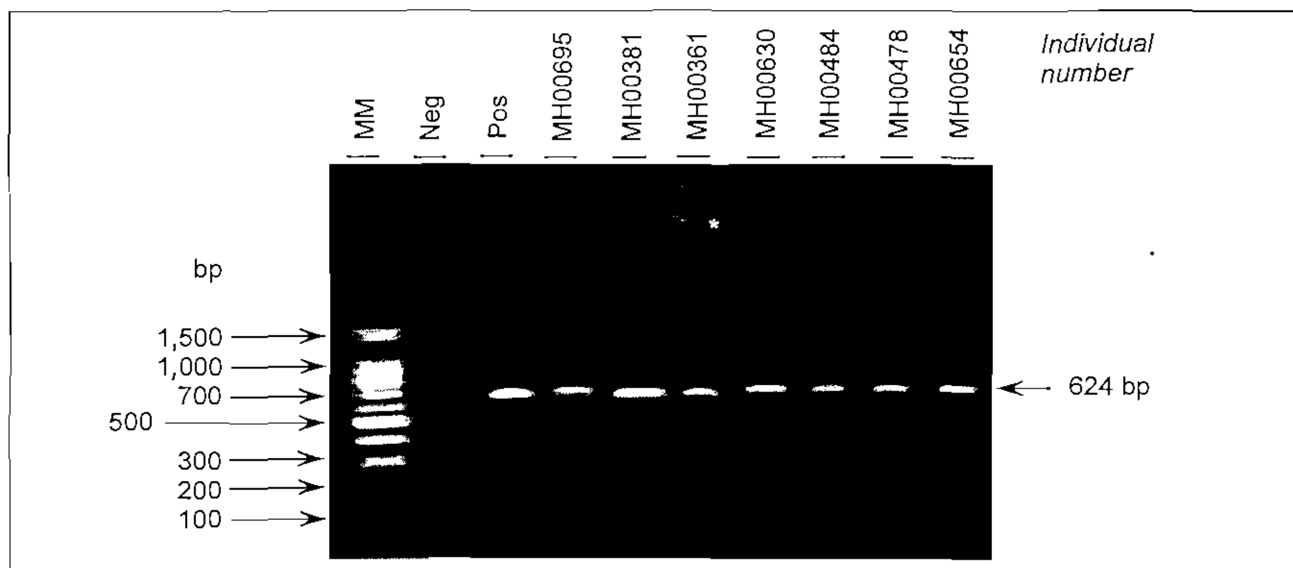
A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.20 Exons 48 and 49 of the RYR1 gene

The standard PCR protocol was used to amplify a 624 bp region, encompassing exons 48 and 49 of the RYR1 gene, as discussed in Section 4.2 (page 159). The results of the PCR

amplification encompassing exons 48 and 49 are depicted in Figure 4.151. This region of the RYR1 gene harbours two reported alterations, Arg2591Gly and Val2627Leu, which have both been reported in one Italian family diagnosed with MH (Galli *et al.*, 2006). In addition, the Glu2545Asp alteration has been identified in a proband diagnosed with CCD (Wu *et al.*, 2006). The amplified region is located outside the mutational hotspots, in close proximity to the second hotspot.

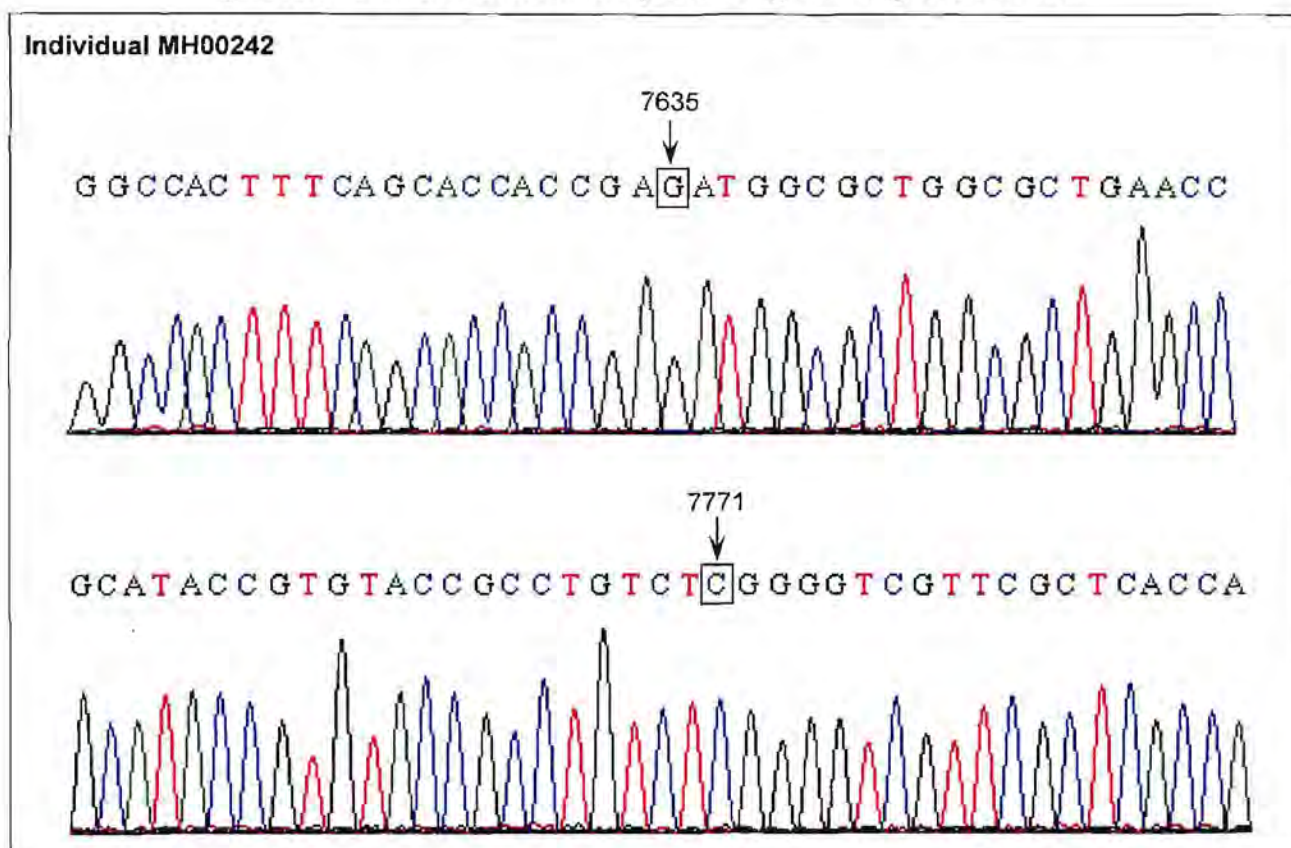
Figure 4.151: Photographic representation of amplified PCR products encompassing exons 48 and 49



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Variation in amplification efficiency between exonic regions and MM distortion were observed, as discussed in Sections 4.2 and 4.3. An artefact in the gel matrix, as indicated by the white asterisk (*) was noted, as discussed in Section 4.3.

Sequencing of the amplified region was conducted using the forward primer (RYRex48F). A representative electropherogram obtained for individual MH00242, illustrating the nucleotide positions of the reported alterations previously observed in exon 48, is depicted in Figure 4.152.

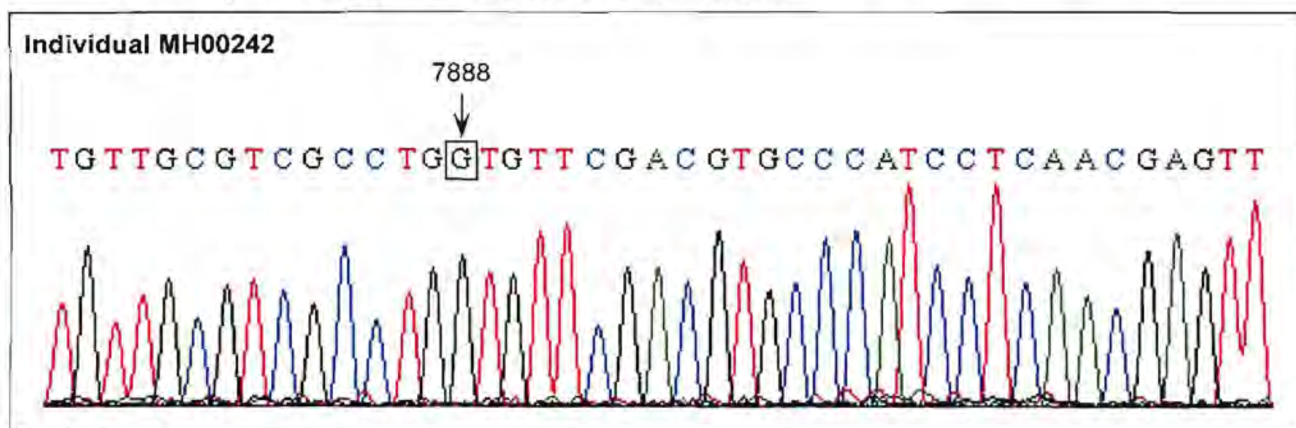
Figure 4.152: Representative electropherogram of exon 48 indicating the nucleotide positions of the Glu2545Asp and Arg2591Gly alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Glu2545Asp at nucleotide 7635 and Arg2591Gly at nucleotide 7771.

A representative electropherogram obtained for individual MH00242, illustrating the nucleotide position of the reported Val2627Leu alteration in exon 49, is depicted in Figure 4.153. To date three alterations have been reported to occur in this region of the RYR1 gene. Each of the Glu2545Asp, Arg2591Gly and Val2627Leu alterations has been reported in single families diagnosed with MH or CCD (Galli *et al.*, 2006; Wu *et al.*, 2006). The frequency of these alterations in other populations has not yet been determined, and the alterations may be specific to the above-mentioned families or may occur more frequently, as discussed in Section 4.10.2 (page 275). Although these alterations, as well as novel alterations, were not observed in any of the 15 MH probands that were sequenced in Phase 3, novel alterations are continually being reported and should be analysed in each population to determine their frequency.

Figure 4.153: Representative electropherogram of exon 49 indicating the nucleotide position of the Val2627Leu alteration

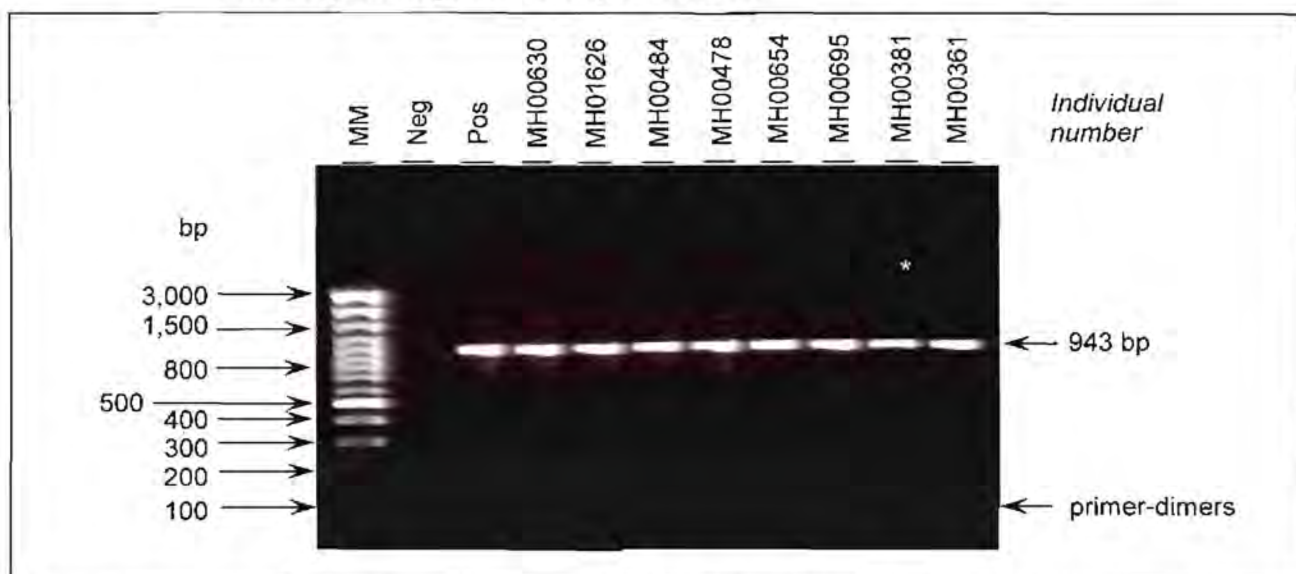


A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Val2627Leu at nucleotide 7888.

4.10.21 Exons 50, 51 and 52 of the RYR1 gene

In order to identify novel or reported alterations associated with MHS in exons 50, 51 and 52 simultaneously, a 943 bp region was amplified, as described in Section 4.2 (page 159) and subsequently sequenced. Results of the amplified PCR product encompassing exons 50, 51 and 52 are presented in Figure 4.154.

Figure 4.154: Photographic representation of amplified PCR products encompassing exons 50, 51 and 52



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Primer-dimers, non-specific amplification, background smear, variation in amplification efficiency and distortion of the MM and amplified fragments were observed, as discussed in Sections 4.2 and 4.3. In addition, an artefact in the gel matrix, as indicated by the white asterisk (*), was noted, as discussed in Section 4.3.

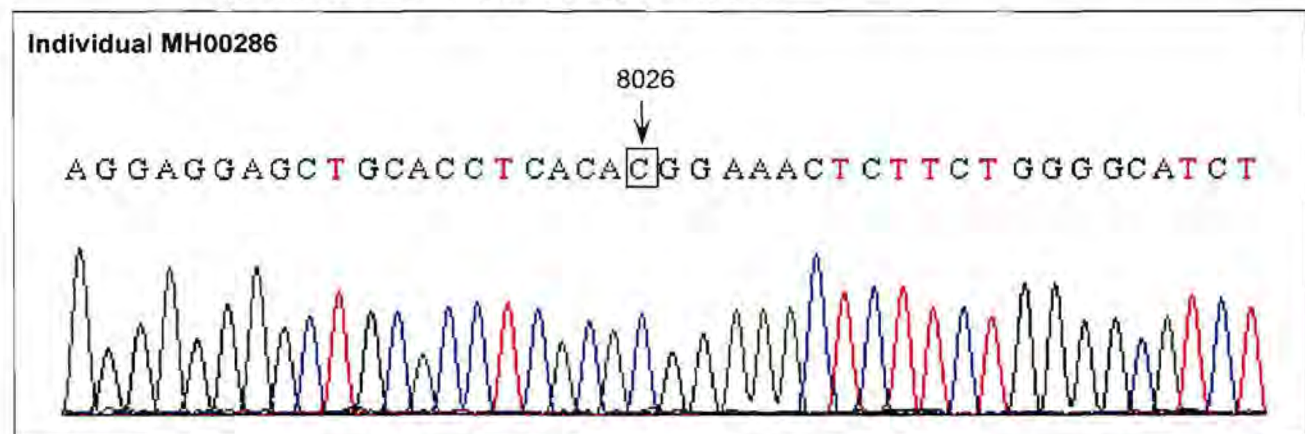
Amplification of this region of the RYR1 gene encompassing the three exons was obtained for all 15 samples obtained from the South African probands. Following PCR amplification, the PCR product was sequenced in order to investigate for the presence of novel or reported alterations that may occur in exons 50, 51 and 52. Table 4.15 lists alterations that have previously been reported in the amplified region in probands diagnosed with MH.

Table 4.15: Reported alterations in exons 50, 51 and 52 of the RYR1 gene

Amino acid change	Nucleotide change	Exon	Reference
Arg2676Trp	C8026T	50	Guis *et al.*, 2004
Asp2730His	G8188C	51	Ibarra *et al.*, 2006
Gly2733Asp	G8198A	51	Sambuughin *et al.*, 2005
Glu2764Lys	G8290A	52	Galli *et al.*, 2006

Sequencing was conducted using the forward primer (RYRex50F), according to the standard protocol. A representative electropherogram obtained for individual MH00286, illustrating the nucleotide position of the reported Arg2626Trp alteration previously observed in exon 50, is presented in Figure 4.155.

Figure 4.155: Representative electropherogram of exon 50 indicating the nucleotide position of the Arg2626Trp alteration



A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Arg2676Trp at nucleotide 8026.

A representative electropherogram obtained for individual MH00286, illustrating the nucleotide positions of the reported Asp2730His and Gly2733Asp alterations previously observed in exon 51, is presented in Figure 4.156. In addition, a representative electropherogram obtained for individual MH00286, illustrating the nucleotide position of the reported Glu2764Lys alteration previously observed in exon 52, is presented in Figure 4.157. Four alterations have been reported to occur in this region of the RYR1 gene (Guis *et al.*, 2004; Sambuughin *et al.*, 2005; Ibarra *et al.*, 2006; Galli *et al.*, 2006).

The Arg2676Trp alteration 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 in a single North American MH pedigree (Sambuughin *et al.*, 2005) and an Asp2730His alteration has been reported in one MH family (Ibarra *et al.*, 2006). In addition, the Glu2764Lys alteration was reported to occur in exon 52 in a single Italian family diagnosed with MH (Galli *et al.*, 2006).

Figure 4.156: Representative electropherogram of exon 51 indicating the nucleotide positions of the Asp2730His and Gly2733Asp alterations

A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Asp2730His at nucleotide 8188 and Gly2733Asp at nucleotide 8198.

The Arg2676Trp alteration has been reported in a single family (Guis *et al.*, 2004), the Gly2733Asp alteration was identified in a single North American MH pedigree (Sambuughin *et al.*, 2005), the Glu2764Lys alteration was reported to occur in a single Italian family (Galli *et al.*, 2006) and the Asp2730His alteration has been reported in one MH family from Japan (Ibarra *et al.*, 2006). None of the 15 individuals that were sequenced in the study presented here harboured any of the reported alterations. All four alterations have been observed in single families and may therefore indicate mutations that are family-specific. As these alterations are newly reported, their frequencies would have to be determined, as discussed in Section 4.10.2 (page 275). The presence of four different alterations from four different populations indicates that alterations outside the mutational hotspots play a role in the development of the MH phenotype. Therefore, all exons of the RYR1 gene should be investigated in order to identify all causative MH alterations.

Figure 4.157: Representative electropherogram of exon 52 indicating the nucleotide position of the Glu2764Lys alteration

A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Glu2764Lys at nucleotide 8290.

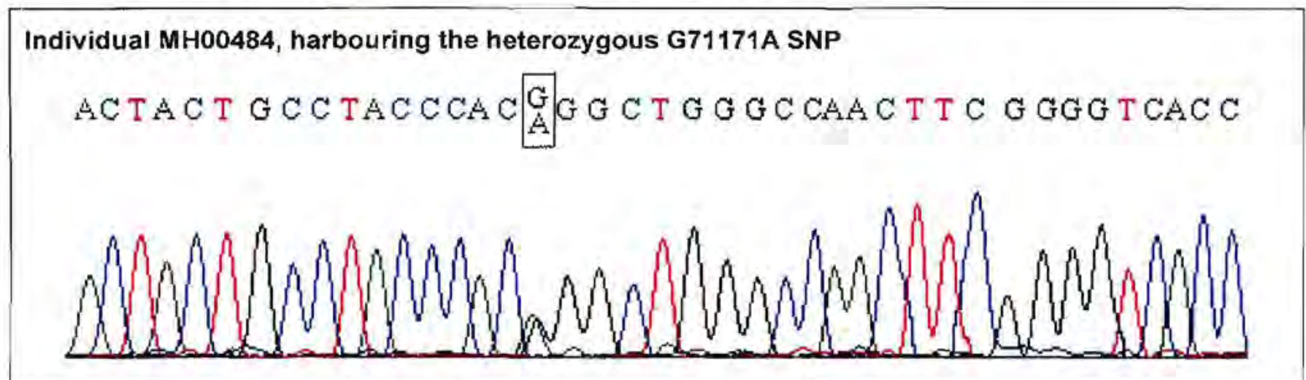
4.10.21.1 Synonymous substitutions in the amplified region of exons 50, 51 and 52 of the RYR1 gene

Within the amplified region of exons 50, 51 and 52, five SNPs (G71171A, G71413A, A71494G, T71699C and T71771C) were observed, as listed in Appendices B and C (pages 447 and 451). Although SNPs observed in the RYR1 gene may not play a primary role in the development of the MH phenotype, they may play an important secondary role, as discussed in Section 4.7.4.1 (page 185). Susceptibility to MH may therefore occur due to epistasis, in which several different alterations and SNPs contribute to the MH phenotype. The effect of all these determinants would have to be analysed further in order to resolve each of their contributions to the MH phenotype.

4.10.21.1.1 SNP G71171A

In three South African probands, as listed in Appendix A (page 443), the heterozygous G71171A SNP was detected. The SNP occurs in the coding region of exon 50 and retains a Thr amino acid. The sequence illustrated in Figure 4.158 is a representative electropherogram depicting the heterozygous G71171A SNP. The synonymous substitution has been reported as a SNP in Genbank® (International Human Genome Sequencing Consortium, 2004; with accession number rs2915951) and the genotype frequencies have been determined in two different populations, i.e. North American and African American. In both populations, the average frequency of the SNP genotypes was as follows: 0.58 for G/G, 0.26 for A/G and 0.16 for A/A (International Human Genome Sequencing Consortium, 2004; with accession number rs2915951).

Figure 4.158: Representative electropherogram indicating the G71171A SNP observed in exon 50 of the RYR1 gene

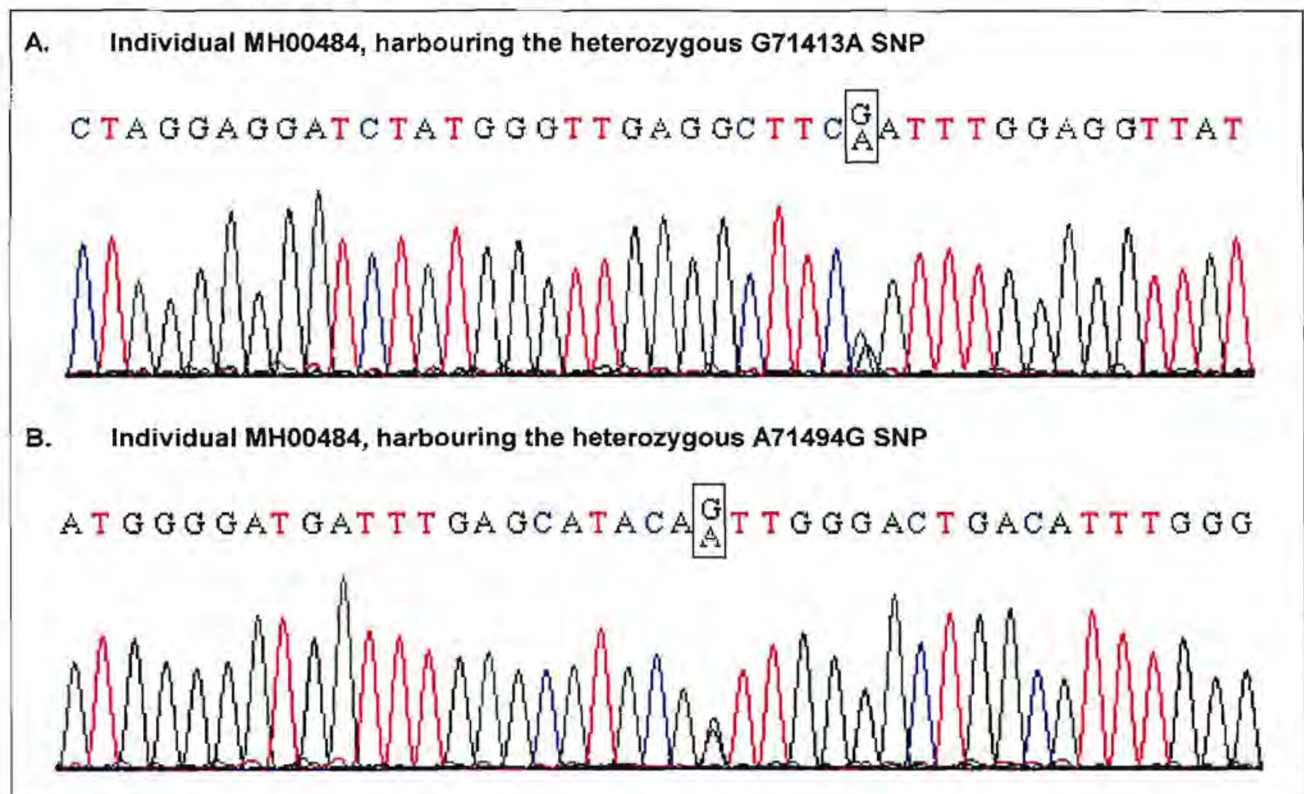


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.21.1.2 <u>SNP G71413A</u>

Within the intron sequence of exons 50 and 51, a heterozygous G71413A SNP was identified in three South African probands analysed in the study presented here. The sequence illustrated in Figure 4.159A is a representative electropherogram depicting the heterozygous G71413A SNP. The frequency data for this SNP has not been reported worldwide and the synonymous substitution has been reported as a SNP in Genbank® (International Human Genome Sequencing Consortium, 2004; with accession number rs2915949).

Figure 4.159: Representative electropherograms indicating the G71413A and A71494G SNPs observed in the intron sequence between exons 50 and 51 of the RYR1 gene



A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.21.1.3 SNP A71494G

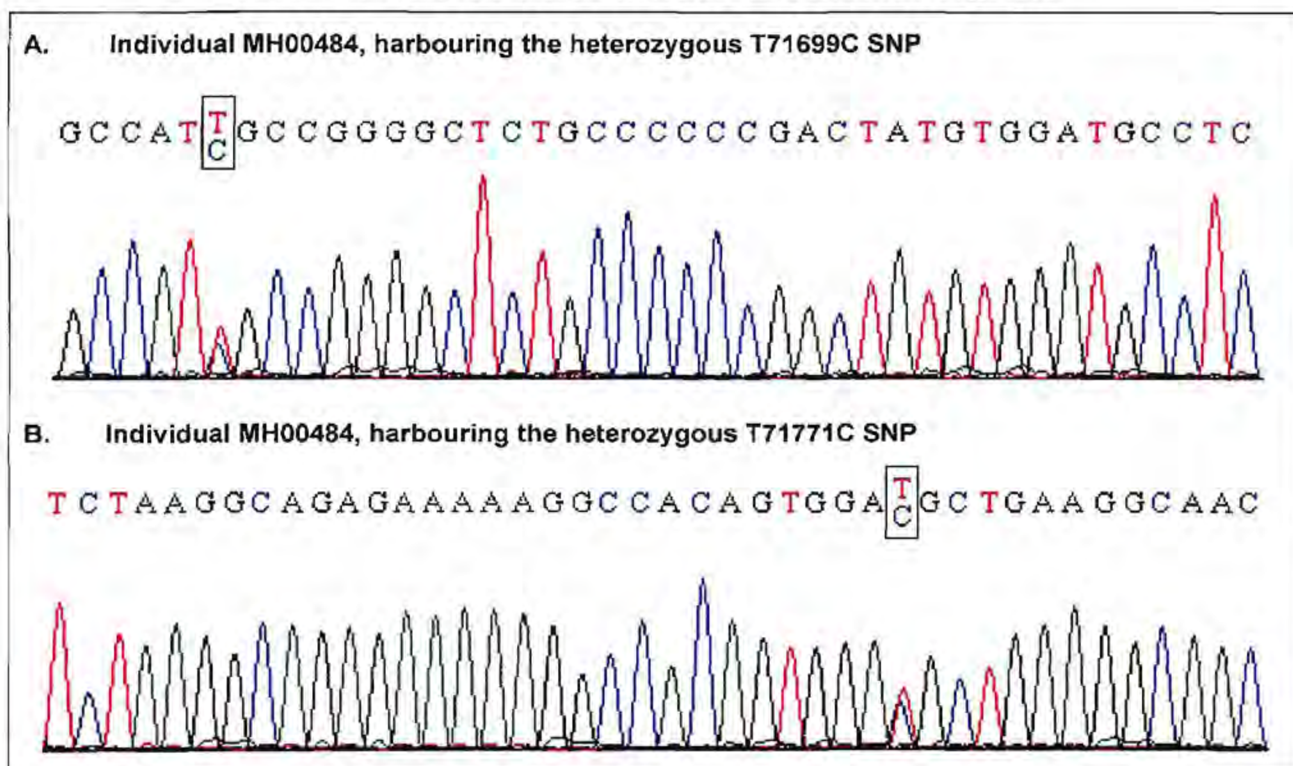
The heterozygous A71494G SNP was identified in five South African probands in the intron sequence between exons 50 and 51. The sequence demonstrated in Figure 4.159B is a representative electropherogram illustrating the heterozygous A71494G SNP. The synonymous substitution has been reported as a SNP in Genbank® (International Human Genome Sequencing Consortium, 2004; with accession number rs2915950). However, to date frequency data for this SNP have not been reported worldwide.

4.10.21.1.4 SNP T71699C

In four South African probands, a heterozygous T71699C SNP was observed in exon 51 of the RYR1 gene. The remaining individuals analysed in the study presented here, did not harbour the SNP. In addition, the homozygous T71699C SNP was not observed. The synonymous substitution has been reported as a SNP in Genbank® (International Human Genome Sequencing Consortium, 2004; with accession number rs2960340) and retains the Ile amino acid. The sequence demonstrated in Figure 4.160A is a representative

electropherogram illustrating the heterozygous T71699C SNP. The allele and genotype frequencies have been determined (International Human Genome Sequencing Consortium, 2004; with accession number rs2960340) in two populations (African American and North American) and are as follows: 0.48 for T/T, 0.38 for C/T and 0.14 for C/C.

Figure 4.160: Representative electropherograms indicating the T71699C and T71771C SNPs observed in exon 51 of the RYR1 gene



A = adenine; C = cytosine; G = guanine; T = thymine.

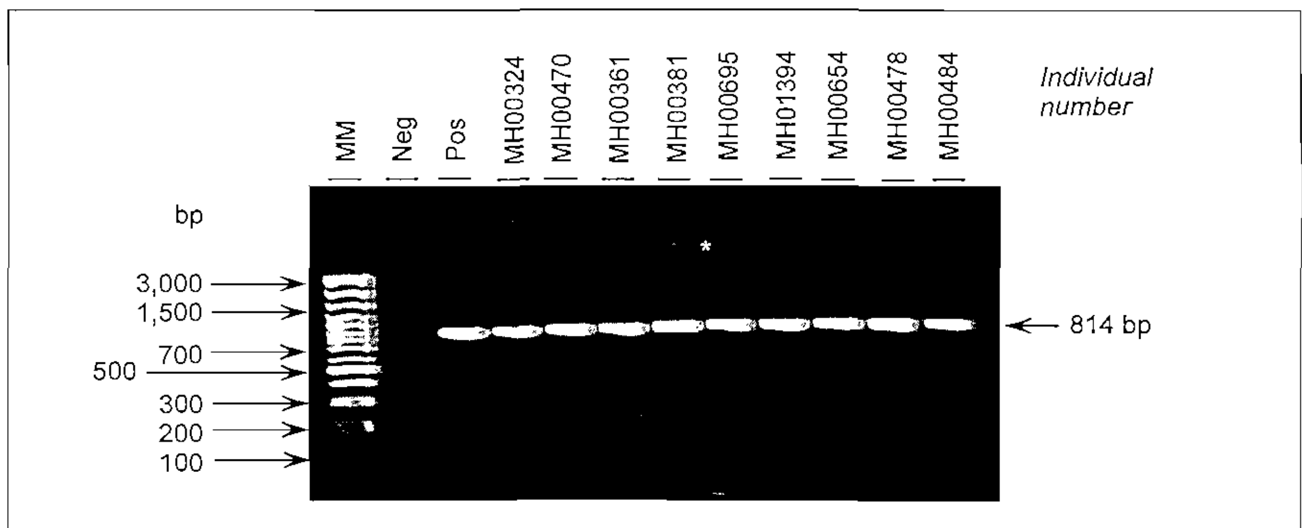
4.10.21.1.5 SNP T71771C

In exon 51 of the RYR1 gene, five South African probands were observed to harbour the heterozygous T71771C alteration. The synonymous substitution has been reported as a SNP in Genbank® (International Human Genome Sequencing Consortium, 2004; with accession number rs2915951) and retains the Asp amino acid. The sequence demonstrated in Figure 4.160B is a representative electropherogram illustrating the heterozygous T71771C SNP. Population diversity studies in two different populations (International Human Genome Sequencing Consortium, 2004; with accession number rs2915951), North American and African American, have indicated the genotype frequencies as follows: 0.46 for T/T, 0.41 for C/T and 0.13 for C/C.

4.10.22 Exons 53 and 54 of the RYR1 gene

Simultaneous amplification of a 814 bp region encompassing exons 53 and 54 was performed as discussed in Section 4.2 (page 159) and Figure 4.161 is a photographic representation of the amplicon encompassing exons 53 and 54. Although both exons occur outside the mutational hotspots, two alterations have been reported in exons 53 and 54. Monnier *et al.* (2005) observed a Thr2787Ser alteration, which occurs due to a C8360G nucleotide transition in one MHS individual from France. Ibarra *et al.* (2006) observed an Arg2840Trp alteration in one MH family that resides in exon 54.

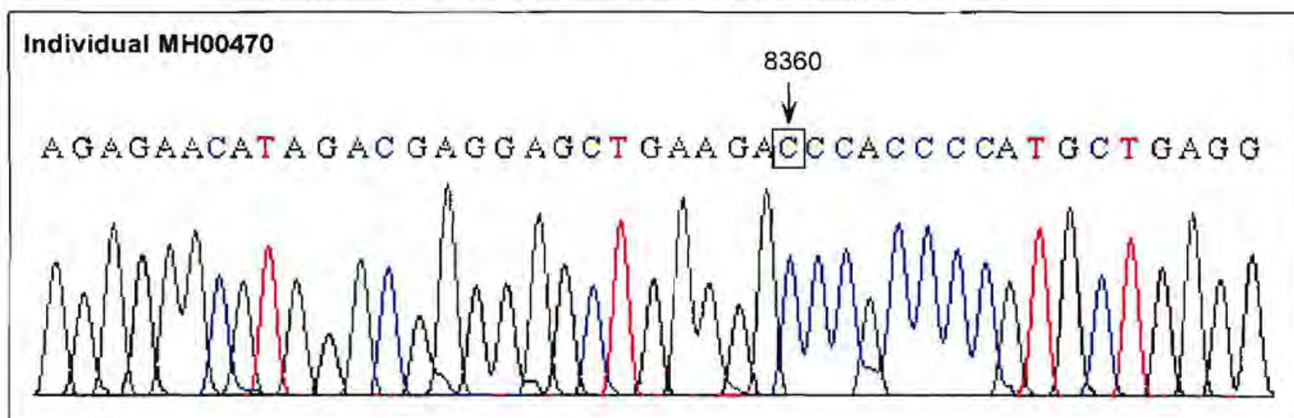
Figure 4.161: Photographic representation of amplified PCR products encompassing exons 53 and 54



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. An artefact in the gel matrix, as indicated by the white asterisk (*) was observed, as discussed in Section 4.3. In addition, the gel exhibited non-specific amplification, background smear and distortion of fragments, as discussed in Sections 4.2 and 4.3.

The amplified region was successfully sequenced, in order to investigate the presence of novel or reported mutations in this region of the RYR1 gene. Sequencing was conducted using the forward primer (RYRex53F). A representative electropherogram obtained for individual MH00470, illustrating the nucleotide position of the Thr2787Ser alteration, previously reported to occur in exon 53, is depicted in Figure 4.162.

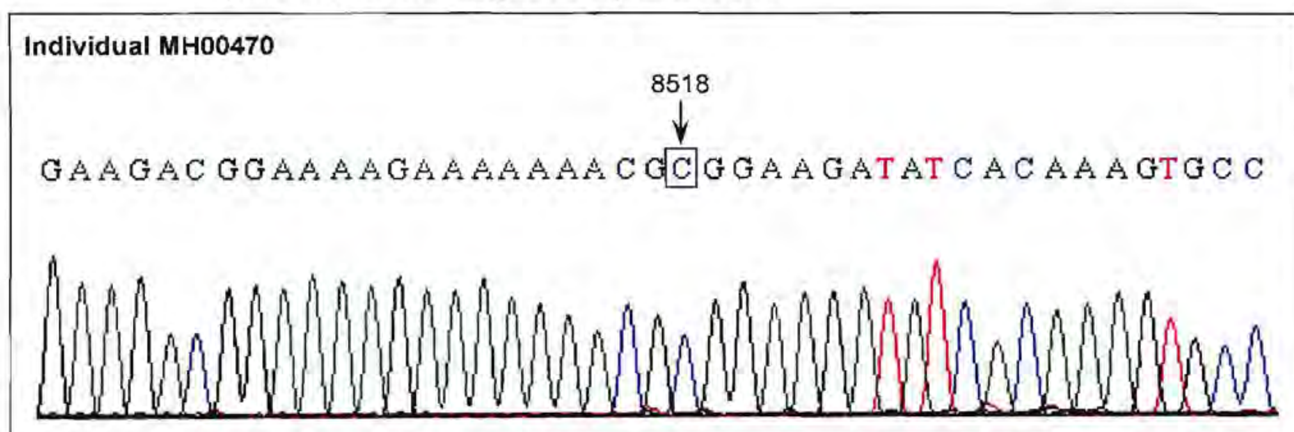
Figure 4.162: Representative electropherogram of exon 53 indicating the nucleotide position of the Thr2787Ser alteration



A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Thr2787Ser at nucleotide 8360.

A representative electropherogram illustrating the nucleotide position of the Arg2840Trp alteration, previously reported to occur in exon 54, is depicted in Figure 4.163. The Thr2787Ser and Arg2840Trp alterations were not detected in any of the MH individuals analysed in Phase 3. The alterations were originally reported in single MH families (Monnier *et al.*, 2005; Ibarra *et al.*, 2006) and they could represent private mutations, which are only present in the above-mentioned families. However, as the alterations have only recently been identified, their frequency has not yet been determined in other populations, as discussed in Section 4.10.2 (page 275).

Figure 4.163: Representative electropherogram of exon 54 indicating the nucleotide position of the Arg2840Trp alteration



A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Arg2840Trp at nucleotide 8518.

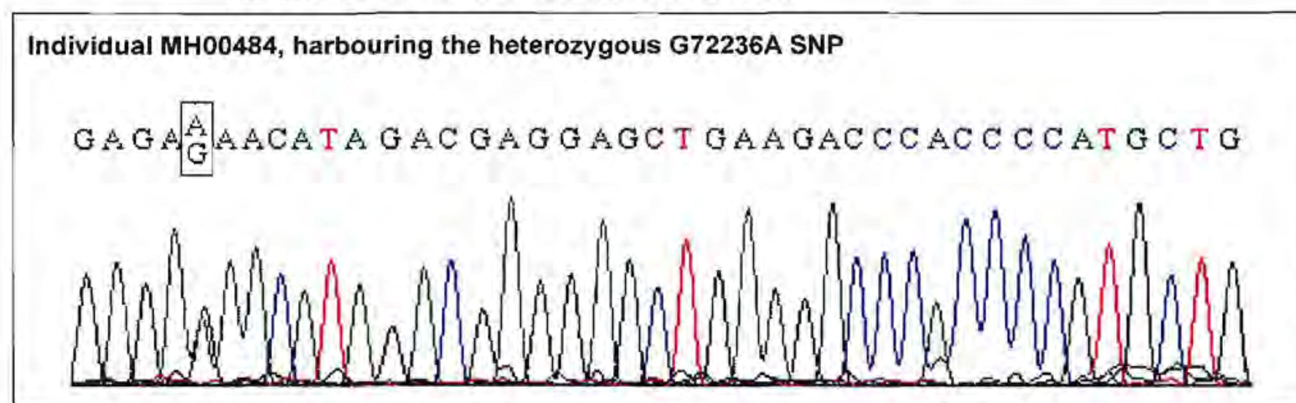
4.10.22.1 Synonymous substitution in the amplified region of exon 53 of the RYR1 gene

In four South African probands, a heterozygous G72236A SNP was observed in exon 53 of the RYR1 gene, as listed in Appendix B (page 447). All four individuals harboured two peaks representing two different nucleotides namely G and A. The remaining individuals analysed in the study presented here did not harbour the SNP. As discussed in Section 4.7.4.1 (page 185), the SNP may play a role in the development of MH, in which both synonymous and non-synonymous SNPs contribute to the phenotype. As discussed in Section 4.7.4.1 (page 185), further studies would have to be conducted to determine if SNPs in the exon sequence of the RYR1 gene contribute to the development of this disorder.

4.10.22.1.1 SNP G72236A

The sequence illustrated in Figure 4.164 is a representative electropherogram illustrating the heterozygous G72236A SNP. The synonymous substitution retains the Glu amino acid and has been reported as a SNP in Genbank® (International Human Genome Sequencing Consortium, 2004; with accession number rs2915952). The genotype frequencies have been determined in the North American and African American populations (International Human Genome Sequencing Consortium, 2004; with accession number rs2915952). The allele and genotype frequencies (International Human Genome Sequencing Consortium, 2004; with accession number rs2915952) in the two populations (African American and North American) are as follows: 0.46 for G/G, 0.43 for A/G and 0.11 for A/A.

Figure 4.164: Representative electropherogram indicating the G72236A SNP observed in exon 53 of the RYR1 gene

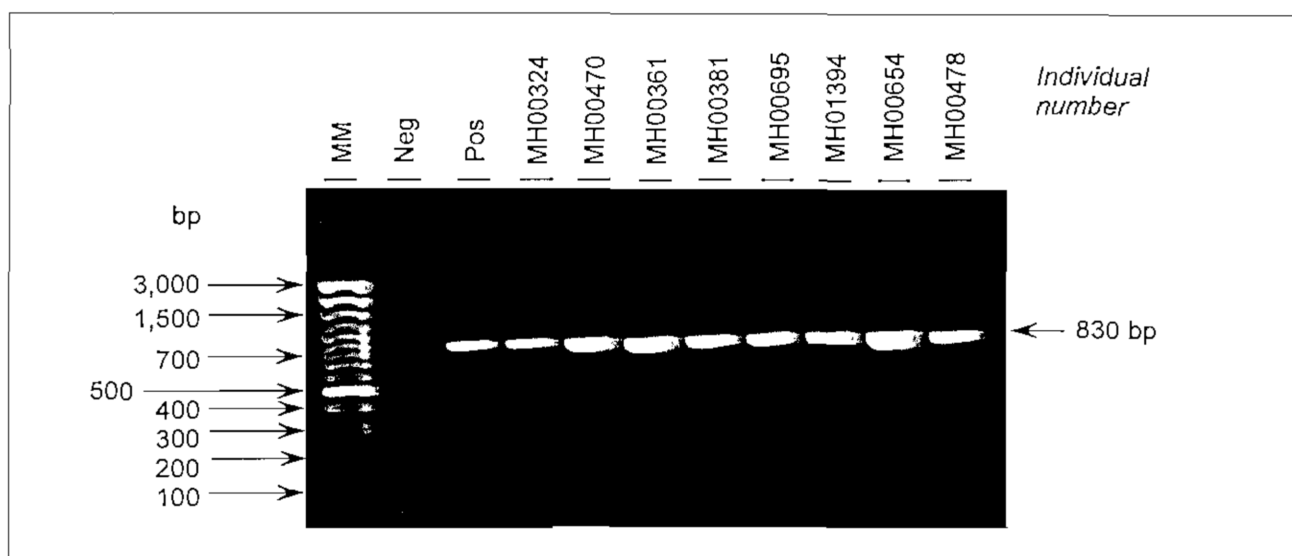


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.23 Exons 55, 56 and 57 of the RYR1 gene

An 830 bp product encompassing exons 55, 56 and 57 was amplified via PCR, as discussed in Section 4.2 (page 159), in order to identify novel or reported alterations. Galli *et al.* (2006) identified a single alteration, Leu2867Gly, in exon 55 of the RYR1 gene in a single MH individual from Italy. Zhou *et al.* (2005) detected an Arg2939Ser alteration in one proband diagnosed with CCD. Successful amplification was achieved for all the samples investigated and Figure 4.165 is a photographic representation of the amplicon encompassing exons 55, 56 and 57.

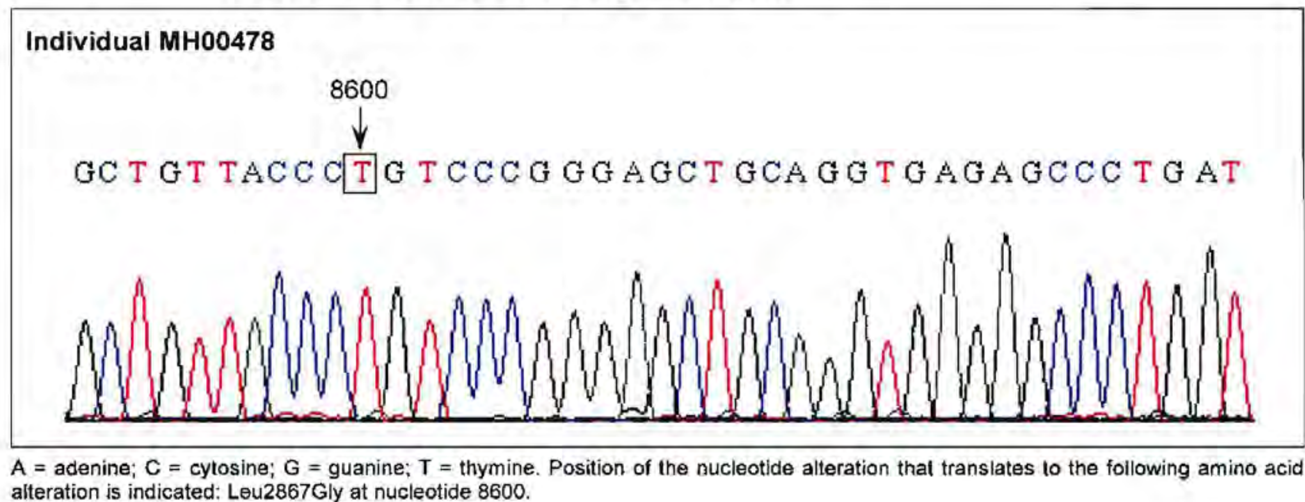
Figure 4.165: Photographic representation of amplified PCR products encompassing exons 55, 56 and 57



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Variation in amplification efficiency, distortion of the MM and amplified fragments were observed, as discussed in Sections 4.2 and 4.3.

Sequencing was conducted using the standard protocol. Both the forward and reverse primers were used in the sequencing reaction in order to analyse the entire coding region of exons 55, 56 and 57. A representative electropherogram illustrating the nucleotide position of the reported Leu2867Gly alteration in exon 55 of the RYR1 gene is depicted in Figure 4.166.

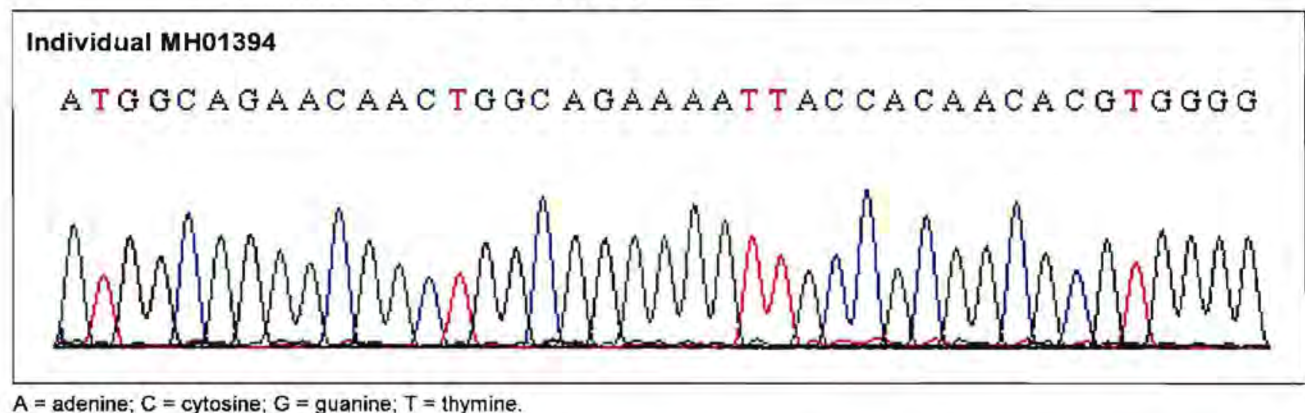
Figure 4.166: Representative electropherogram of exon 55 indicating the nucleotide position of the Leu2867Gly alteration



A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Leu2867Gly at nucleotide 8600.

A representative electropherogram illustrating a portion of the amplified region of exon 56 is depicted in Figure 4.167. In addition, a representative electropherogram illustrating the nucleotide position of the reported Arg2939Ser alteration in exon 57 of the RYR1 gene is depicted in Figure 4.168.

Figure 4.167: Representative electropherogram illustrating a portion of the amplified region of exon 56



A = adenine; C = cytosine; G = guanine; T = thymine.

The Leu2867Gly alteration was not detected in any of the 15 MH probands analysed. As the alteration has thus far only been identified in a single MH family, it is likely that it is specific to that particular family. Although the frequency of each alteration in the RYR1 gene varies, all alterations should initially be screened for in the different populations to determine the frequency of each alteration, as discussed in Section 4.10.2 (page 275). In addition, the Arg2939Ser alteration was not identified in any of the South African probands analysed. The absence of the Arg2939Ser alteration would be expected, as the alteration has thus far only been identified in a proband diagnosed with CCD.

Figure 4.168: Representative electropherogram of exon 57 indicating the nucleotide position of the Arg2939Ser alteration

A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Arg2939Ser at nucleotide 8817.

4.10.23.1 Synonymous substitutions in the amplified region of exons 55, 56 and 57 of the RYR1 gene

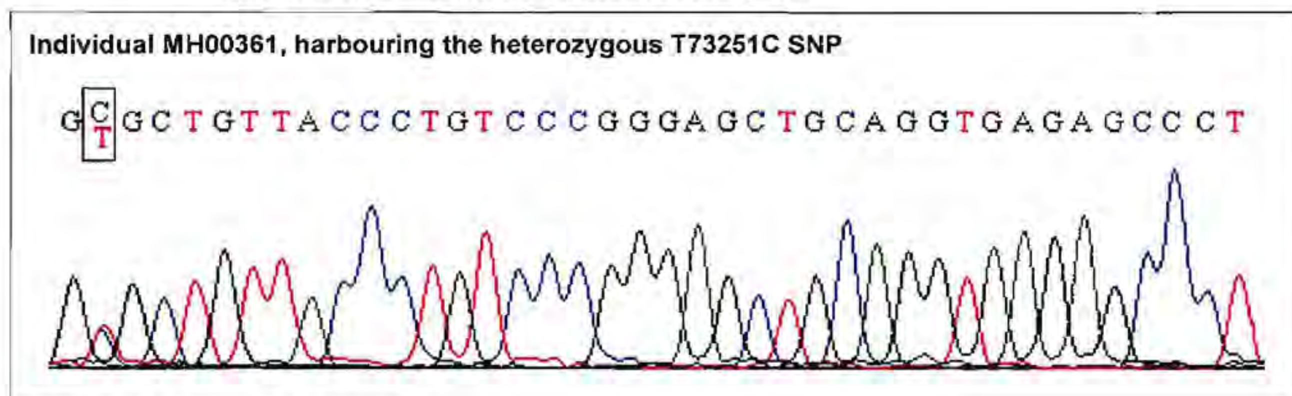
In the amplified region of exons 55, 56 and 57, six SNPs were identified. In exon 55, a synonymous T73251C SNP was observed. Within the intron sequence between exons 55 and 56 a C73337T SNP was observed in three individuals. Within the intron sequence of exons 56 and 57, three SNPs were identified, namely T73475G, T73584C and G73720C. In addition, a T73870A SNP was observed in the intron sequence of the RYR1 gene between exons 57 and 58. As none of the SNPs described in the amplified region of exons 55, 56 and 57 result in a change in the amino acid of the protein or encode for a stop codon, it is unlikely that they play a primary role in susceptibility to MH. However, they may play an important secondary role via epistasis, as discussed in Section 4.7.4.1 (page 185).

4.10.23.1.1 SNP T73251C

In four South African probands, namely MH00478, MH00324, MH00361 and MH00242, two peaks representing two different nucleotide bases, i.e. C and T, were identified. The T73251C SNP is a synonymous substitution, as it does not result in an alteration in the amino acid and was identified in the coding region of the RYR1 gene. The remaining individuals included in the study presented here did not harbour this SNP. The T73251C synonymous substitution retains the Ser amino acid and is indicated as a SNP of the RYR1 gene in GenBank® (International Human Genome Sequencing Consortium, 2004; with accession number rs2229146). The sequence depicted on the electropherogram of

Figure 4.169 is a representative electropherogram illustrating the heterozygous T73251C SNP. The genotypes of this SNP have been determined in the European, Sub-Saharan African and Asian populations (International Human Genome Sequencing Consortium, 2004; with accession number rs2229146). In the European and Asian populations the frequencies of the T/T genotype have been identified as 0.50 and 0.48, respectively. In the Sub-Saharan African population the C/T genotype has a frequency of 0.58, whereas the T/T genotype has a frequency of 0.34.

Figure 4.169: Representative electropherogram indicating the T73251C SNP observed in exon 55 of the RYR1 gene

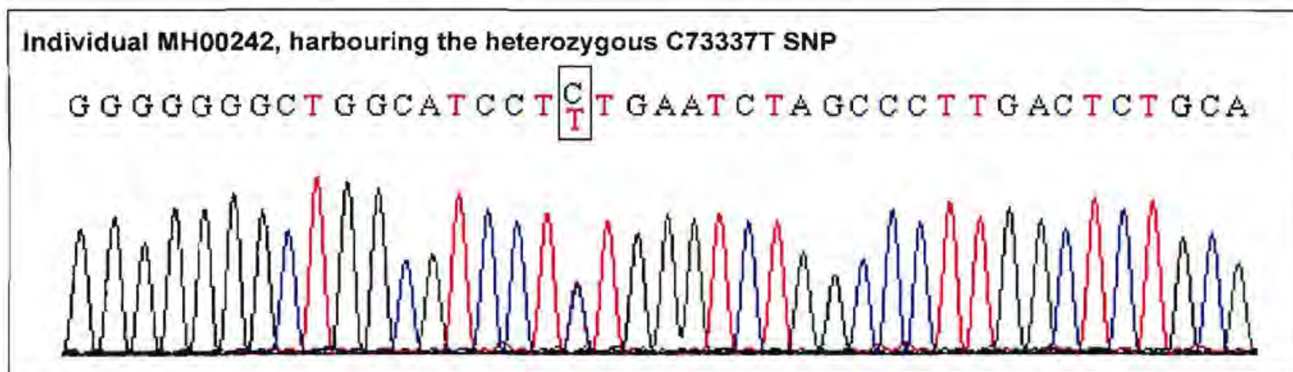


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.23.1.2 SNP C73337T

In three South African probands (MH00478, MH00324 and MH00242), a C73337T SNP was observed. The SNP occurs in the intron sequence between exons 55 and 56. The sequence depicted on the electropherogram of Figure 4.170, indicates two distinct peaks, indicating a heterozygous state of C/T at this position. Therefore, all individuals investigated are heterozygous for the C73337T SNP, as listed in Appendix C (page 451). The remaining individuals included in the study presented here did not exhibit the SNP. To date, the allele and genotype frequencies worldwide for this SNP have not been reported. However, the synonymous substitution has been identified as a SNP of the RYR1 gene in GenBank® (International Human Genome Sequencing Consortium, 2004; with accession number rs2960344).

Figure 4.170: Representative electropherogram indicating the C73337T SNP observed in the intron sequence between exons 55 and 56 of the RYR1 gene

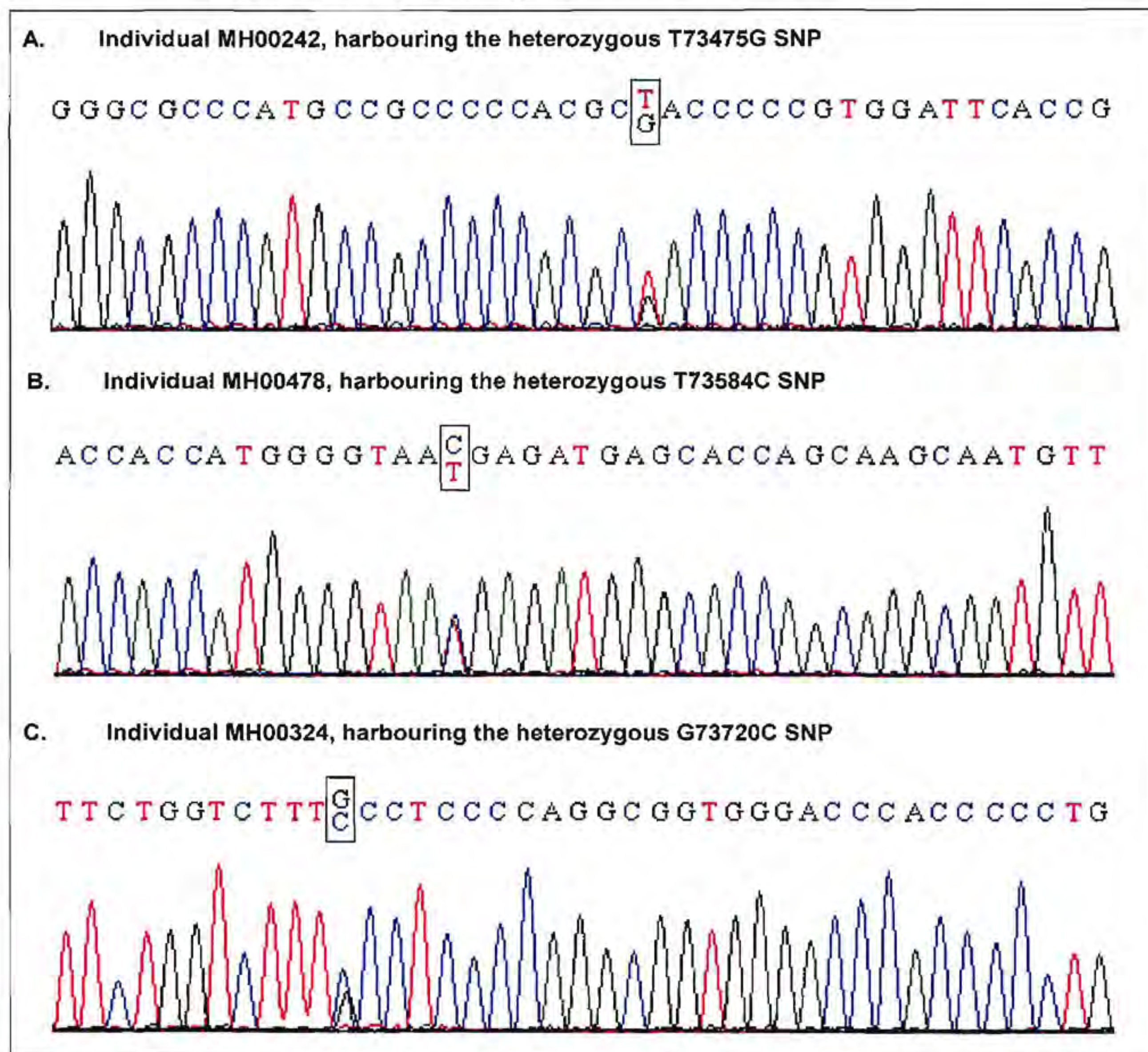


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.23.1.3 SNP T73475G

Within the intron sequence of exons 56 and 57, a heterozygous T73475G SNP has been identified. The synonymous substitution was detected in three individuals (MH00478, MH00324 and MH00242) and is indicated as a SNP of the RYR1 gene in GenBank® (International Human Genome Sequencing Consortium, 2004; with accession number rs2960345). Figure 4.171A depicts the sequence generated for an individual heterozygous for the T73475G SNP.

Figure 4.171: Representative electropherograms indicating the T73475G, T73584C and G73720C SNPs observed in the intron sequence between exons 56 and 57 of the RYR1 gene



A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.23.1.4 SNP T73584C

A second SNP was identified in the intron sequence between exons 56 and 57. The T73584C SNP was identified in three South African probands (MH00478, MH00324 and MH00242). The SNP was heterozygous in all three individuals, and was not observed in the remaining individuals analysed. The sequence depicted in Figure 4.171B is a representative electropherogram illustrating the heterozygous T73584C SNP. The synonymous substitution is indicated as a SNP of the RYR1 gene in GenBank® (International Human Genome Sequencing Consortium, 2004; with accession number

rs2915957). To date, the worldwide allele and genotype frequencies for this SNP have not been reported.

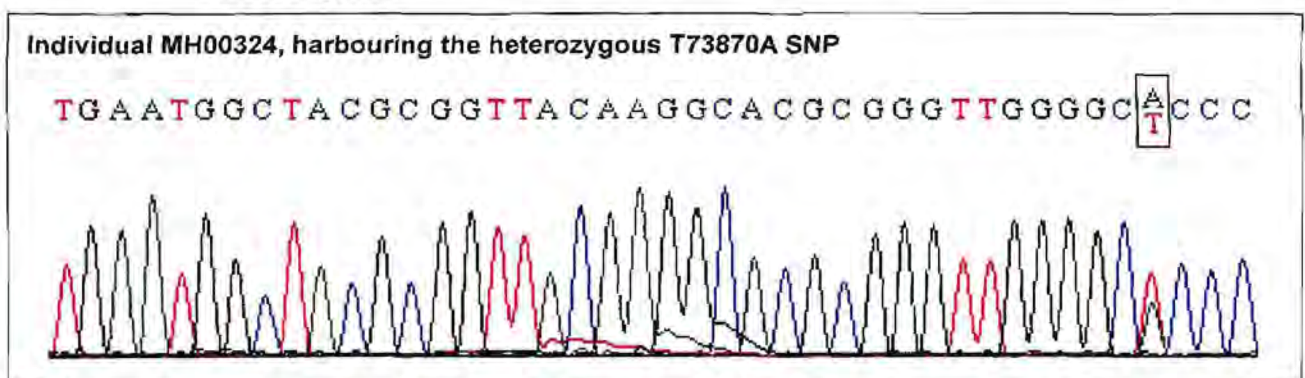
4.10.23.1.5 SNP G73720C

A third SNP was identified in the amplified region between exons 56 and 57. Figure 4.171C depicts the sequence generated for the heterozygous G73720C SNP. The heterozygous SNP was observed in four South African probands (MH00478, MH00484, MH00324 and MH00242) and was not detected in any other individuals analysed. The synonymous substitution is indicated as a SNP of the RYR1 gene in GenBank® (International Human Genome Sequencing Consortium, 2004; with accession number rs2915958) and the allele and genotype frequencies worldwide for this SNP have not been reported.

4.10.23.1.6 SNP T73870A

In three South African probands, MH00478, MH00324 and MH00484, a heterozygous T73870A SNP was identified. The synonymous substitution was identified in the intron sequence of the RYR1 gene between exons 57 and 58 and is indicated as a SNP of the RYR1 gene in GenBank® (International Human Genome Sequencing Consortium, 2004; with accession number rs2915959). The sequence depicted on the electropherogram of Figure 4.172, generated with the forward primer, is a representative electropherogram illustrating the heterozygous T73870A SNP. The remaining individuals included in the study presented here did not exhibit the SNP. The genotype frequencies of this SNP has not been determined in other populations, worldwide.

Figure 4.172: Representative electropherogram indicating the T73870A SNP observed in the intron sequence between exons 57 and 58 of the RYR1 gene

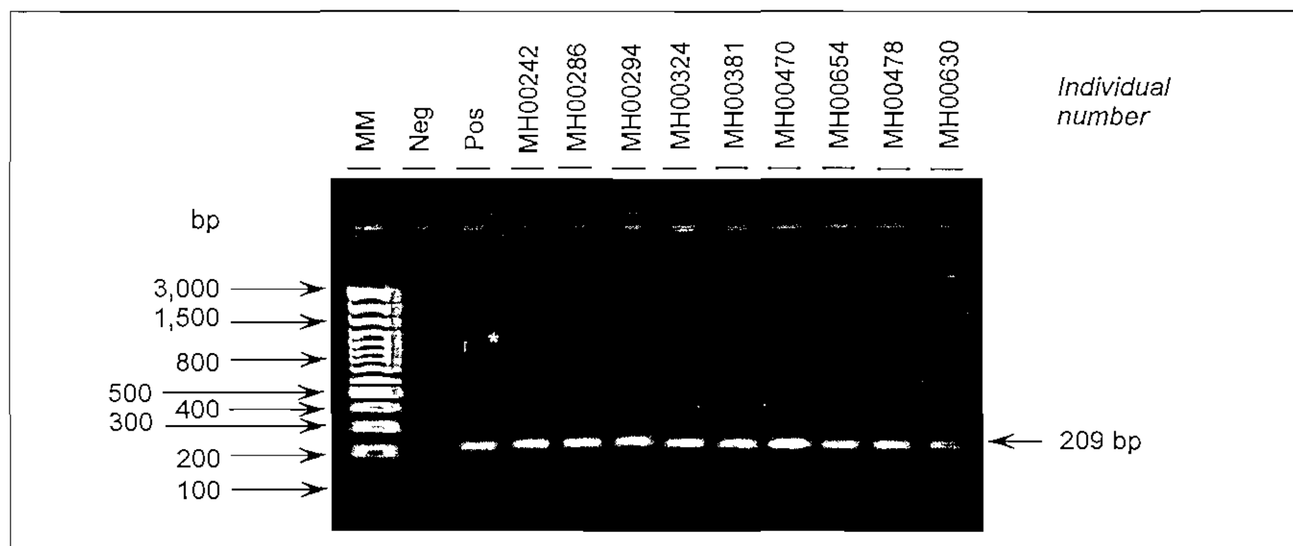


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.24 Exon 58 of the RYR1 gene

Thus far, exon 58 does not harbour any reported alterations associated with MH susceptibility. The PCR reaction was optimised as discussed in Section 4.2 (page 159). The product was electrophoresed on a 2% (w/v) mini agarose gel and the results are presented in Figure 4.173.

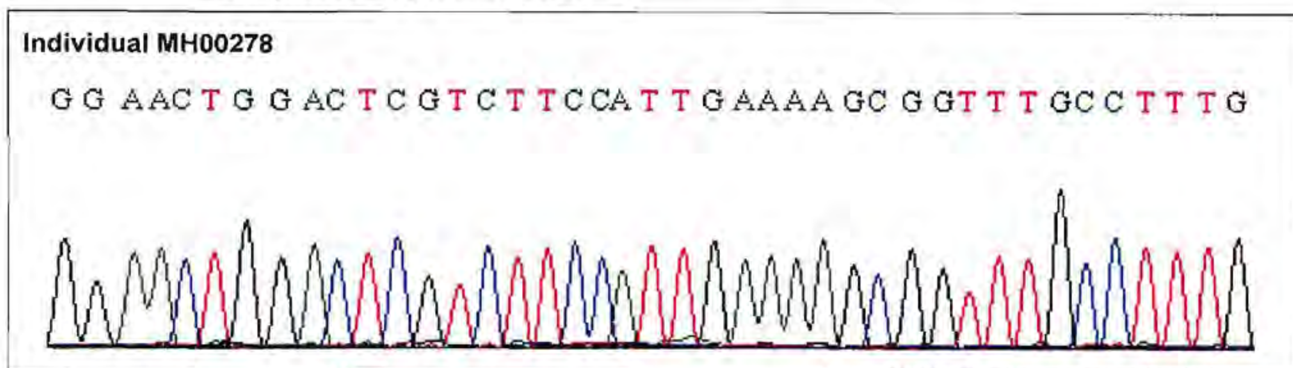
Figure 4.173: Photographic representation of amplified PCR products encompassing exon 58



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. An artefact in the gel matrix, as indicated by the white asterisk (*) was detected, as discussed in Section 4.3. Variation in the amplification efficiency was observed between samples, as discussed in Section 4.2. The depicted amplified fragments do not appear linear, as discussed in Section 4.2 and the MM appears distorted, as discussed in Section 4.3.

All fifteen samples obtained from MH probands that were successfully amplified via PCR were subsequently sequenced using the standard protocol, using the forward primer (RYRex58F). A representative electropherogram obtained for individual MH00278, illustrating a portion of exon 58, is indicated in Figure 4.174. None of the 15 MHS probands analysed in the study presented here harboured any novel alterations in this region. In addition, the region did not harbour any polymorphisms. Therefore, alterations in exon 58 do not result in the MH phenotype in the cohort of South African probands included in this investigation. However, as this exon has only recently been screened for MH mutations, it may harbour alterations associated with MH that have not been detected in any individual analysed to date, as discussed in Section 4.10.2 (page 275).

Figure 4.174: Representative electropherogram illustrating a portion of the amplified region of exon 58

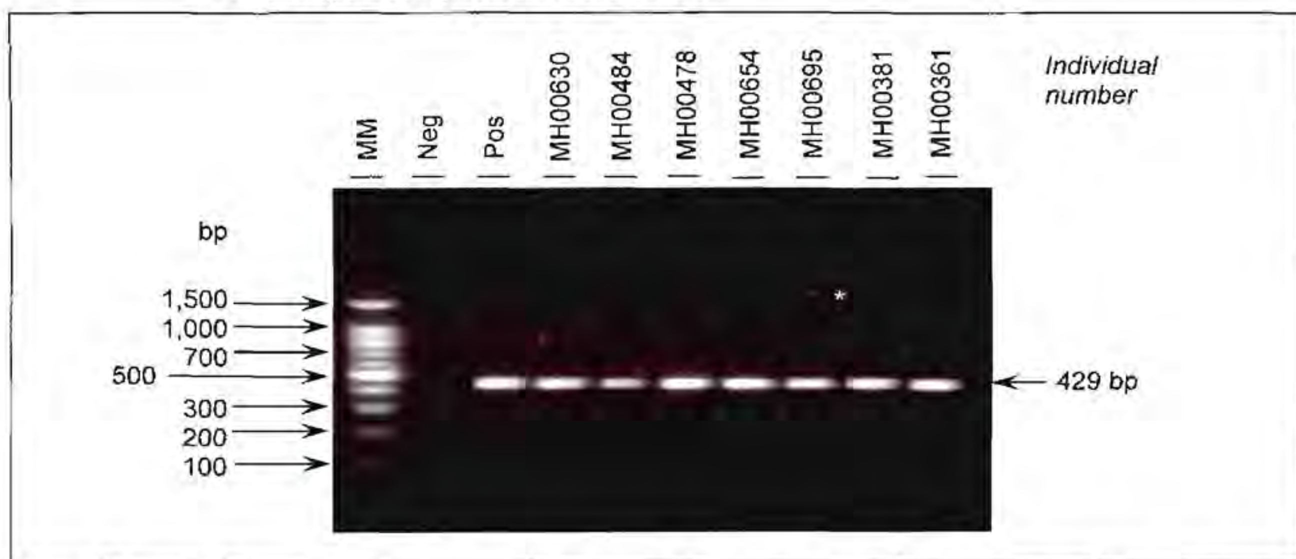


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.25 Exons 59 and 60 of the RYR1 gene

A 429 bp product encompassing exons 59 and 60 was amplified via PCR in order to identify novel alterations or polymorphisms, as discussed in Section 4.2 (page 159). This region of the RYR1 gene does not currently harbour any alterations associated with the MH phenotype. Successful amplification was achieved for all the samples investigated. Figure 4.175 is a photographic representation of the amplicon encompassing exons 59 and 60. The amplified region was subsequently sequenced in order to identify possible novel mutations that may result in MHS.

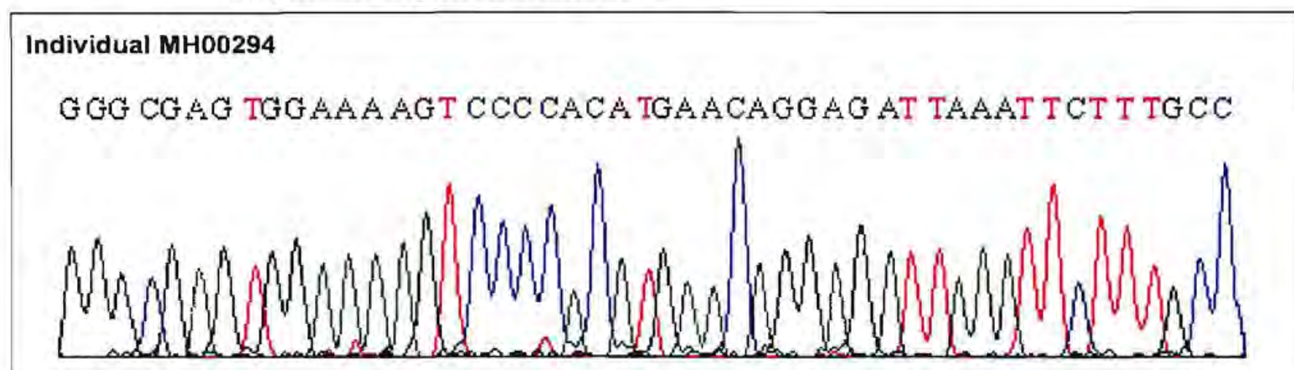
Figure 4.175: Photographic representation of amplified PCR products encompassing exons 59 and 60



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. An artefact in the gel matrix, as illustrated by the white asterisk (*), was detected, as discussed in Section 4.3. As discussed in Sections 4.2 and 4.3, variation in amplification efficiency, background smear and MM distortion were observed.

Sequencing was conducted using the standard protocol. As the reverse primer was used (RYRex59R) in the sequencing reaction, sequence data obtained for individual MH00294 are indicated as the reverse complement. A representative electropherogram illustrating a portion of the amplified region of exon 59 is depicted in Figure 4.176 and a portion of the amplified region of exon 60 is depicted in Figure 4.177.

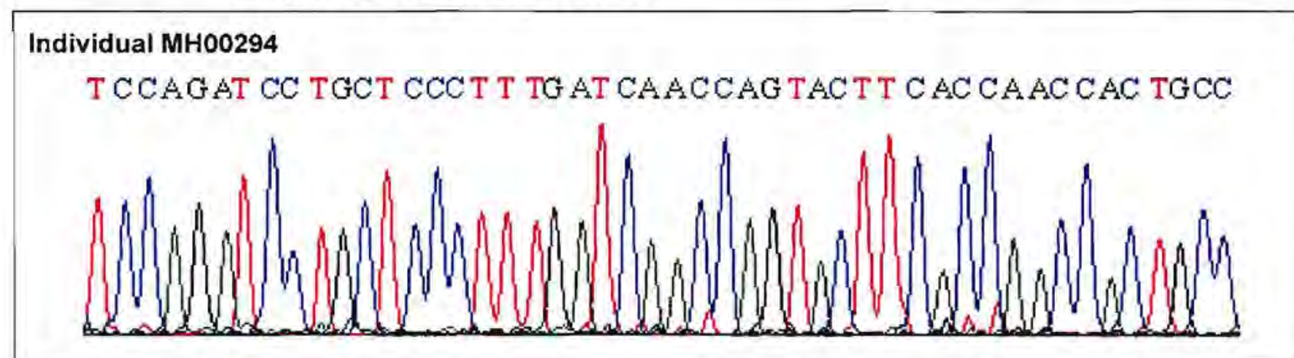
Figure 4.176: Representative electropherogram illustrating a portion of the amplified region of exon 59



A = adenine; C = cytosine; G = guanine; T = thymine.

Alterations associated with MH were not observed in the amplified region of either exons 59 or 60 in any of the South African probands analysed. However, both exons encode residues that play a functional role in the RyR1 protein. Buratti *et al.* (1995) indicated that this region of the RyR1, residues 2937 - 3225 (coded by exons 59 to 64) and residues 3546 - 3655 (coded by exons 71 to 74), contained a Ca^{2+}-dependent binding site for CaM. The authors indicated that CaM is involved in RyR1 channel activation. Therefore, although alterations were not detected in the study presented here, further mutation analysis would determine if these functionally significant exons harbour alterations that play a role in the MH phenotype, as discussed in Section 4.10.2 (page 275).

Figure 4.177: Representative electropherogram illustrating a portion of the amplified region of exon 60

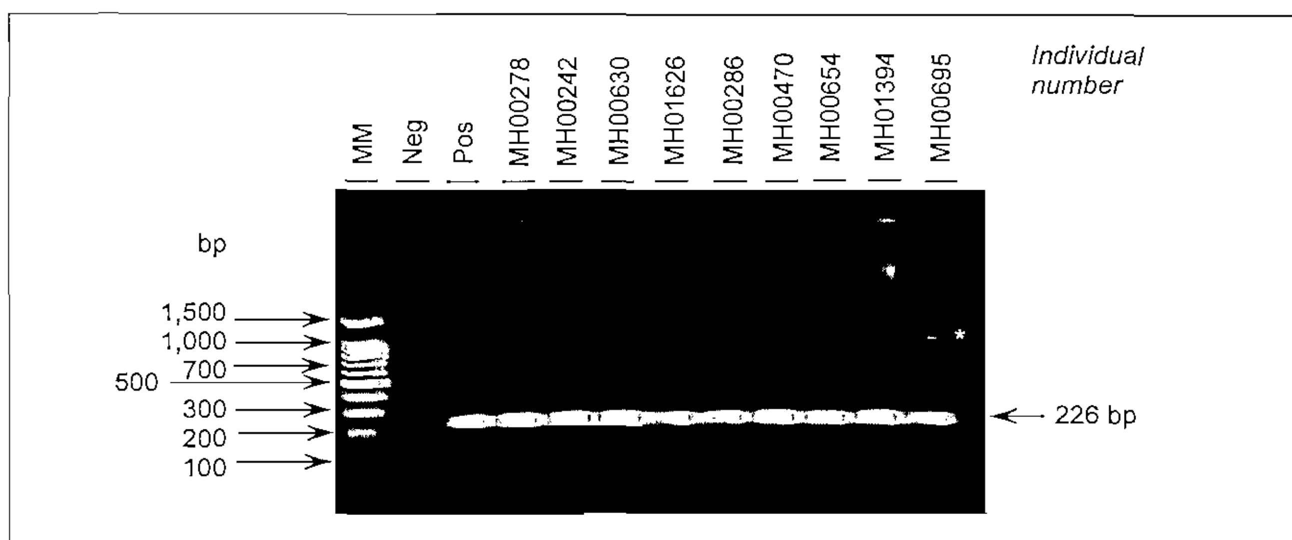


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.26 Exon 61 of the RYR1 gene

The standard PCR protocol was used to amplify a region of 226 bp encompassing exon 61 of the RYR1 gene, as listed in Table 4.1 (page 161). Thus far, alterations associated with MHS have not been reported to occur in exon 61. However, a SNP has been described for this region. The amplified region is located between the second and third mutation hotspots. PCR amplification was successful for all samples analysed and the results of PCR amplification of this region encompassing exon 61 are depicted in Figure 4.178.

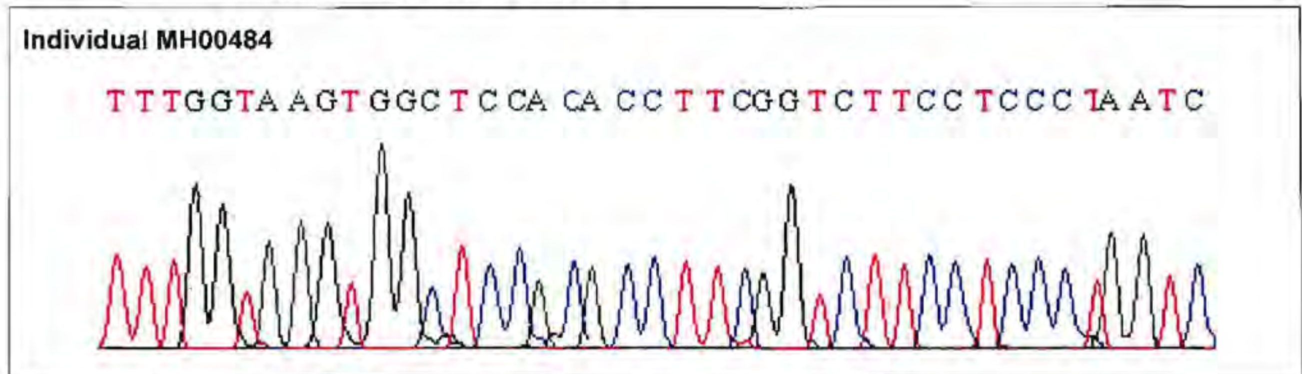
Figure 4.178: Photographic representation of amplified PCR products encompassing exon 61



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. An agarose artefact, as indicated by the white asterisk (*) was observed in the agarose gel, as discussed in Section 4.7. In addition, non-specific amplification, variation in amplification efficiency, background smear and distortion of fragments were detected, as discussed in Sections 4.2 and 4.3.

In order to detect novel alterations that may be associated with MH, the amplified region of exon 61 was sequenced using the forward primer (RYRex61R). A representative electropherogram obtained for individual MH00484, illustrating a portion of the amplified region, is depicted in Figure 4.179.

Figure 4.179: Representative electropherogram illustrating a portion of the amplified region of exon 61



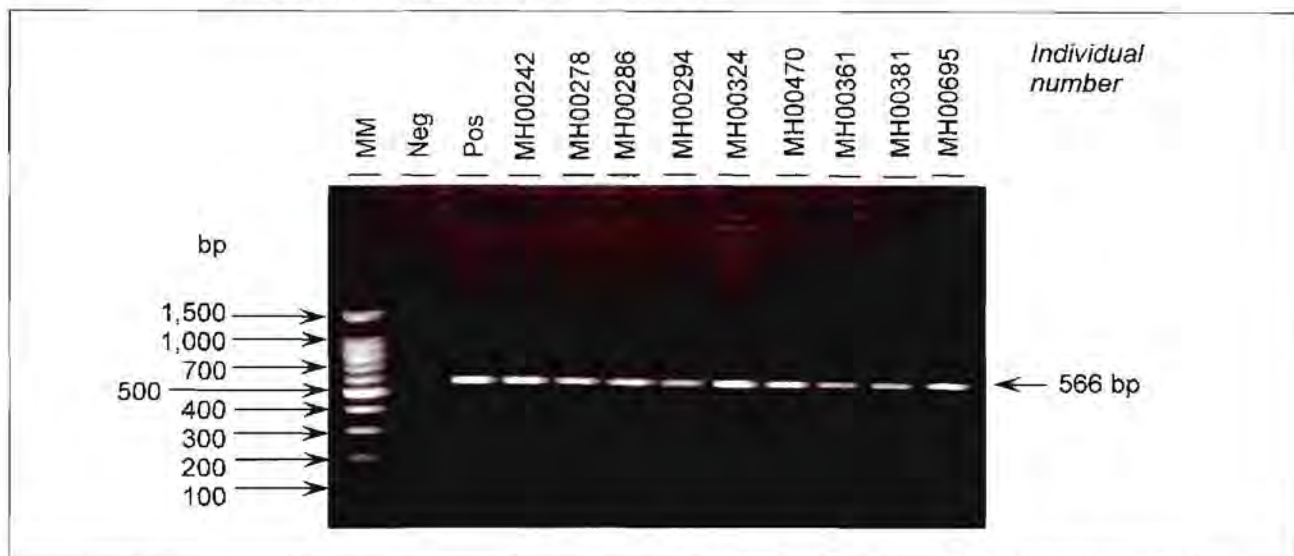
A = adenine; C = cytosine; G = guanine; T = thymine.

None of the 15 individuals that were sequenced for novel alterations in exon 61 harboured any novel alterations in exon 61 of the RYR1 gene. As discussed in Section 4.10.2 (page 275), exon 61 has only recently been analysed in certain populations (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006), therefore the presence of potentially causative alterations in this exon has not been determined. As discussed in Section 4.10.25 (page 343), exon 61 may play a role in the development of the MH phenotype since it contains functionally significant domains.

4.10.27 Exons 62 and 63 of the RYR1 gene

A region of 566 bp encompassing exons 62 and 63 was amplified via PCR in order to identify novel and reported alterations. This region of the RYR1 gene harbours one alteration that occurs in exon 63. Ibarra *et al.* (2006) identified an Arg3119His alteration in one MH family from Japan. The PCR reaction was optimised as discussed in Section 4.2 (page 159). Figure 4.180 is a photographic representation of the amplicon encompassing exons 62 and 63.

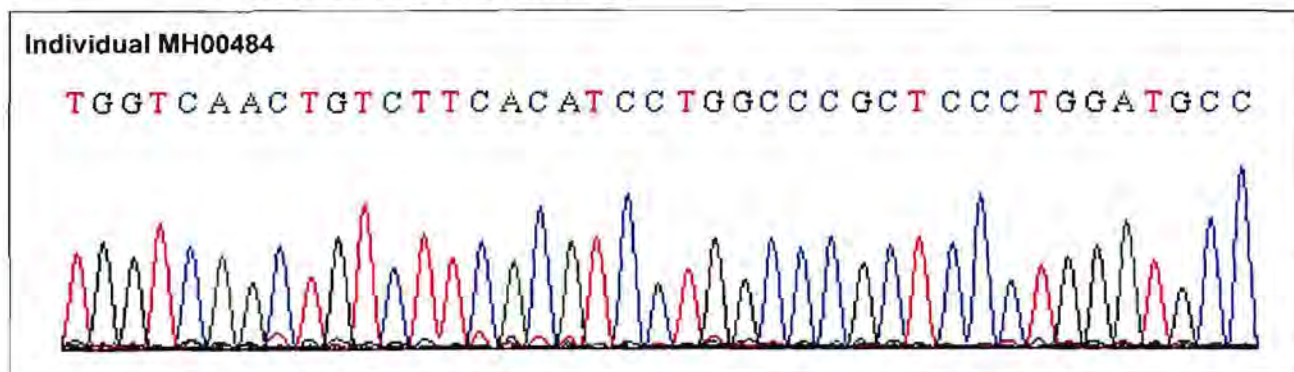
Figure 4.180: Photographic representation of amplified PCR products encompassing exons 62 and 63



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Depicted fragments exhibited non-specific amplification, variation in amplification efficiency, distortion of fragments and appear skew, which could be attributed to reasons listed in Sections 4.2 and 4.3.

Sequencing was conducted using the forward primer and the standard sequencing protocol. The entire exon region encompassing both exons 62 and 63 was successfully sequenced, which allowed for the accurate assessment of novel and reported mutations and polymorphisms. Alterations associated with MH have not been reported in exon 62. A representative electropherogram obtained for individual MH00484, illustrating a portion of the amplified region of exon 62, is depicted in Figure 4.181.

Figure 4.181: Representative electropherogram illustrating a portion of the amplified region of exon 62

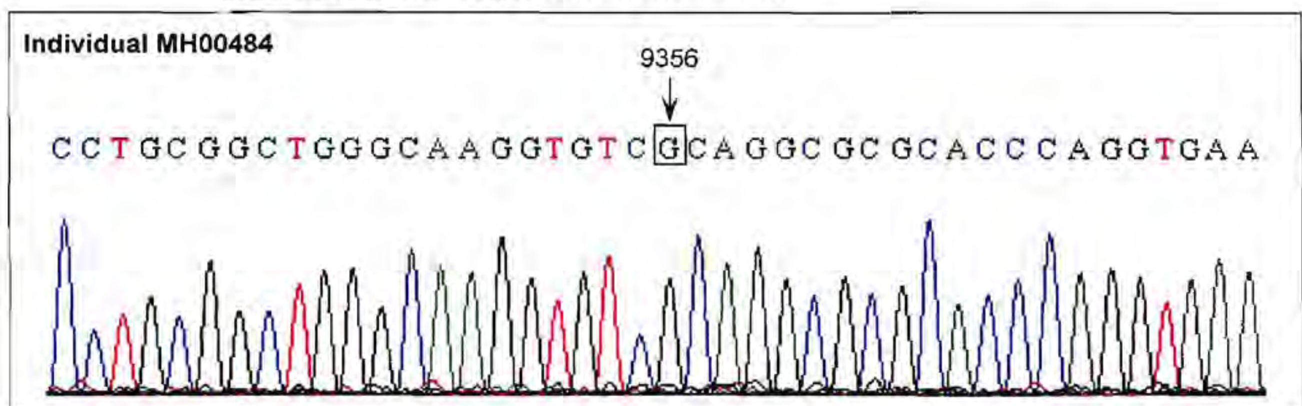


A = adenine; C = cytosine; G = guanine; T = thymine. Background peaks were observed, as discussed in Section 4.5.

A representative electropherogram obtained for individual MH00484, illustrating the nucleotide position of the reported Arg3119His alteration in the amplified region of exon 63, is depicted in Figure 4.182. In the study presented here, alterations associated with

MH were not observed in any of the South African MH probands. Thus far, only certain populations from North America, France, Italy and Japan (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006), as discussed in Section 4.10.2 (page 275), have been screened for alterations outside the RYR1 gene mutation hotspots. Therefore additional studies would have to be conducted, which include adequate sample numbers in order to determine if alterations associated with MH reside in either of these two exons. In addition, the Arg3119His alteration may be family-specific or population-specific and may only be observed in the family described by Ibarra *et al.* (2006) or in the Japanese population.

Figure 4.182: Representative electropherogram of exon 63 indicating the nucleotide position of the Arg3119His alteration



A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Arg3119His at nucleotide 9356. Background peaks were observed, as discussed in Section 4.5.

4.10.27.1 <u>Synonymous substitution in the amplified region of exon 62 and 63 of the RYR1 gene</u>

An A78986G SNP was observed in exon 62 of the RYR1 gene in five probands from South Africa, as listed in Appendix B (page 447). As discussed in Section 4.7.4.1 (page 185), the alteration may have occurred because of the high GC content in this genomic region and may play an important secondary role in the development of the MH phenotype.

4.10.27.1.1 <u>SNP A78986G</u>

Four probands were heterozygous for the A78986G alteration and one individual (MH00324) was homozygous for the SNP. The SNP is a synonymous substitution as it occurs at the third codon position and does not result in an alteration in the amino acid Pro. The synonymous substitution was identified in the coding region of the RYR1 gene

and was indicated as a SNP in Genbank® (International Human Genome Sequencing Consortium, 2004; with accession number rs2071089). The frequencies of genotypes vary, and have been determined as 0.09 for G/G, 0.50 for A/A and 0.41 for A/G in the European population (International Human Genome Sequencing Consortium, 2004; with accession number rs2071089). The sequence electropherograms depicted in Figure 4.183 are representative electropherograms illustrating the heterozygous and homozygous A78986G SNP, respectively. The remaining MH probands included in the study presented here did not harbour the SNP.

Figure 4.183: Representative electropherograms indicating the A78986G SNP observed in exon 62 of the RYR1 gene

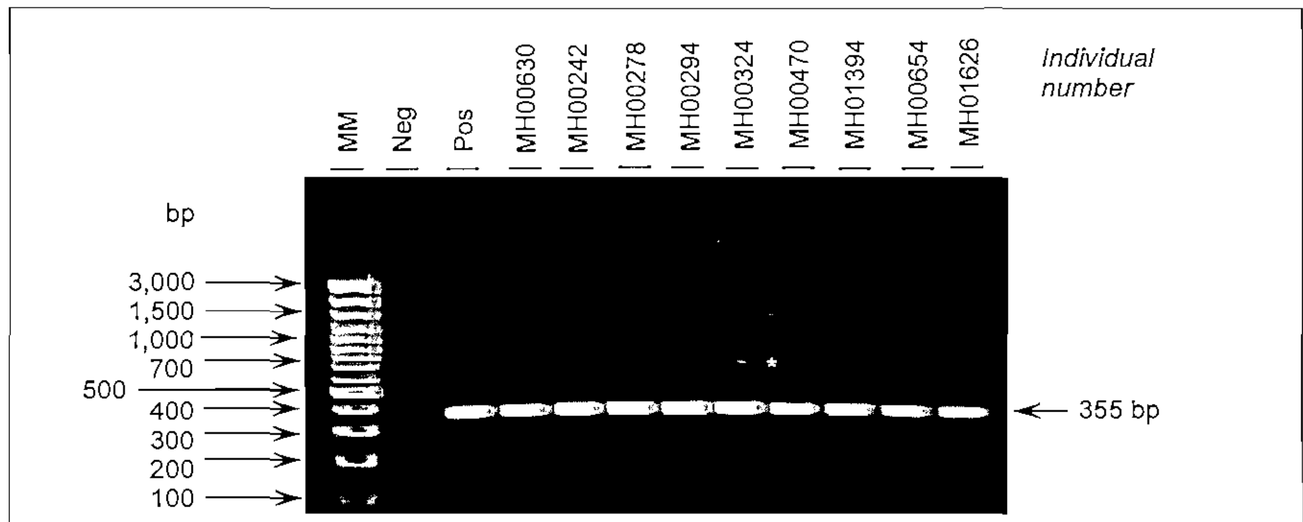
A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.28 Exon 64 of the RYR1 gene

Exon 64 is located outside the mutational hotspot regions of the RYR1 gene. Thus far, alterations associated with the MH phenotype have not been reported in this region of the RYR1 gene. A 355 bp region encompassing this exon was amplified using optimised conditions, listed in Table 4.1 (page 161). Thereafter, the PCR product was purified and sequenced according to the standard protocol. The PCR product was electrophoresed on a 2% (w/v) mini agarose gel as illustrated in Figure 4.184. Sequencing of exon 64 was conducted in order to determine if novel alterations associated with MHS reside in this

region of the RYR1 gene. Sequencing was performed using the reverse primer (RYRex64R) and sequences are illustrated as the reverse complement.

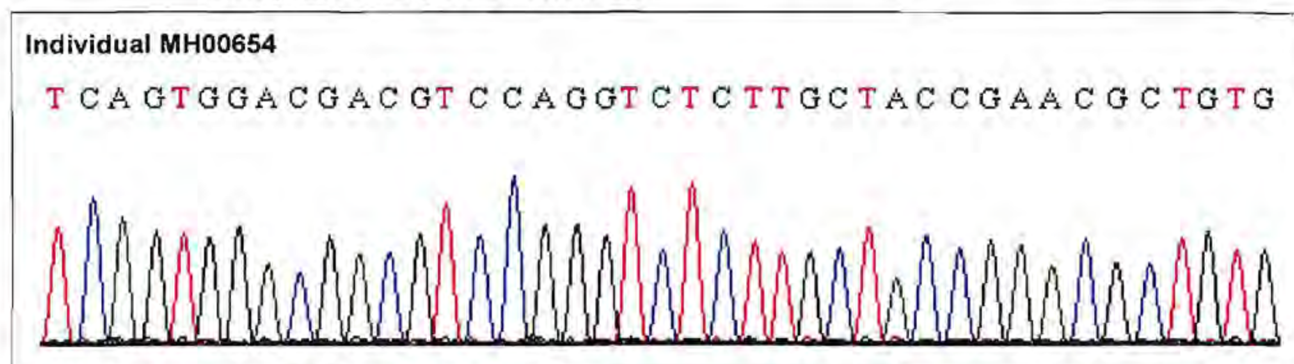
Figure 4.184: Photographic representation of amplified PCR products encompassing exon 64



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. An artefact in the gel matrix, as illustrated by the white asterisk (*) was detected, as discussed in Section 4.3. The amplified fragments do not appear linear, as discussed in Section 4.3 and the MM appeared overloaded, as discussed in Section 4.3.

A representative electropherogram illustrating a portion of the amplified region for exon 64 is indicated in Figure 4.185. Fifteen South African probands were screened for novel alterations that may occur in exon 64. None of the 15 probands harboured any novel alterations. Due to the absence of alterations in exon 64, it could be concluded that this exon does not harbour alterations that result in MHS, in this cohort of South African MH probands. However, as additional alterations are continually being reported in exons outside the mutational hotspots (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006), this exon would have to be analysed further in a larger number of individuals, as discussed in Section 4.10.2 (page 275).

Figure 4.185: Representative electropherogram illustrating a portion of the amplified region of exon 64



A = adenine; C = cytosine; G = guanine; T = thymine.

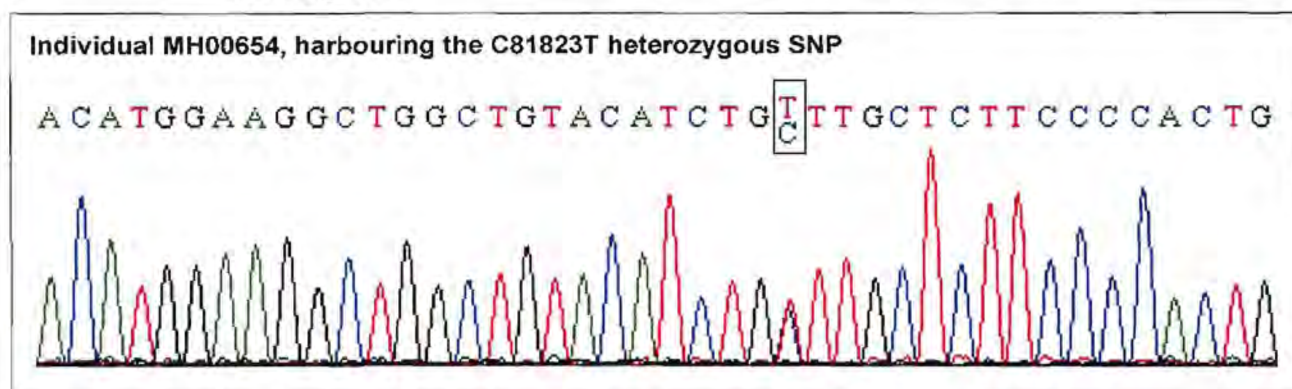
4.10.28.1 Synonymous substitution in the amplified region of exon 64 of the RYR1 gene

Analysis of the amplified region of exon 64 identified a C81823T SNP in one South African MH proband (MH00654). The remaining individuals analysed in the study presented here did not harbour the SNP. As discussed in Section 4.7.8.1 (page 196), intron sequence may play a larger functional role in gene regulation than previously realised. Therefore, although the alteration has been reported and is not directly associated with MH, the SNP may contribute to MH susceptibility via epistasis.

4.10.28.1.1 SNP C81823T

The C81823T SNP occurs in the intron sequence between exons 63 and 64. The sequence depicted in Figure 4.186 is a representative electropherogram illustrating the heterozygous C81823T SNP. The genotype and allele frequencies have thus far not been reported, worldwide. The synonymous substitution has, however, been reported as a SNP in Genbank® (International Human Genome Sequencing Consortium, 2004; with accession number rs12461853).

Figure 4.186: Representative electropherogram indicating the C81823T SNP observed in the intron sequence between exons 63 and 64 of the RYR1 gene

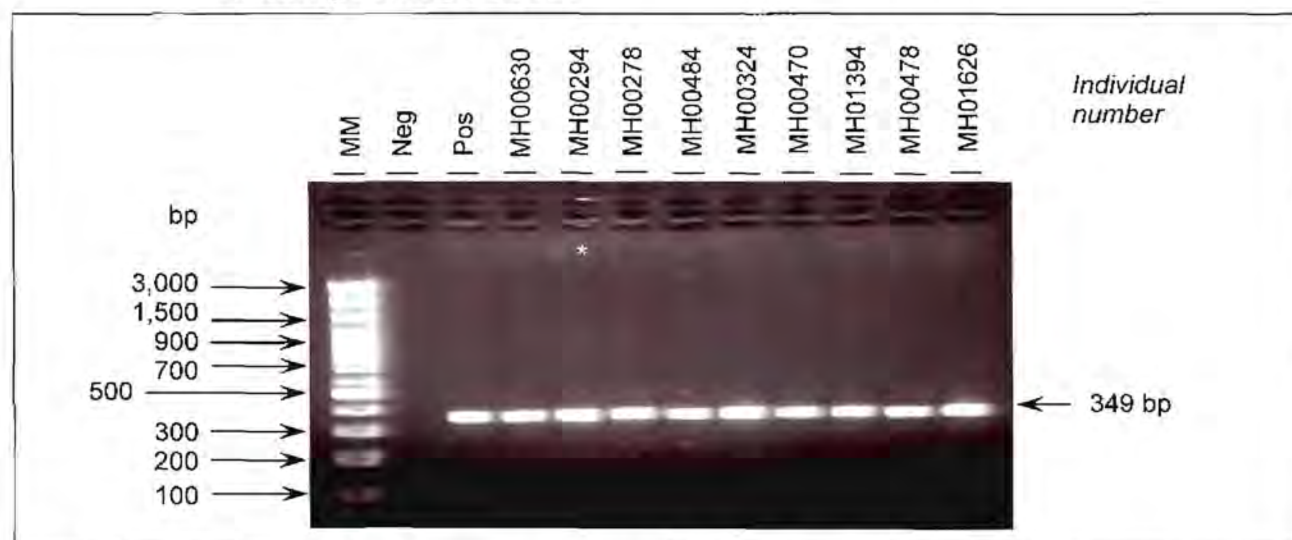


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.29 Exon 65 of the RYR1 gene

PCR was conducted in order to amplify a 349 bp region of exon 65 of the RYR1 gene. The sequence currently does not harbour any reported alterations associated with the MH phenotype. PCR was optimised using conditions listed in Table 4.1 (page 161). The amplified product was subsequently electrophoresed on a 2% (w/v) mini agarose gel (Figure 4.187).

Figure 4.187: Photographic representation of amplified PCR products encompassing exon 65

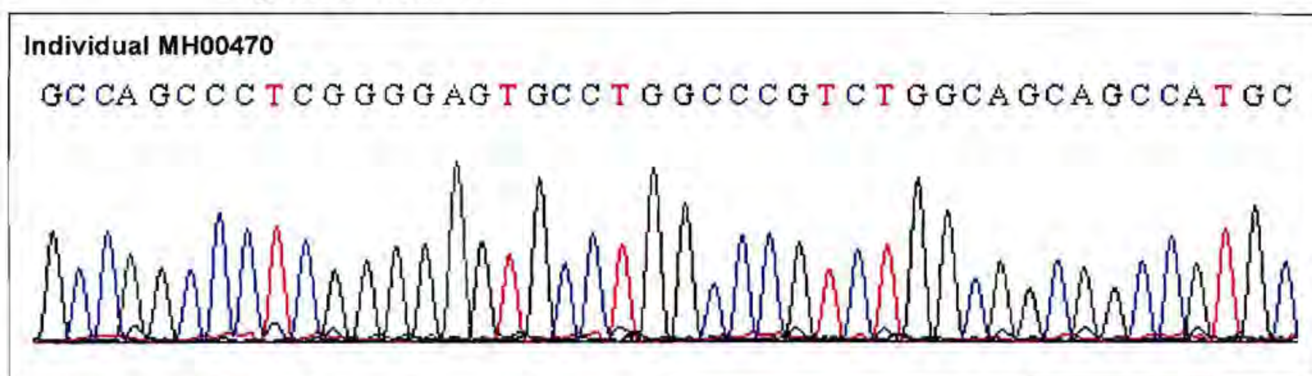


Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. An artefact in the gel matrix, as indicated by the white asterisk (*), was observed, as discussed in Section 4.3 and distortion of fragments, as discussed in Sections 4.2 and 4.3, was observed.

A representative result generated via automated sequencing is presented in Figure 4.188. Sequencing was conducted using the reverse primer (RYRex65R) and sequences are

illustrated in the reverse complement. None of the 15 MHS probands analysed in the study presented here harboured any novel alterations in this region. In addition, the region did not harbour any polymorphisms. Exon 65 has only recently been screened for alterations in other populations (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006), as discussed in Section 4.10.2 (page 275).

Figure 4.188: Representative electropherogram illustrating a portion of the amplified region of exon 65

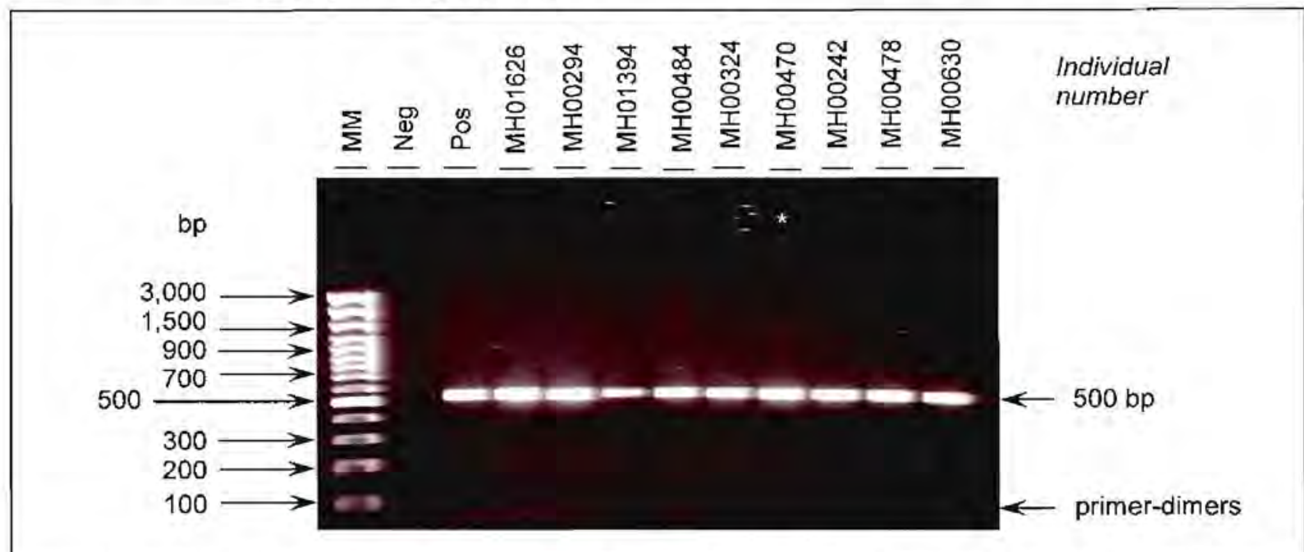


A = adenine; C = cytosine; G = guanine; T = thymine. As discussed in Section 4.5, sequences exhibited a high signal-to-noise ratio.

4.10.30 Exon 66 of the RYR1 gene

Alterations associated with MHS have thus far not been reported to occur in exon 66 of the RYR1 gene. PCR conditions were optimised as discussed in Section 4.2 (page 159). Using these conditions led to the successful amplification and sequencing of all 15 South African MH probands analysed in the study presented here. Figure 4.189 depicts the amplified products prior to sequencing using the optimised PCR protocol.

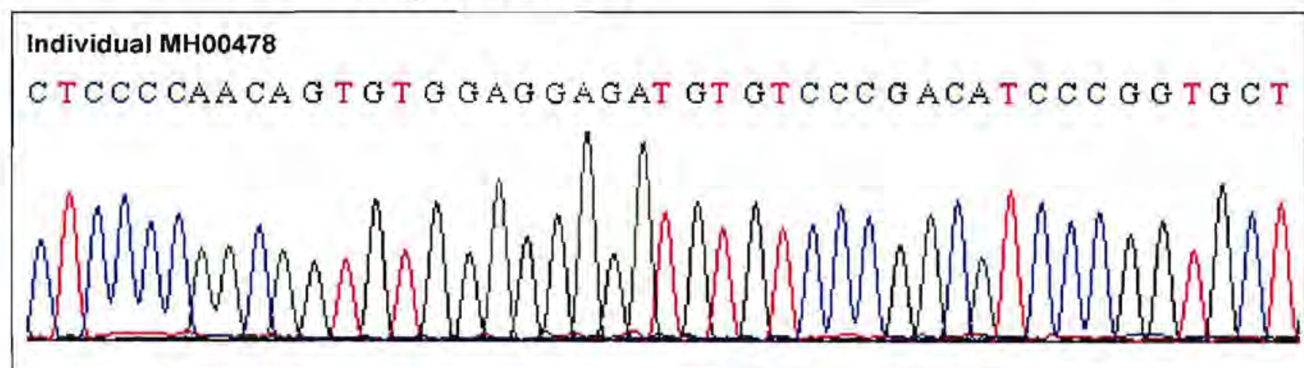
Figure 4.189: Photographic representation of amplified PCR products encompassing exon 66



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. As discussed in Sections 4.2 and 4.3, the presence of an artefact in the gel matrix, as indicated by the white asterisk (*), variation in amplification efficiency, non-specific amplification, background smears, fragment distortion and primer-dimers were observed.

Sequencing was conducted using the forward primer (RYRex66F). A representative sequence electropherogram obtained for individual MH00478, illustrating a portion of the amplified region of exon 66, is depicted in Figure 4.190. Alterations associated with MH were not observed in any of the South African MH probands analysed. In addition, alterations have never been reported to occur in this exon in other populations. As discussed in Section 4.10.2 (page 275), analysis of this region of the RYR1 gene in a larger group of individuals from various populations would have to be conducted in order to determine whether alterations associated with the MH phenotype reside in exon 66.

Figure 4.190: Representative electropherogram illustrating a portion of the amplified region of exon 66



A = adenine; C = cytosine; G = guanine; T = thymine.

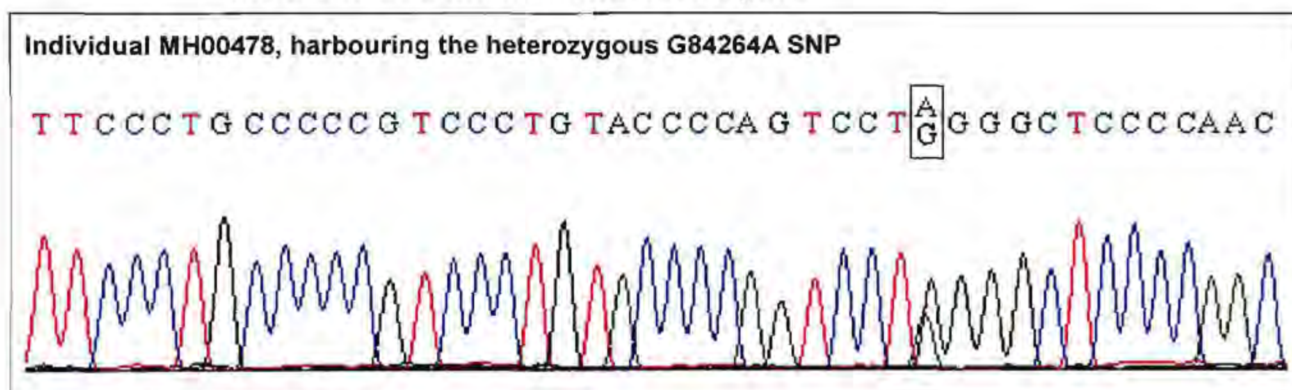
4.10.30.1 Synonymous substitution in the amplified region of exon 66 of the RYR1 gene

The heterozygous G84264A SNP was identified in the coding region of exon 66 in two South African probands (MH00478 and MH00324). As discussed in Section 4.7.4.1 (page 185), the SNP may affect gene expression by altering binding site sequences or may occur in order to increase translational efficiency or to maintain proper pre-mRNA processing or optimal base composition. In this manner, SNPs observed in the RYR1 gene may not be directly associated with MH. However, via epistasis a SNP may contribute a portion of the effect and may do so additively as well as interactively, as discussed in Section 5.1 (page 393).

4.10.30.1.1 SNP G84264A

The G84264A SNP has been identified as a synonymous substitution of the RYR1 gene in GenBank® (International Human Genome Sequencing Consortium, 2004; with accession number rs2304151) and retains the Leu amino acid. Figure 4.191 depicts the sequence generated for the heterozygous G84264A SNP. Population data reporting the frequency of this SNP have not been published worldwide.

Figure 4.191: Representative electropherogram indicating the G84264A SNP observed in exon 66 of the RYR1 gene



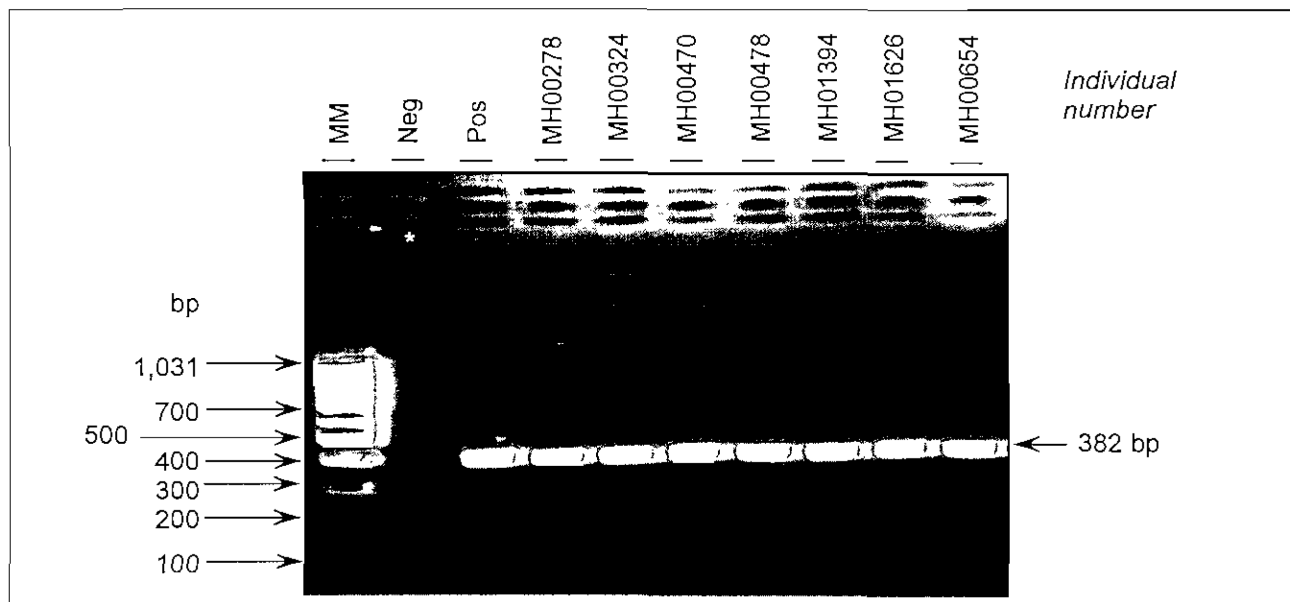
A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.31 Exon 67 of the RYR1 gene

The Arg3348His alteration is located outside the mutational hotspot, between the second and third hotspots of the RYR1 gene in exon 67. Sambuughin *et al.* (2005) reported the Arg3348His alteration in one MH family from North America. In addition, Wu *et al.* (2006) identified a Lys3367Arg alteration in one proband diagnosed with CCD. The 382 bp region

encompassing the mutation was amplified using optimised PCR conditions, as discussed in Section 4.2 (page 159). Thereafter, the PCR product was purified (Figure 4.192) and sequenced according to the standard protocol.

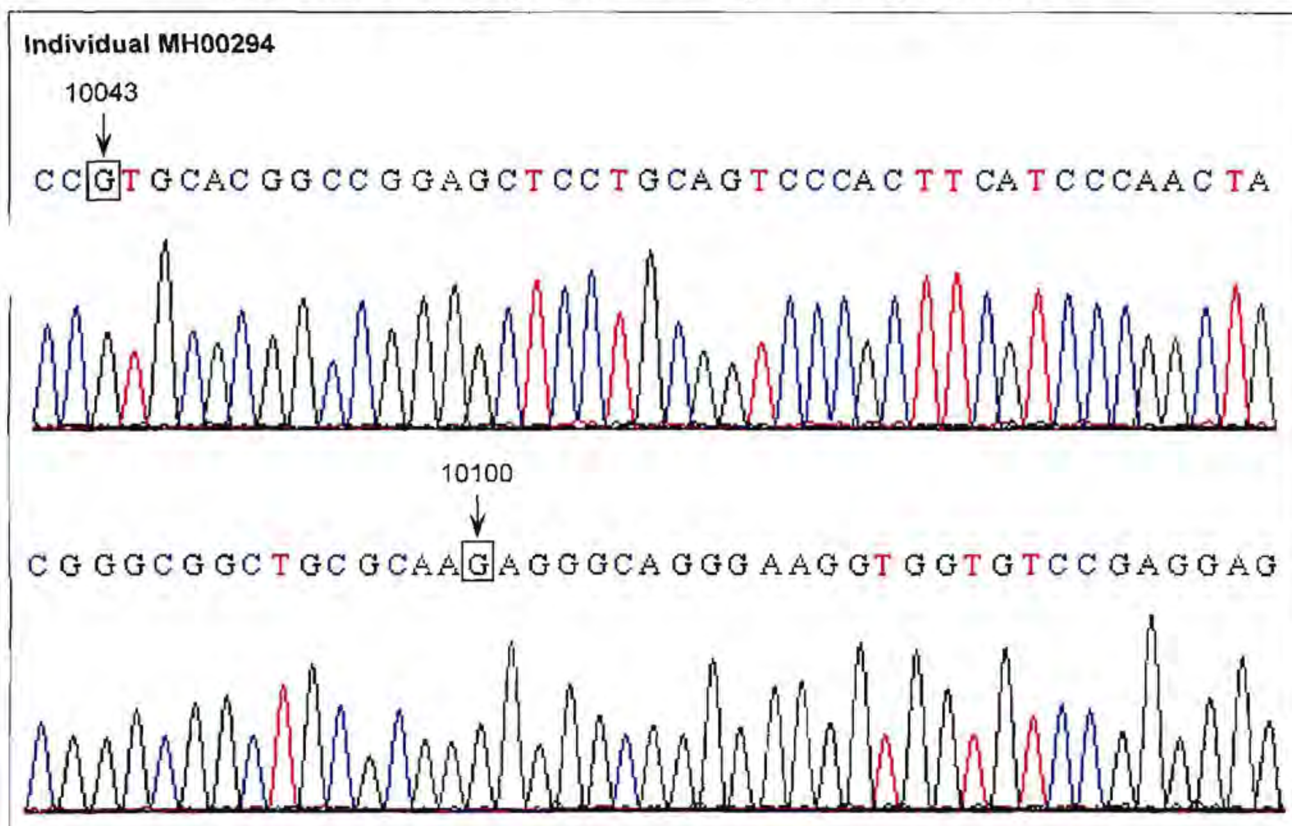
Figure 4.192: Photographic representation of amplified PCR products encompassing exon 67



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. An artefact in the gel matrix, as indicated by the white asterisk (*), was detected, the MM appeared overloaded and the EtBr migration front is visible, as discussed in Sections 4.2 and 4.3.

The forward primer (RYRex67F) was used in the sequencing reaction. A representative electropherogram obtained for individual MH00294, illustrating the nucleotide positions of the reported Arg2248His and Lys3367Arg alterations in exon 67 of the RYR1 gene, is depicted in Figure 4.193. None of the 15 MH probands from South Africa harboured the Arg3348His or the Lys3367Arg alterations or any other novel mutations that may be responsible for the MH phenotype. As these alterations have only been observed recently, the frequencies of the alterations in other populations have not been reported, as discussed in Section 4.10.2 (page 275). In addition, the Arg3348His alteration may represent a mutation that is private to the specific family in which it was first described (Sambuughin *et al.*, 2005) and the Lys3367Arg may also be family-specific and could only be observed in probands diagnosed with CCD (Wu *et al.*, 2006).

Figure 4.193: Representative electropherogram of exon 67 indicating the nucleotide positions of the Arg2248His and Lys3367Arg alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Arg3348His at nucleotide 10043 and Lys3367Arg at nucleotide 10100.

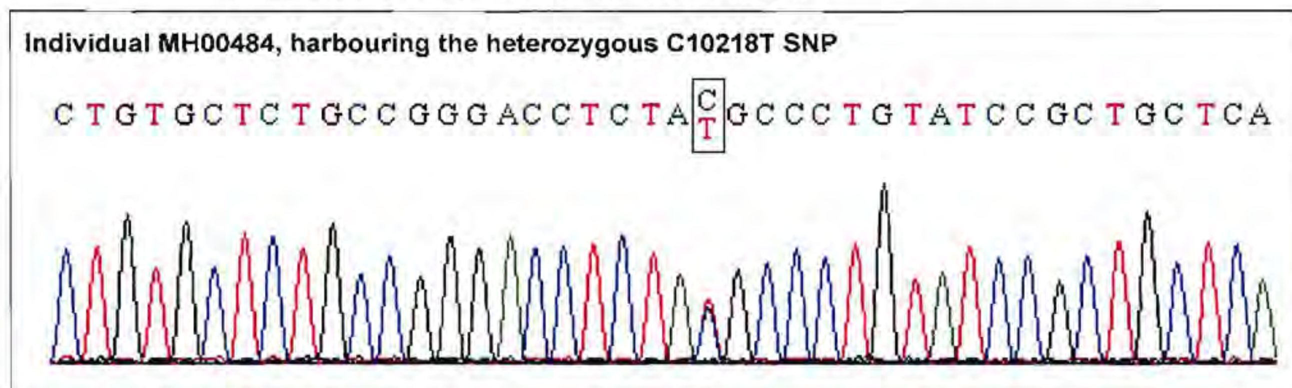
4.10.31.1 <u>Synonymous substitution in the amplified region of exon 67 of the RYR1 gene</u>

A C10218T SNP observed in exon 67 was identified in one MHS South African proband (MH00484). The remaining individuals analysed did not harbour the SNP. As discussed in Section 4.7.4.1 (page 185), the SNP may not result in MH in this individual. However, it may contribute to the MH phenotype via epistasis.

4.10.31.1.1 <u>SNP C10218T</u>

The C10218T SNP was reported recently by Galli *et al.* (2006). The SNP is synonymous as it retains the Tyr amino acid. Galli *et al.* (2006) calculated the minor allele frequency from the analysis of 100 chromosomes as 0.02. Figure 4.194 depicts the sequence generated for the heterozygous SNP.

Figure 4.194: Representative electropherogram indicating the C10218T SNP observed in exon 67 of the RYR1 gene

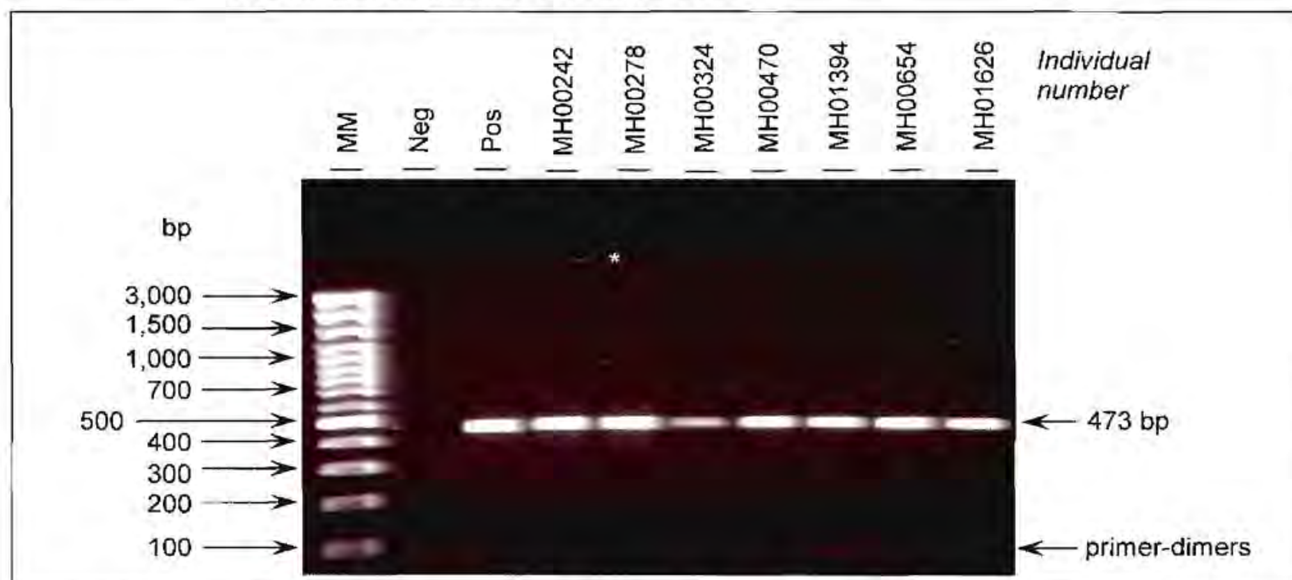


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.32 Exons 68 and 69 of the RYR1 gene

In order to investigate the presence of novel and reported alterations in exons 68 and 69, a 437 bp product was amplified, as discussed in Section 4.2 (page 159) and subsequently sequenced. Zhou *et al.* (2005) reported a Ser3446Phe alteration in one proband diagnosed with CCD. Figure 4.195 depicts the amplified products prior to sequencing using the optimised PCR protocol.

Figure 4.195: Photographic representation of amplified PCR products encompassing exons 68 and 69

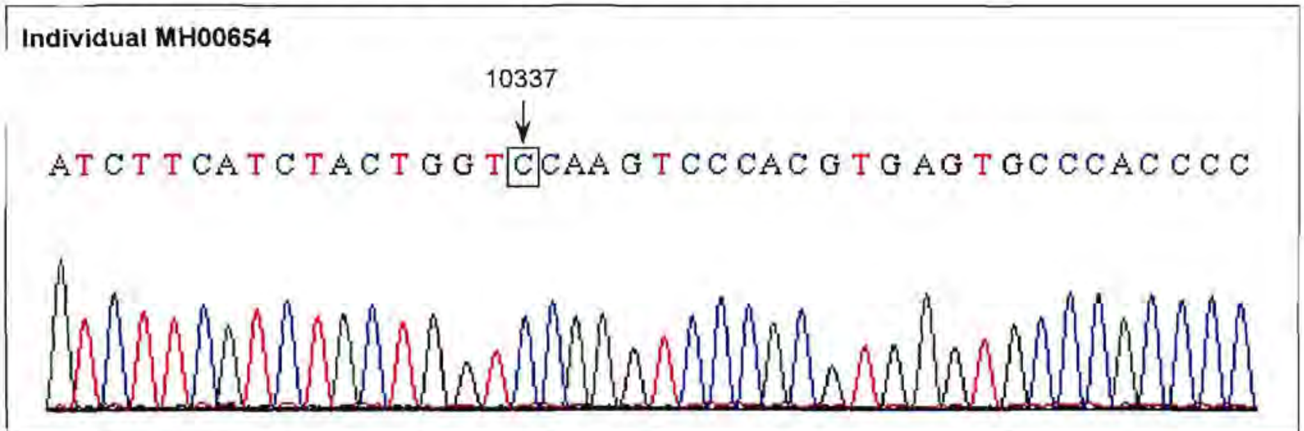


Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. An artefact in the gel matrix, as indicated by the white asterisk (*), variation in amplification efficiency, primer-dimers and MM distortion were observed and could be attributed to reasons discussed in Sections 4.2 and 4.3.

Of the 15 samples amplified, all samples were successfully sequenced according to the standard protocol. A representative electropherogram obtained for individual MH00654,

illustrating the nucleotide position of the reported Ser3446Phe alteration in exon 68 of the RYR1 gene, is depicted in Figure 4.196.

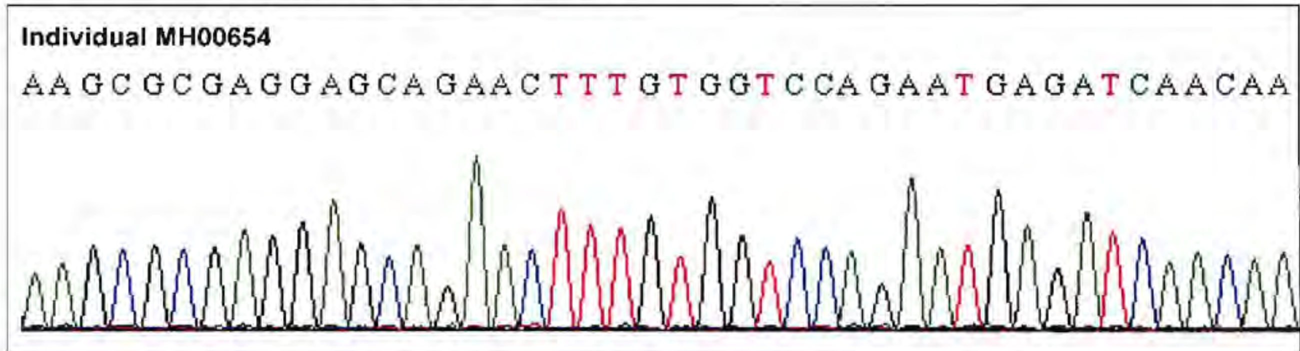
Figure 4.196: Representative electropherogram of exon 68 indicating the nucleotide position of the Ser3446Phe alteration



A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Ser2446Phe at nucleotide 10337.

A representative electropherogram illustrating a portion of the amplified region of exon 69 is depicted in Figure 4.197. None of the 15 MHS probands analysed in the study presented here harboured any novel alterations in this region of the RYR1 gene. Due to the absence of alterations in exons 68 and 69 in the South African individuals included in this investigation, it was concluded that these exons do not harbour alterations that add to the list of published MH mutations in the cohort presented here. However, as these exons have only recently been screened for MH mutations, alterations associated with MH may still be observed in these exons in subsequent studies, as discussed in Section 4.10.2 (page 275). It could be expected that the Ser3446Phe alteration would not be detected in this cohort of South African individuals, as the alteration has thus far only been reported in a proband diagnosed with CCD (Zhou *et al.*, 2005). Therefore, the alteration may be family-specific or only be observed in probands diagnosed with CCD.

Figure 4.197: Representative electropherogram illustrating a portion of the amplified region of exon 69

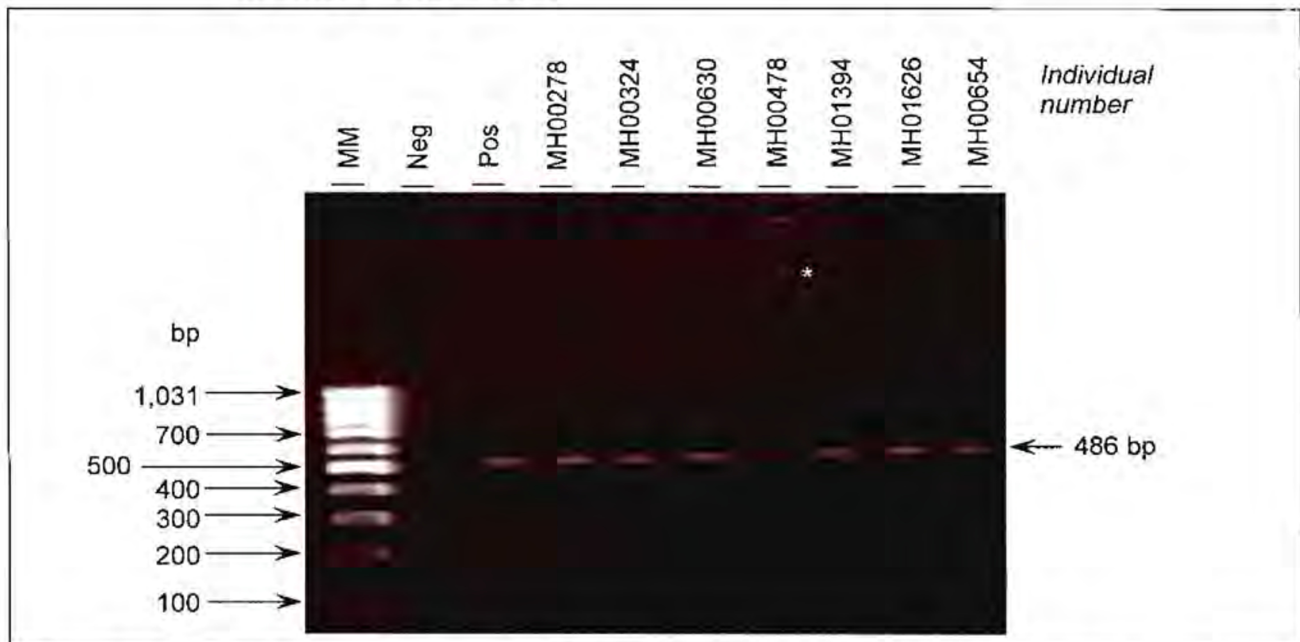


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.33 Exon 70 of the RYR1 gene

Exon 70 of the RYR1 gene currently does not harbour any reported alterations associated with the MH phenotype. PCR was performed according to optimised conditions listed in Table 4.1 (page 161). Figure 4.198 is a photographic representation of the amplicon encompassing exon 70.

Figure 4.198: Photographic representation of amplified PCR products encompassing exon 70

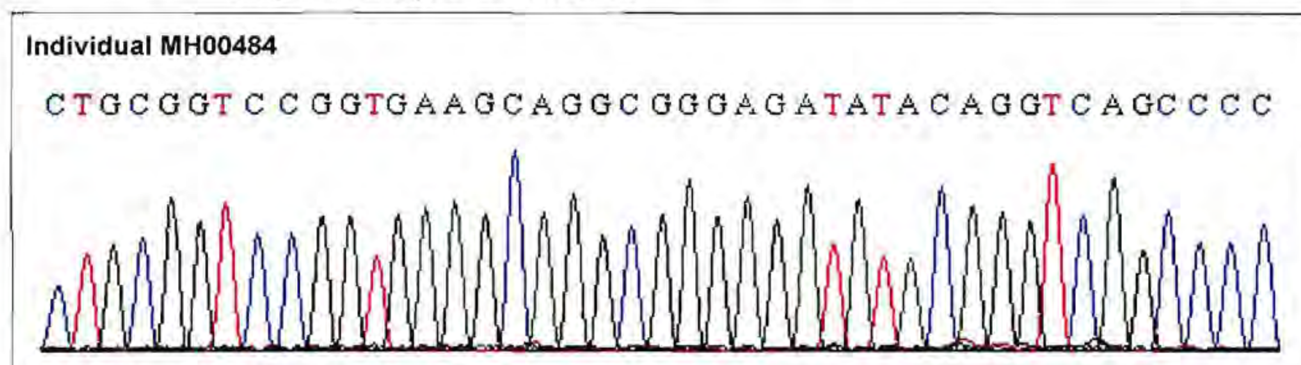


Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. The agarose gel exhibited an artefact in the gel matrix, as indicated by the white asterisk (*), variation in exonic amplification efficiency, distortion of the MM and non-linear migration, as discussed in Sections 4.2 and 4.3.

A representative electropherogram obtained for individual MH00484, depicting a portion of exon 70, is illustrated in Figure 4.199. Novel alterations in exon 70 were not observed in

any of the 15 MH probands analysed in the study presented here. As discussed in Section 4.10.2 (page 275), additional alterations outside the mutational hotspots have only been observed recently (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006). Therefore, further analysis of this exon in additional MH probands from various populations may identify alterations that result in susceptibility to MH.

Figure 4.199: Representative electropherogram illustrating a portion of the amplified region of exon 70

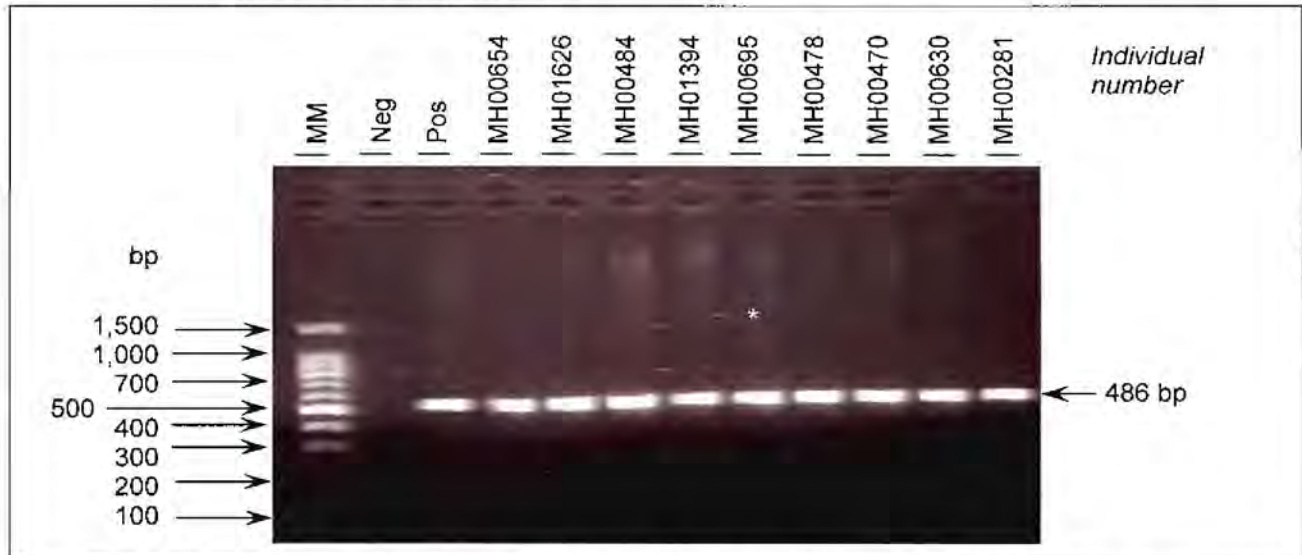


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.34 Exon 71 of the RYR1 gene

To date, one alteration has been reported for this region of the RYR1 gene. The Pro3527Ser alteration was observed in one proband diagnosed with CCD (Zhou *et al.*, 2005). A 486 bp product encompassing exon 71 was amplified via PCR, as discussed in Section 4.2 (page 159). The product was subsequently electrophoresed on a 2% (w/v) mini agarose gel and Figure 4.200 is a photographic representation of the amplicon encompassing exon 71.

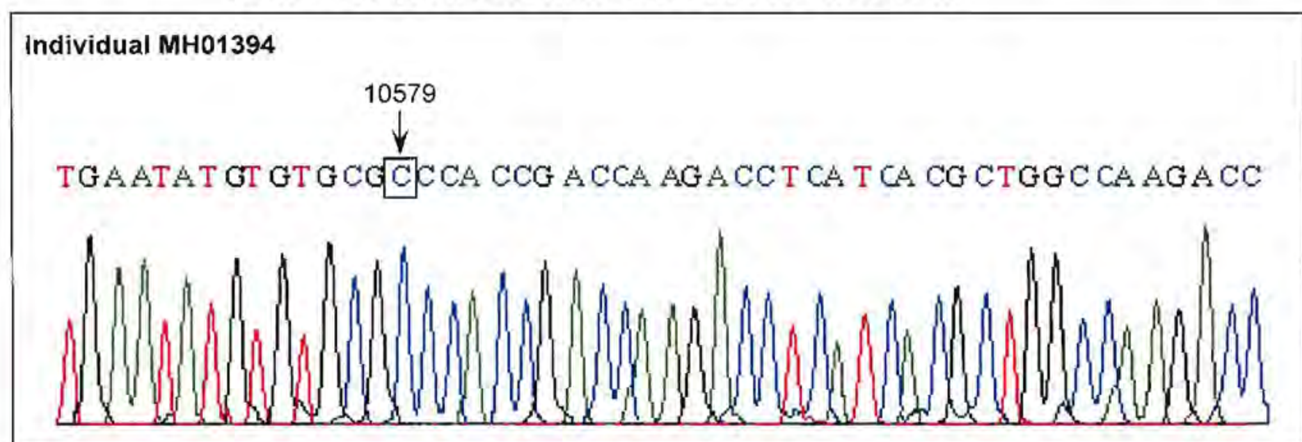
Figure 4.200: Photographic representation of amplified PCR products encompassing exon 71



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. An artefact in the gel matrix was observed above the amplified products, as indicated by the white asterisk (*), background smear, fragment distortion and non-linear migration were observed, as discussed in Sections 4.2 and 4.3.

All 15 samples were successfully sequenced using the reverse primer (RYRex71R). Therefore, sequences are illustrated as the reverse complement and a representative result generated via automated sequencing depicting the nucleotide position of the reported Pro3526Ser alteration is presented in Figure 4.201.

Figure 4.201: Representative electropherogram of exon 71 indicating the nucleotide position of the Pro3526Ser alteration



A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Pro3527Ser at nucleotide 10579.

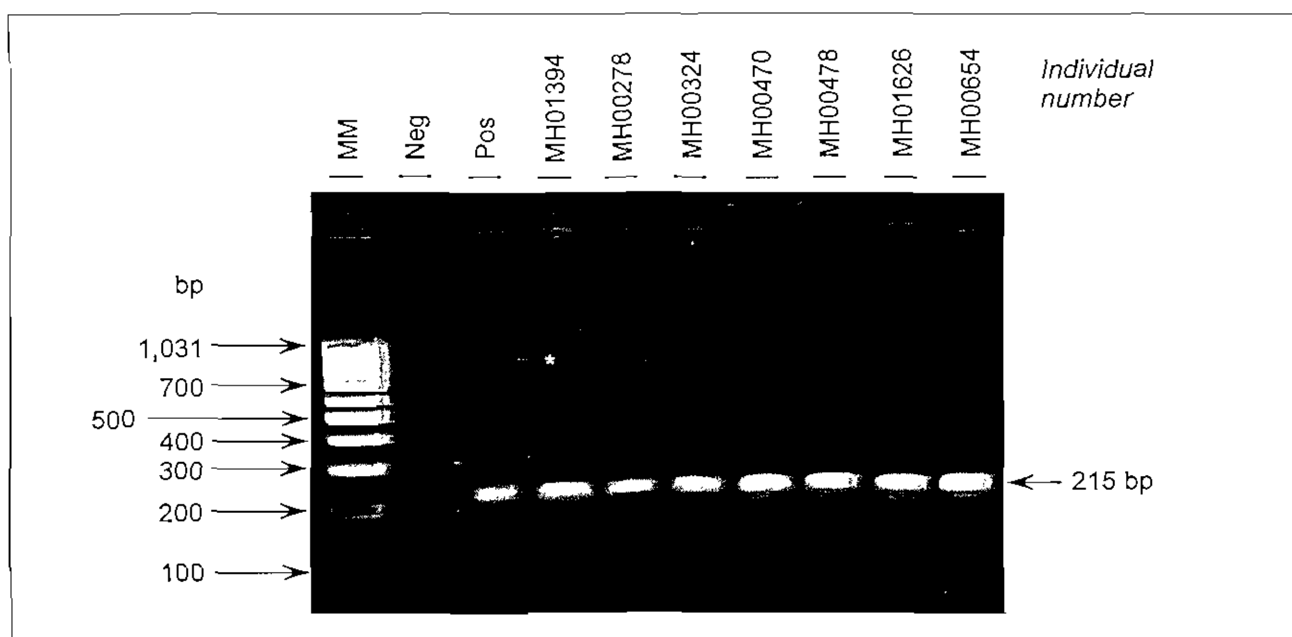
None of the 15 MHS probands analysed in the study presented here harboured any novel alterations in this region. The Pro3527Ser alteration has thus far only been observed in one proband diagnosed with CCD (Zhou *et al.*, 2005). Therefore, the Pro3527Ser alteration may be specific to CCD or may be family-specific. At the present time, the

presence of alterations in exon 71 in all populations has not yet been determined, as discussed in Section 4.10.2 (page 275). Therefore, further analysis will have to be conducted in order to determine the role of this exon in the development of the disorder.

4.10.35 Exon 72 of the RYR1 gene

Thus far, exon 72 does not harbour any reported alterations associated with MH susceptibility. PCR was conducted as discussed in Section 4.2 (page 159) and the results are presented in Figure 4.202.

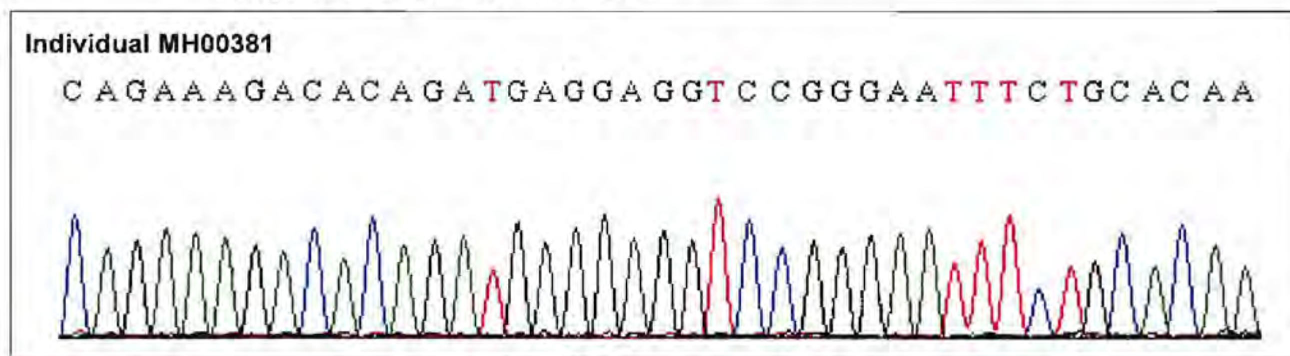
Figure 4.202: Photographic representation of amplified PCR products encompassing exon 72



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. An artefact in the gel matrix, as indicated by the white asterisk (*), non-specific amplification, background smear, distortion of fragments and variation in amplification efficiency were observed, as discussed in Sections 4.2 and 4.3.

A representative result generated via automated sequencing, depicting a portion of the amplified region of exon 72, is presented in Figure 4.203. Sequencing was conducted using the reverse primer (RYRex72R) and sequences are thus depicted as the reverse complement. None of the 15 MHS probands analysed in the study presented here harboured any novel alterations in this region. To date, this exon has never been screened for alterations associated with MH in the South African population. Although alterations have never been reported to occur in this exon, further analysis of this region of the RYR1 gene may yield alterations associated with MH, as discussed in Section 4.10.2 (page 275).

Figure 4.203: Representative electropherogram illustrating a portion of the amplified region of exon 72

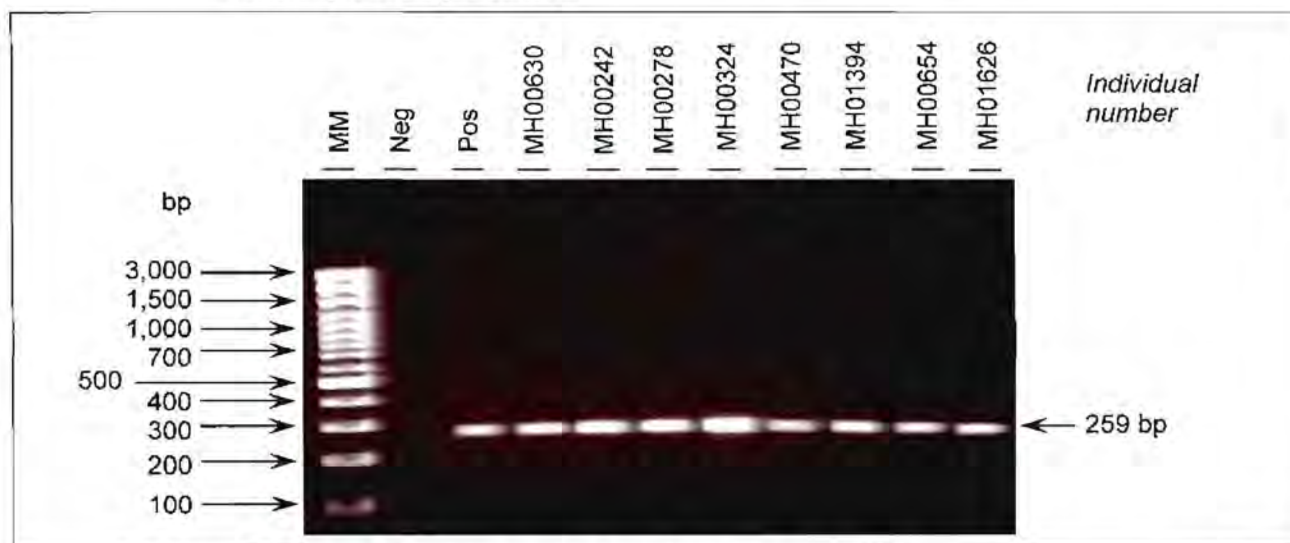


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.36 Exon 73 of the RYR1 gene

Fifteen MH probands that have previously not been analysed for novel alteration in this region of the RYR1 gene were screened during the Phase 3 study. The PCR conditions were optimised using conditions listed in Table 4.1 (page 161). The amplified product was electrophoresed on a 2% (w/v) mini agarose gel (Figure 4.204). A single Leu3606Pro alteration has been observed previously in a proband diagnosed with CCD (Wu *et al.*, 2006).

Figure 4.204: Photographic representation of amplified PCR products encompassing exon 73

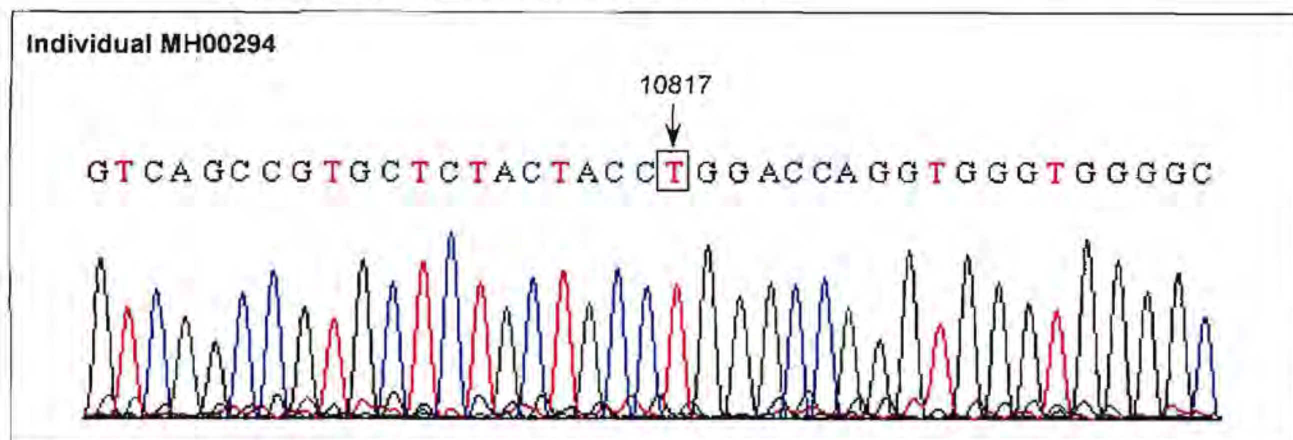


Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Variation in amplification efficiency and overloading of the MM were observed, as discussed in Sections 4.2 and 4.3.

Fifteen MH probands amplified via PCR were successfully sequenced using the protocol outlined in Section 3.7 (page 94). A representative electropherogram obtained for individual MH00294 illustrating the nucleotide position of the previously reported

Leu3606Pro alteration in exon 73 is indicated in Figure 4.205. A single novel alteration was observed in exon 73 in one MH proband (MH01626) and is discussed in Section 4.10.36.1 (page 365). The remaining 14 South African probands did not harbour the alteration.

Figure 4.205: Representative electropherogram of exon 73 indicating the nucleotide position of the Leu3606Pro alteration



A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Leu3606Pro at nucleotide 10817. Background peaks were observed, as discussed in Section 4.5.

4.10.36.1 The Glu3583Gln alteration in the RYR1 gene

In one South African proband (MH01626), a single novel alteration was observed in exon 73 of the RYR1 gene. Table 4.16 depicts the partial amino acid sequences of exon 73 of the RyR1 protein obtained from all three RyR isoforms and other vertebrate *ryr1* proteins. Multiple alignments encompassing the position of the alteration were obtained from GenBank® (P21817, Q92736, NP_001027, P16960 and P11716), and the position of the alteration detected in this study is indicated in blue.

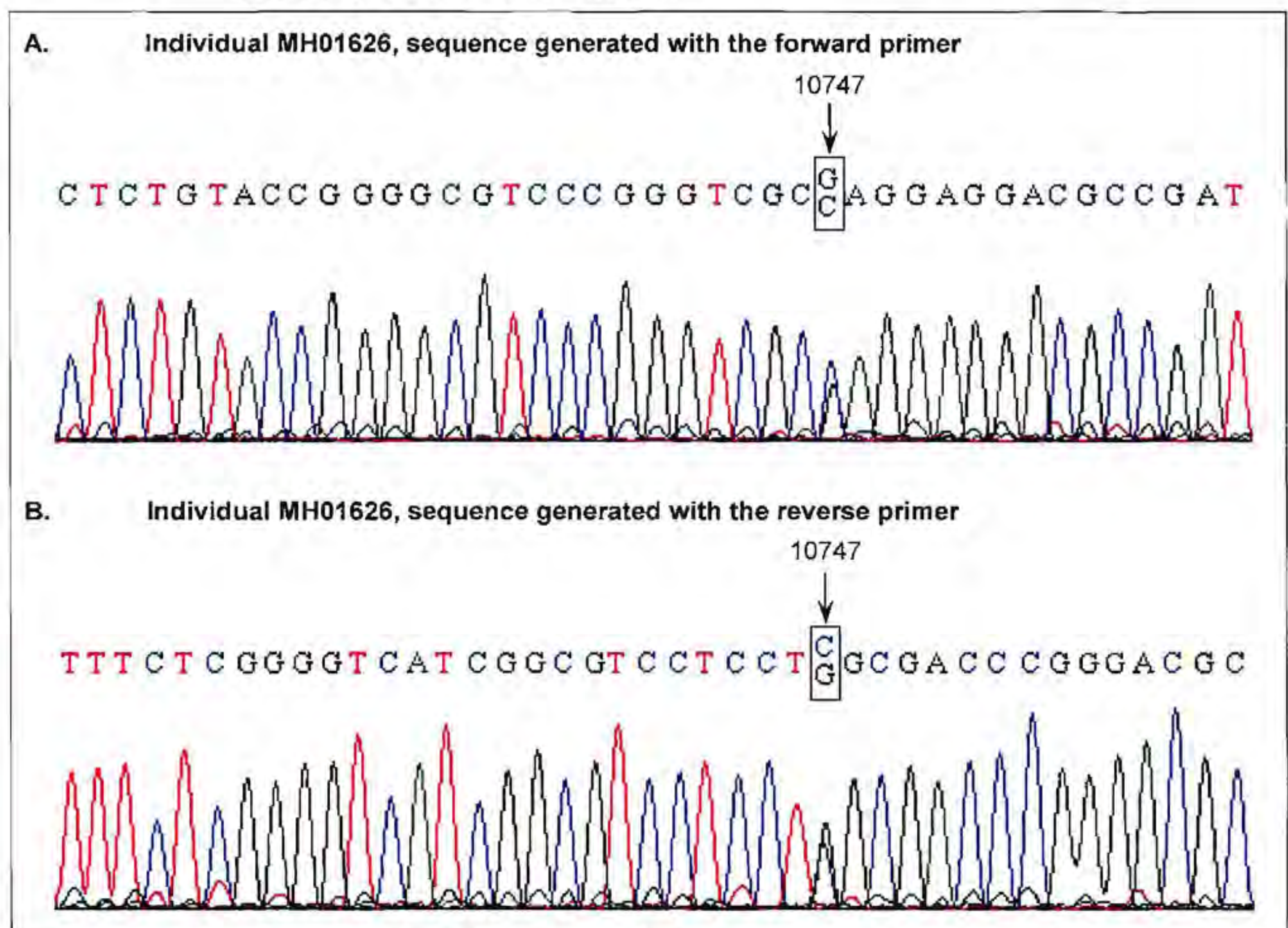
Table 4.16: Conserved amino acids from different RyR isoforms and species surrounding the novel and reported mutations in exon 73 of the RyR1 protein

Isoform	Species	Alignment of RyR protein sequences
RyR1	Human	GKVEGSPSLR WQMALYRGV PGR**E**EDADD PEKIVRRVQE VSAVLYY**L**DQ TEHPYTS
RyR2	Human	GKLE-DPAIR WQMALYKDL PNR**T**DDTSD PEKTVERVLD IANVLFH**L**EQ KSKRVTS
RyR3	Human	------PAVK WQLNLYKDV L-K**S**EEPFN PEKTVERVQR ISAAVFH**L**EQ TEHPYTS
ryr1	Pig	GKVEGSPSLR WQMALYRGL PGR**E**EDADD PEKIVRRVQE VSAVLYH**L**DE MEHPYTS
ryr1	Rabbit	GKVEGSPSLR WQMALYRGL PGR**E**EDADD PEKIVRRVQE VSAVLYH**L**DE TEHPYTS

RyR1 = RyR expressed in human skeletal muscle; RyR2 = RyR expressed in human cardiac muscle; RyR3 = RyR expressed in human brain; *ryr1* = RyR1 protein expressed in animals. Amino acid residues that are not conserved among different isoforms and species are highlighted in grey. The site of the conserved novel Glu amino acid at nucleotide 10747 is indicated in blue. The site at which the previously reported alteration that occurs in the depicted region is indicated as follows: Leu3606Pro = red. The accession numbers are as follows: RyR1 human = P21817; RyR2 human = Q92736; RyR3 human = NP_001027; *ryr1* pig = P16960 and *ryr1* rabbit = P11716.

The variant affects a well conserved amino acid among RYR1 species. However, the region is not highly conserved among RYR isoforms. As discussed in Section 4.10.15.1 (page 305), alterations that result in disease may not reside in highly conserved regions. At nucleotide position 10747, two peaks were observed that indicated the presence of two nucleotide bases G and C. The different bases resulted in the expression of two amino acids, namely Glu and Gln, respectively. The sequences depicted on the electropherograms of Figure 4.206 indicate the alteration observed using the forward primer and reverse primer, respectively.

Figure 4.206: Representative electropherograms indicating the Glu3583Gln alteration observed in exon 73



A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Glu3583Gln at nucleotide 10747.

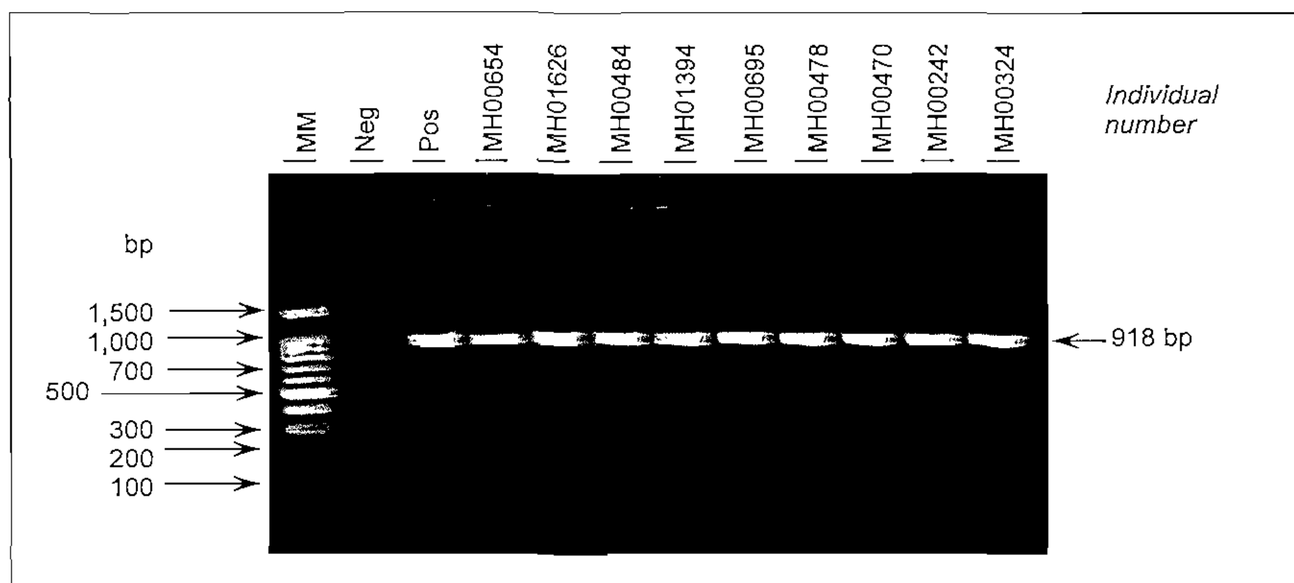
The Glu3583Gln alteration occurs due to a G10747C nucleotide transition and has never been reported in any other population. The Glu3583Gln alteration was, however, not observed in any other family members of this pedigree, which indicates that the alteration was not transmitted. Since the alteration was not observed in any other family members, DNA from MH01626 was re-amplified and sequenced to verify the result. The alteration

was confirmed in the re-amplified sequence, indicating that an error had not occurred during the amplification process. This alteration represents the first mutation in exon 73 observed in an MHS individual from South Africa. The Glu3583Gln alteration is the second alteration identified in family MH125-1. The Phe2364Val alteration was identified in exon 44 of the RYR1 gene in this family, as discussed in Section 4.8.5.2 (page 231). As the Glu3583Gln alteration does not occur in a conserved region of the RyR1 protein and does not segregate with the MH phenotype, it may represent a polymorphism. However, functional studies would have to be conducted in order to clarify the role of this alteration. As discussed in Section 5.4 (page 406), the Glu3583Gln alteration may contribute to the MH phenotype via epistasis.

4.10.37 Exons 74, 75 and 76 of the RYR1 gene

In order to identify novel alterations that may occur in exons 74, 75 or 76, a region of 918 bp was amplified, as discussed in Section 4.2 (page 159) and subsequently sequenced. The amplified product was electrophoresed on a 2% (w/v) mini agarose gel and results depicting the amplified region of exons 74, 75 and 76 are illustrated in Figure 4.207.

Figure 4.207: Photographic representation of amplified PCR products encompassing exons 74, 75 and 76

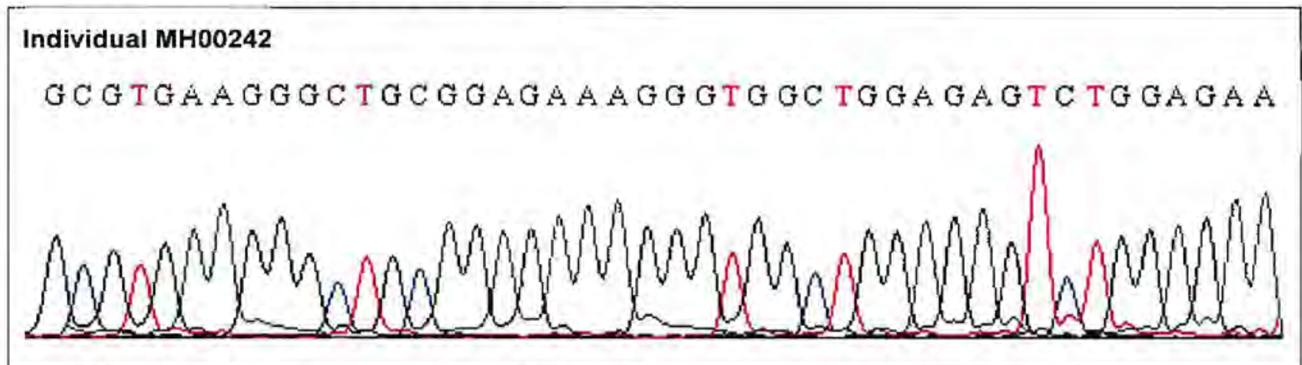


Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. The depicted fragments exhibited non-specific amplification, background smear, variation in amplification efficiency and fragment distortion, as discussed in Sections 4.2 and 4.3.

The amplified region was subsequently sequenced in order to screen for novel mutations that might be present in this region of the RYR1 gene. Sequencing was conducted using

the reverse primer (RYRex74R). A representative electropherogram depicted as the reverse complement, obtained for individual MH00242, illustrating a portion of the amplified region of exon 74, is depicted in Figure 4.208.

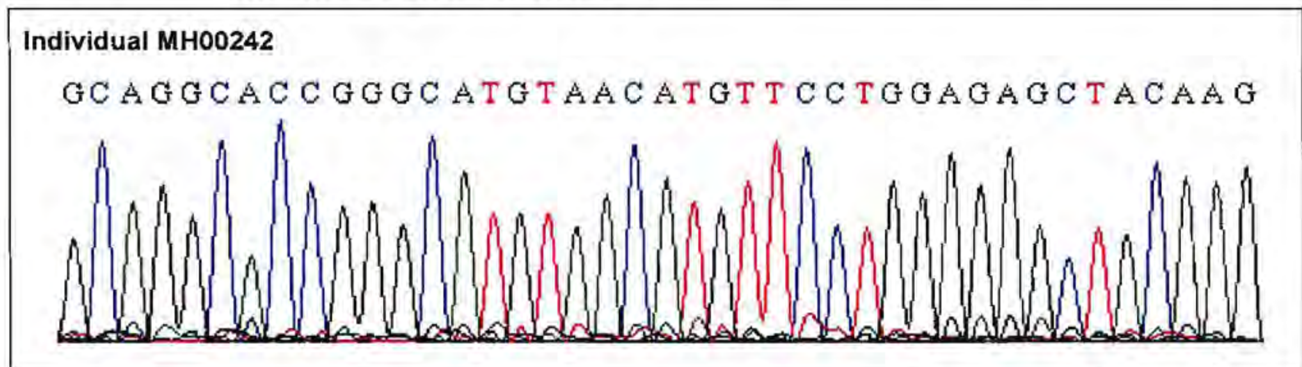
Figure 4.208: Representative electropherogram illustrating a portion of the amplified region of exon 74



A = adenine; C = cytosine; G = guanine; T = thymine. As discussed in Section 4.5, low background peaks were observed.

A representative electropherogram obtained for individual MH00242, illustrating a portion of the amplified region of exon 75, is depicted in Figure 4.209. In addition, a representative electropherogram illustrating a portion of the amplified region of exon 76, is depicted in Figure 4.210. To date, alterations associated with MHS have never been reported for this region of the RYR1 gene.

Figure 4.209: Representative electropherogram illustrating a portion of the amplified region of exon 75

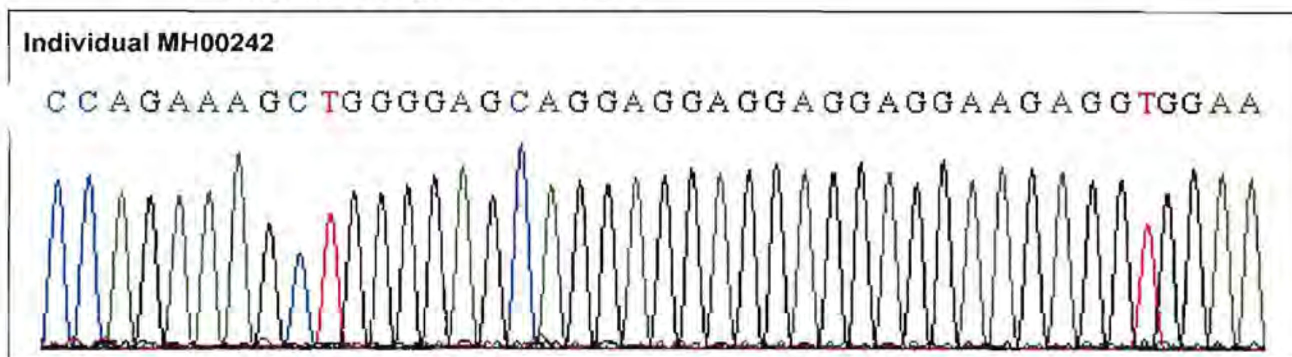


A = adenine; C = cytosine; G = guanine; T = thymine. As discussed in Section 4.5, low background peaks were observed.

In the study presented here, novel alterations associated with MH were not observed in any of the South African MH probands. However, a small group of individuals was screened in the study presented here. Thus far complete screening of the coding region of the RYR1 gene has only been conducted in a few populations (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006), as

discussed in Section 4.10.2 (page 275). Therefore, further analysis would have to be conducted in order to clarify the role of exons 74, 75 and 76 in the development of MHS.

Figure 4.210: Representative electropherogram illustrating a portion of the amplified region of exon 76

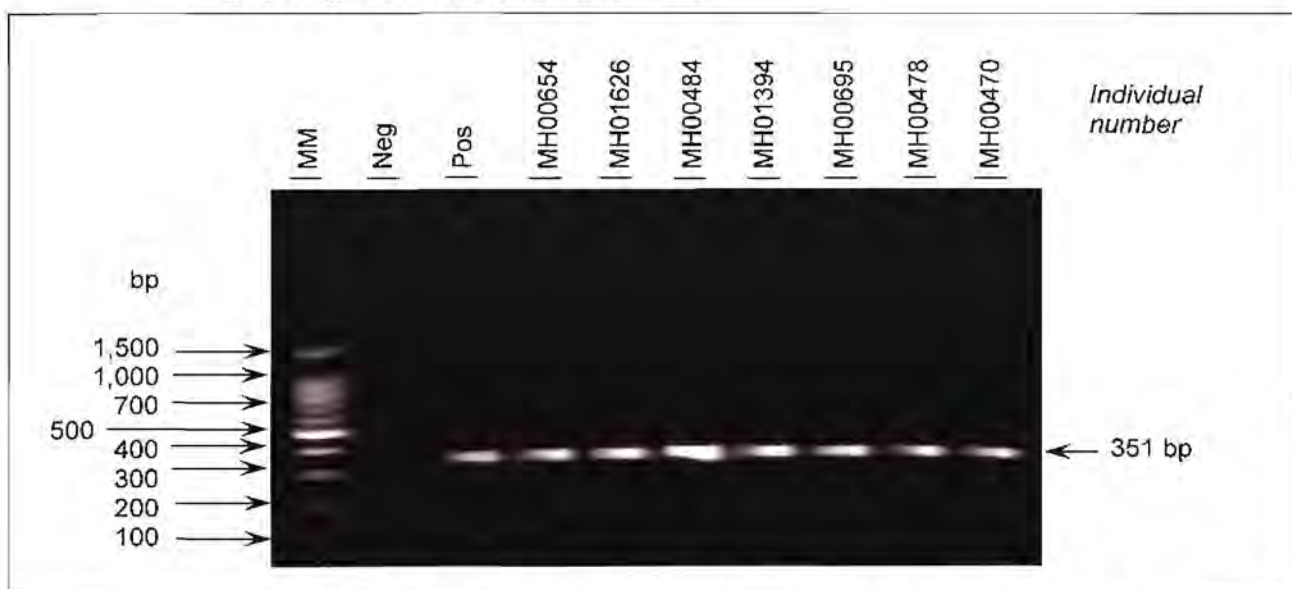


A = adenine; C = cytosine; G = guanine; T = thymine. As discussed in Section 4.5, low background peaks were observed.

4.10.38 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 novel and reported polymorphisms that may occur in this region. PCR was optimised using conditions described in Section 4.2 (page 159). The amplified region encompassing exons 77 and 78 is illustrated in Figure 4.211. Alterations associated with MHS have not been reported for exons 77 and 78.

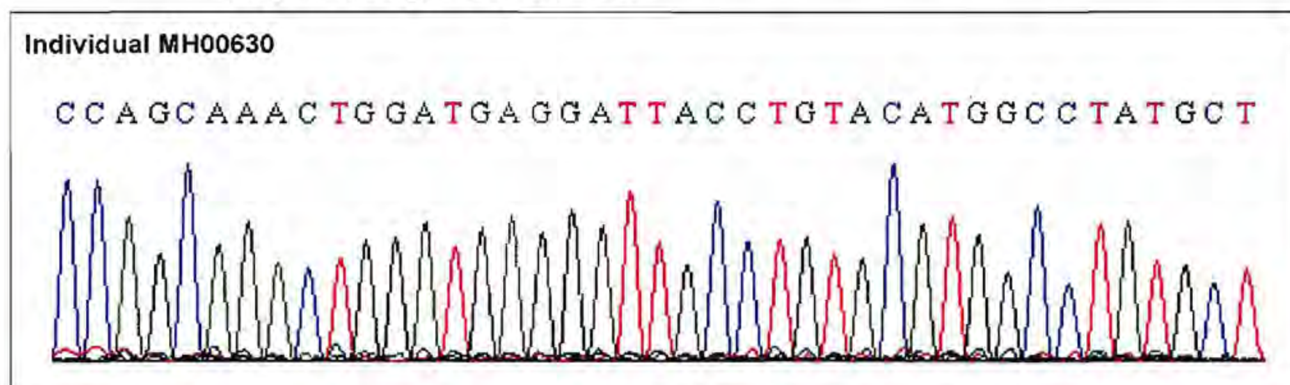
Figure 4.211: Photographic representation of amplified PCR products encompassing exons 77 and 78



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Fragment distortion and variation in the amplification efficiency were observed, as discussed in Sections 4.2 and 4.3.

A representative result generated via automated sequencing for exon 77 is presented in Figure 4.212. In addition, a representative electropherogram for exon 78 is presented in Figure 4.213. Sequencing was conducted using the reverse primer (RYRex77R), therefore, sequences are depicted as the reverse complement.

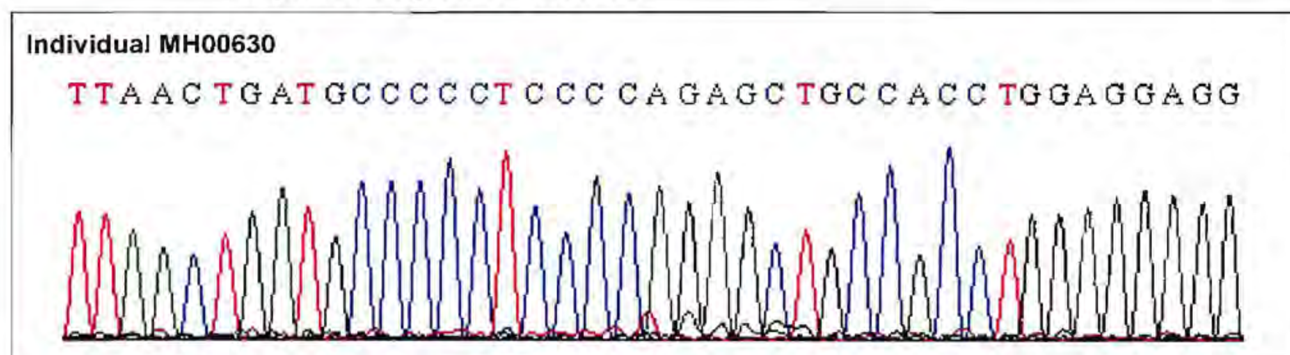
Figure 4.212: Representative electropherogram illustrating a portion of the amplified region of exon 77



A = adenine; C = cytosine; G = guanine; T = thymine. As discussed in Section 4.5, low background peaks were observed.

Fifteen individuals were screened for alterations in exon 77 and exon 78. Thus far, alterations associated with MH in other populations have not been detected in this region of the RYR1 gene in the South African probands investigated. Novel alterations were also not observed in any of the individuals analysed. However, regions outside the RYR1 mutational hotspots have been only investigated recently, as discussed in Section 4.10.2 (page 275). Further analysis of these exons should be conducted in all populations in order to identify novel alterations associated with MHS.

Figure 4.213: Representative electropherogram illustrating a portion of the amplified region of exon 78

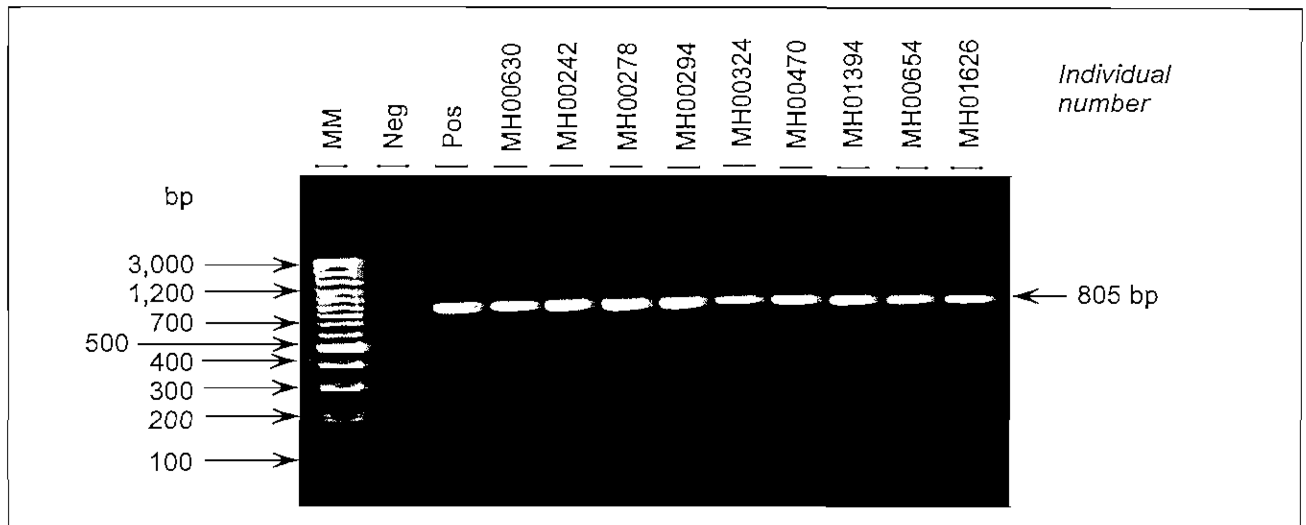


A = adenine; C = cytosine; G = guanine; T = thymine. As discussed in Section 4.5, low background peaks were observed.

4.10.39 Exons 79, 80 and 81 of the RYR1 gene

A region of 805 bp encompassing exons 79, 80 and 81 was amplified via PCR, as discussed in Section 4.2 (page 159). Successful amplification was achieved for all the samples investigated. Figure 4.214 is a photographic representation of the electrophoresed amplicon.

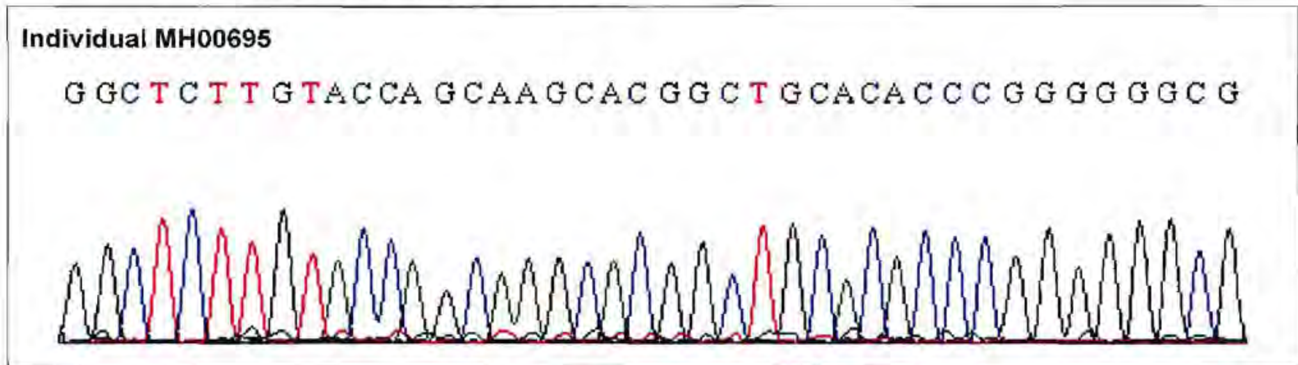
Figure 4.214: Photographic representation of amplified PCR products encompassing exons 79, 80 and 81



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Variation in amplification efficiency and MM overloading were observed, as discussed in Sections 4.2 and 4.3.

Fifteen MH probands that were amplified via PCR were successfully sequenced using the protocol outlined in Section 3.5 (page 92). The amplified region was sequenced in order to identify possible novel mutations that may result in MHS. A representative electropherogram obtained for individual MH00695, illustrating a portion of exon 79, is indicated in Figure 4.215.

Figure 4.215: Representative electropherogram illustrating a portion of the amplified region of exon 79



A = adenine; C = cytosine; G = guanine; T = thymine. Background peaks, as discussed in Section 4.5 were observed.

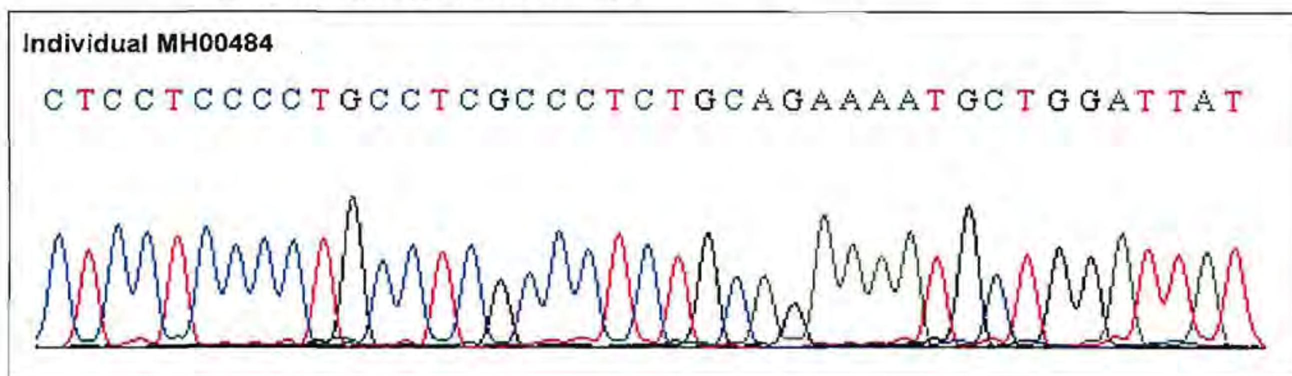
This region of the RYR1 gene resides between the second and third mutational hotspots. A representative electropherogram obtained for individual MH00695, illustrating a portion of the amplified region for exon 80, is presented in Figure 4.216.

Figure 4.216: Representative electropherogram illustrating a portion of the amplified region of exon 80

A = adenine; C = cytosine; G = guanine; T = thymine.

In the study presented here, alterations that may be associated with MH were not observed in any of the 15 South African MH probands analysed. A representative electropherogram obtained for individual MH00695, illustrating a portion of the amplified region for exon 81, is presented in Figure 4.217. As discussed in Section 4.10.2 (page 275), additional alterations outside the mutational hotspots have only been observed recently. Therefore, further analysis of these exons may identify alterations that result in susceptibility to MH.

Figure 4.217: Representative electropherogram illustrating a portion of the amplified region of exon 81

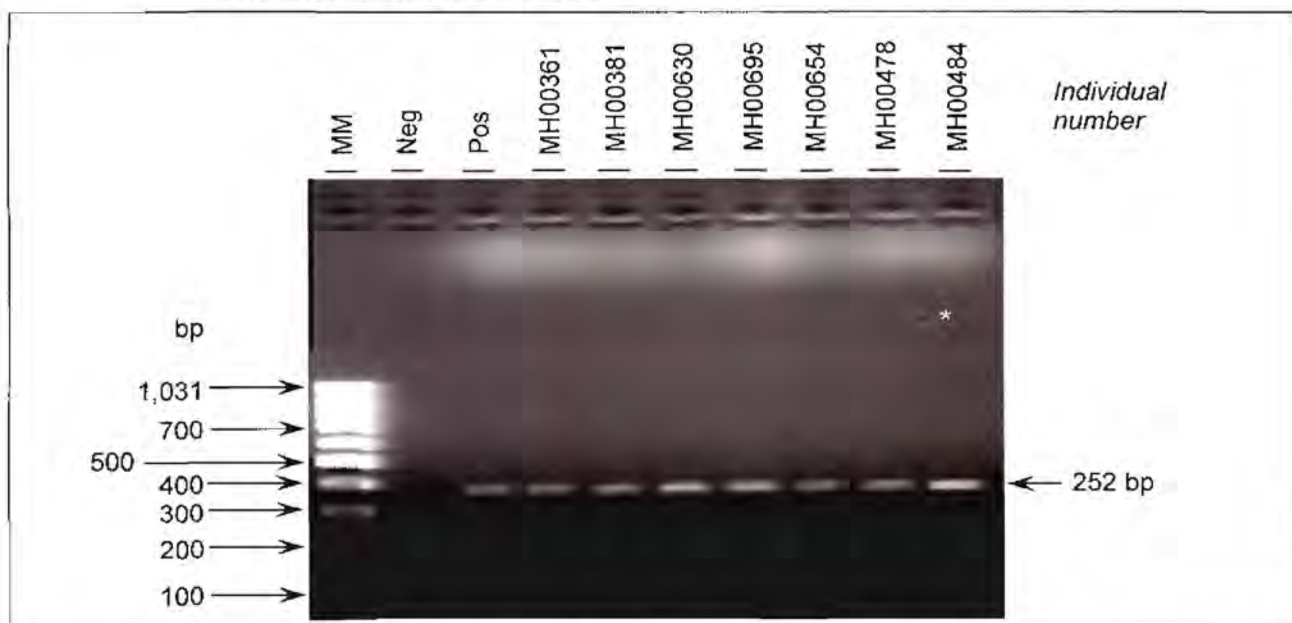


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.40 Exon 82 of the RYR1 gene

Thus far, exon 82 harbours one reported alteration. The Val3840Ile alteration was observed in one MH family from Japan (Ibarra *et al.*, 2006). PCR was conducted as described in Section 4.2 (page 159). The product was electrophoresed on a 2% (w/v) mini agarose gel, and the results are presented in Figure 4.218.

Figure 4.218: Photographic representation of amplified PCR products encompassing exon 82

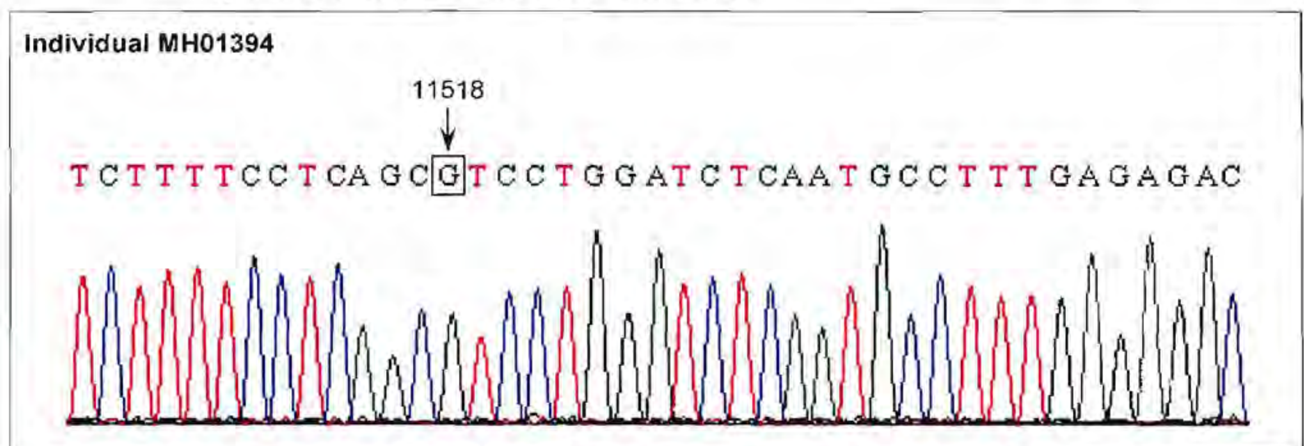


Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. An artefact in the gel matrix, as indicated by the white asterisk (*), variation in amplification efficiency between exonic region, MM overloading and the EtBr migration front were observed, as discussed in Sections 4.2 and 4.3.

Sequencing was conducted using the forward primer (RYRex82F). A representative electropherogram indicating the nucleotide position of the previously reported Val3840Leu

alteration observed in exon 82 for individual MH01394 is illustrated in Figure 4.219. Novel or reported alterations were not detected in any of the 15 South African MH probands analysed. Further analysis in a large group of individuals from various populations would have to be conducted in order to determine if exon 82 plays a role in the development of MH, as discussed in Section 4.10.2 (page 275). Previous identification of the Val3840Ile alteration (Ibarra *et al.*, 2006) in a proband diagnosed with MH may indicate that this region, if mutated, may result in the MH phenotype. However, the causative status of this alteration via functional studies has not been determined. In addition, the alteration may either be population- or family-specific.

Figure 4.219: Representative electropherogram of exon 82 indicating the nucleotide position of the Val3840Ile alteration



A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Val3840Ile at nucleotide 11518.

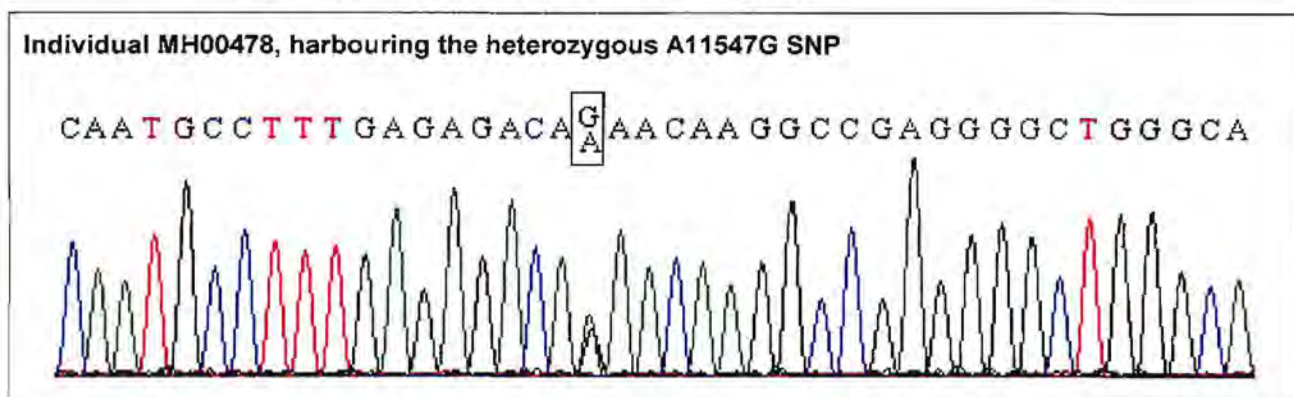
4.10.40.1 Synonymous substitution in the amplified region of exon 82 of the RYR1 gene

An A11547G SNP was identified in one South African proband (MH00478) in exon 82. The remaining individuals analysed did not harbour the SNP. As discussed in Section 4.7.4.1 (page 185), the SNPs may not result in MH in these individuals, however, they may contribute to the MH phenotype via epistasis.

4.10.40.1.1 SNP A11547G

The A11547G SNP was reported recently by Galli *et al.* (2006). The SNP is synonymous as it retains the Gln amino acid. Galli *et al.* (2006) calculated the minor allele frequency from analysis of 100 chromosomes as 0.15. Figure 4.220 depicts the sequence generated for the heterozygous SNP.

Figure 4.220: Representative electropherogram indicating the A11547G SNP observed in exon 82 of the RYR1 gene

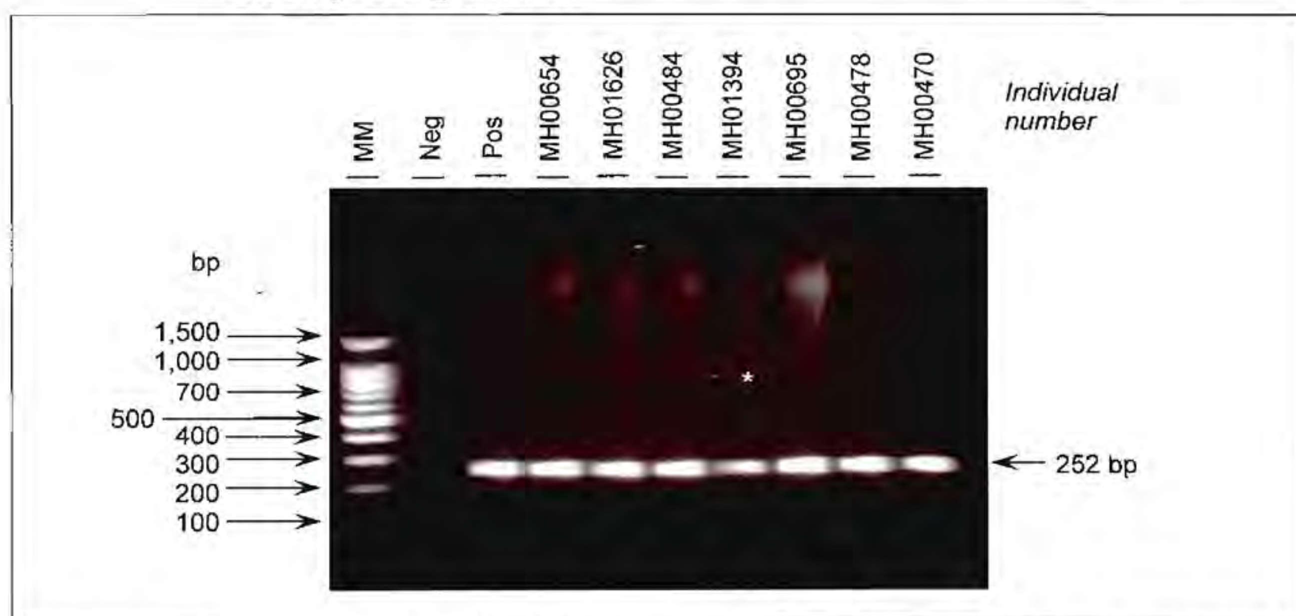


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.41 Exon 83 of the RYR1 gene

PCR was used to amplify a 252 bp product of exon 83, in order to identify novel alterations that may occur in this region of the RYR1 gene. The PCR conditions were optimised using conditions outlined in Section 4.2 (page 159). Both primers for this exon had a moderately high T_m, therefore the two-step fast PCR protocol was used as discussed in Section 3.5 (page 92). The amplified product was electrophoresed on a 2% (w/v) mini agarose gel (Figure 4.221).

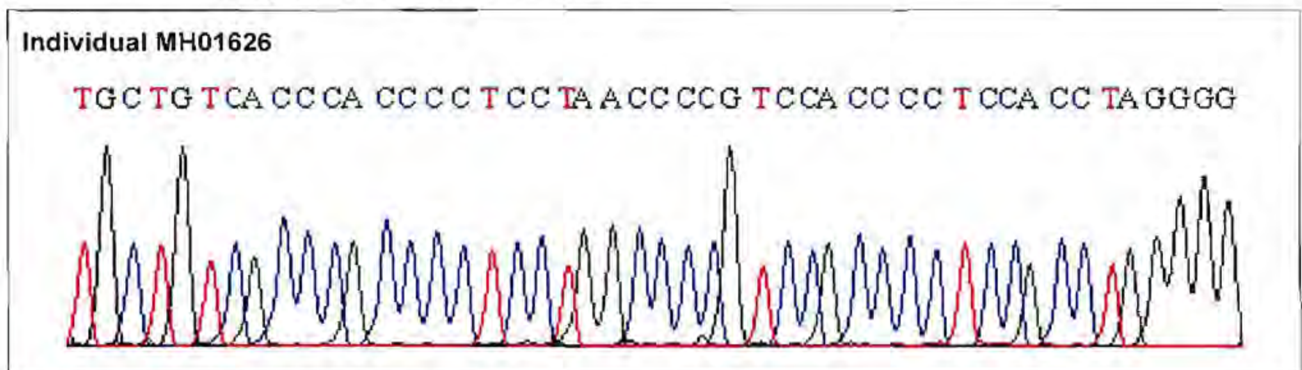
Figure 4.221: Photographic representation of amplified PCR products encompassing exon 83



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. An artefact in the gel matrix, as indicated by the whit asterisk (*), non-specific amplification, background smear, variation in amplification efficiency and fragment distortion were observed, as described in Sections 4.2 and 4.3.

This exon occurs just outside the third mutational hotspot and to date alterations resulting in the MH phenotype have not been reported in this region of the RYR1 gene. In addition, polymorphisms have not been described for exon 83. A standard sequencing protocol was conducted using the forward primer (RYRex83F). A representative electropherogram obtained for individual MH01626, illustrating a portion of the amplified region of exon 83, is depicted in Figure 4.222.

Figure 4.222: Representative electropherogram illustrating a portion of the amplified region of exon 83



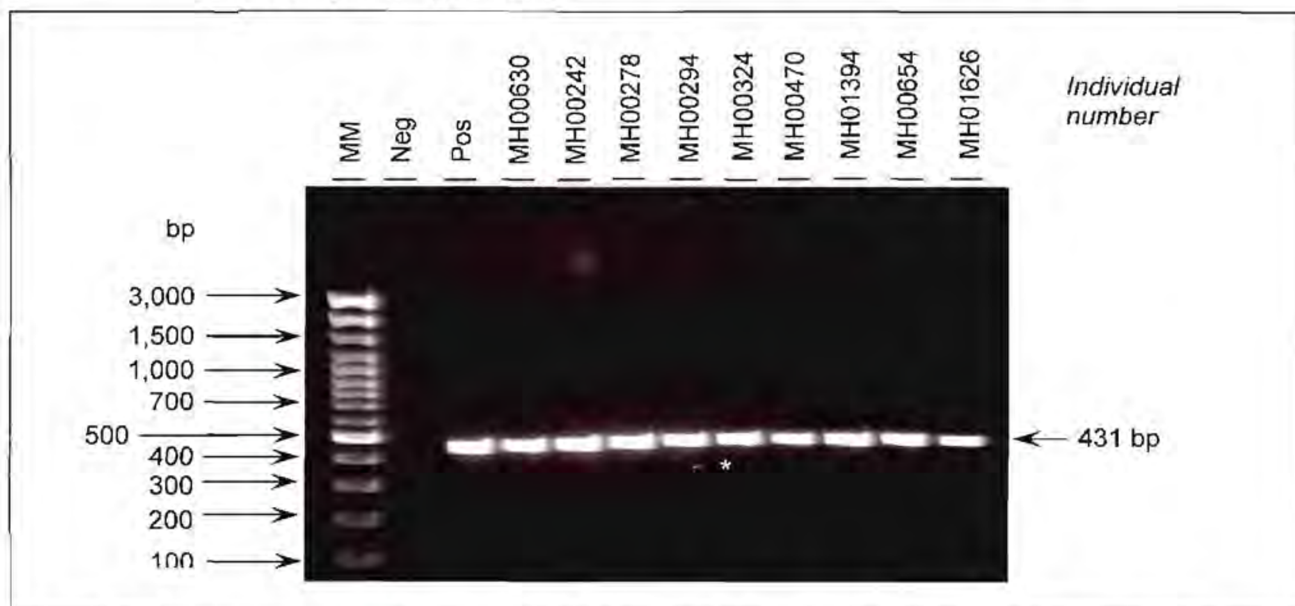
A = adenine; C = cytosine; G = guanine; T = thymine.

Alterations that may result in the MH phenotype were not observed in the amplified region for any of the 15 MHS probands studied. Exon 83 is one of the smallest exons in the RYR1 gene and encompasses only 18 bp. The exon contains a second alternatively spliced sequence, which encodes a six amino acid insertion (Phillips *et al.*, 1996). Therefore exon 83 exists in a modulatory region of the RyR1 (Futatsugi *et al.*, 1995), and alterations in this region may not be tolerated. As discussed in Section 4.10.2 (page 275), further analysis would clarify the role of exon 83 in the development of the MH phenotype.

4.10.42 Exon 84 of the RYR1 gene

PCR was conducted in order to amplify a 431 bp region of exon 84 of the RYR1 gene. This region currently does not harbour any reported alterations associated with the MH phenotype. PCR was optimised, as discussed in Section 4.2 (page 159). Results obtained for PCR amplification encompassing exon 84 are depicted in Figure 4.223.

Figure 4.223: Photographic representation of amplified PCR products encompassing exon 84



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. In addition to an artefact in the gel matrix, as indicated by the white asterisk (*), non-specific amplification and fragment distortion were observed, as discussed in Sections 4.2 and 4.3.

A representative result generated via automated sequencing is presented in Figure 4.224. Sequencing was conducted using the reverse primer (RYRex84R). None of the 15 MHS probands analysed in the study presented here, harboured any novel alterations in this region. Additional screening of this exon in a larger group of individuals from a variety of populations may be required in order to determine if this exon plays a role in the pathogenesis of the MH phenotype, as discussed in Section 4.10.2 (page 275).

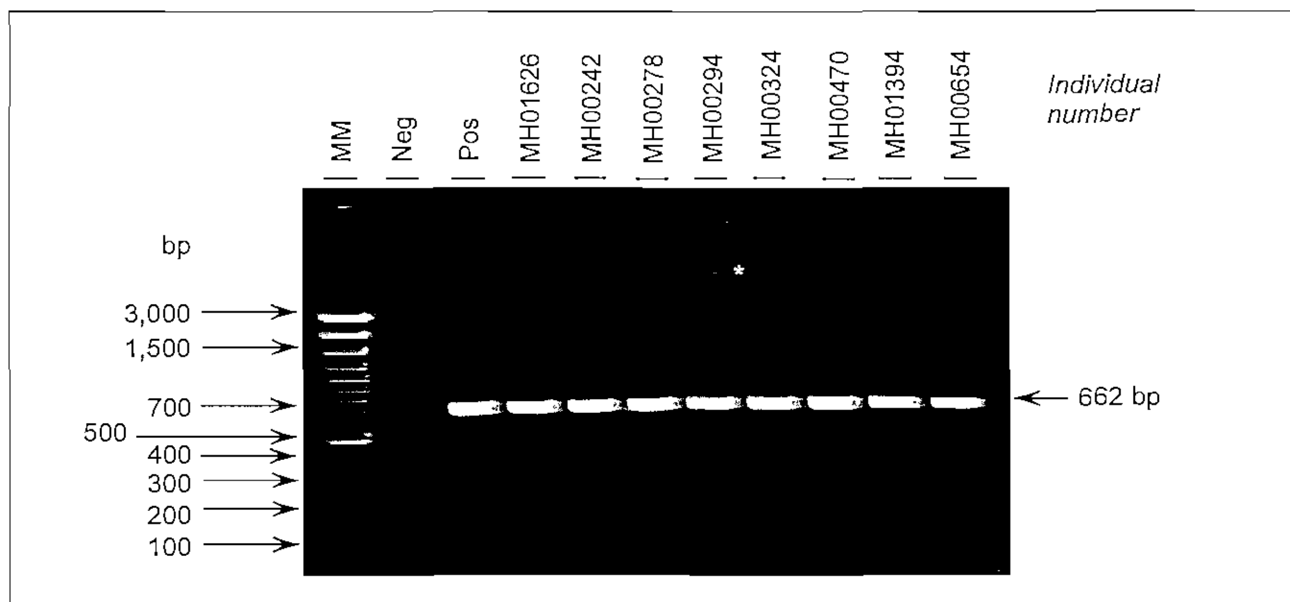
Figure 4.224: Representative electropherogram illustrating a portion of the amplified region of exon 84

A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.43 Exons 85, 86 and 87 of the RYR1 gene

PCR conditions were optimised as discussed in Section 4.2 (page 159). Using the aforementioned conditions, the 662 bp fragment encompassing exons 85, 86 and 87 was successfully amplified, in all 15 samples obtained from the South African MH probands analysed. Figure 4.225 depicts the amplified products prior to sequencing using the optimised PCR protocol.

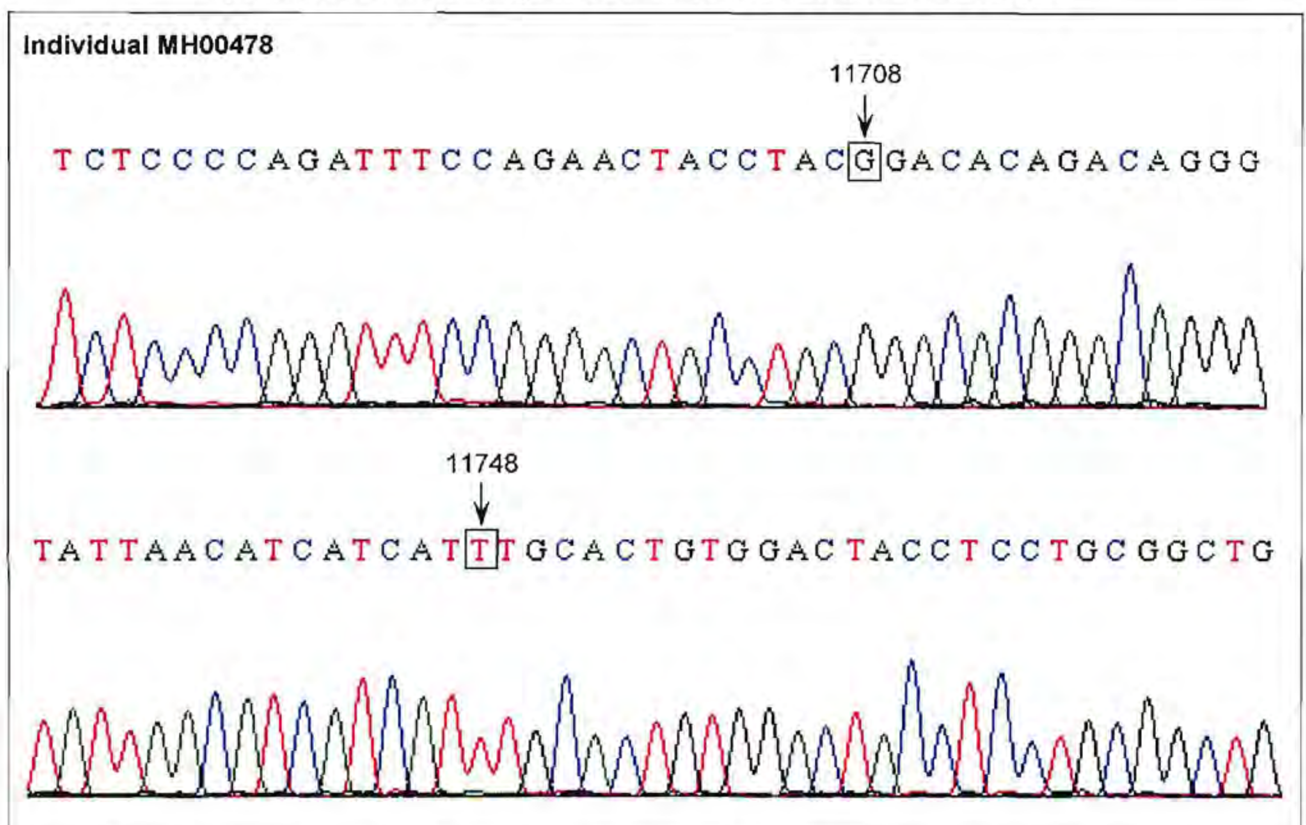
Figure 4.225: Photographic representation of amplified PCR products encompassing exons 85, 86 and 87



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. An artefact in the gel matrix, as indicated by the white asterisk (*), non-specific amplification, variation in amplification efficiency and fragment distortion were observed for the reasons outlined in Sections 4.2 and 4.3.

Sequencing was conducted using the standard protocol. The forward primer (RYRex85F) was used in the sequencing reaction. A representative electropherogram obtained for individual MH00478, illustrating the nucleotide positions of the Arg3903Gln and Ile3913Met alteration previously reported to occur in the amplified region of exon 85, is depicted in Figure 4.226.

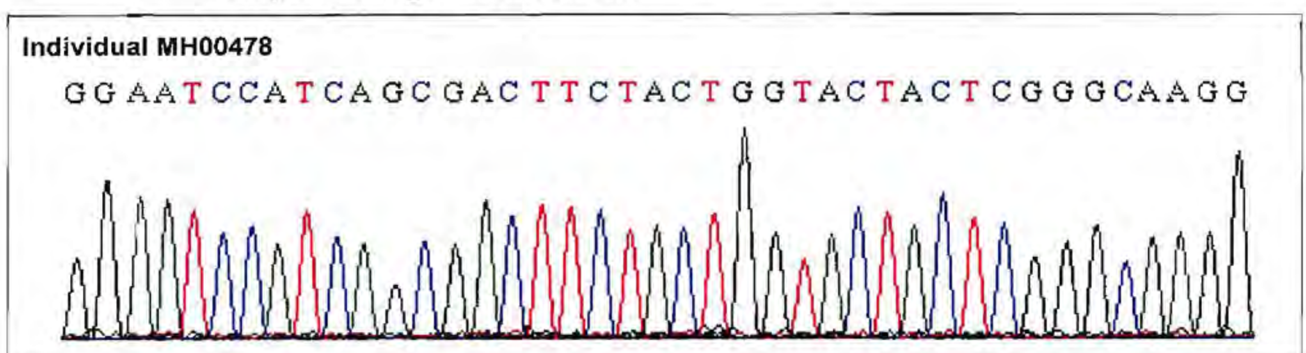
Figure 4.226: Representative electropherogram of exon 85 indicating the nucleotide positions of the Arg3903Gln and Ile3916Met alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Arg3903Gln at nucleotide 11708 and Ile3916Met at nucleotide 11748.

A representative electropherogram obtained for individual MH00478, illustrating a portion of the amplified region for exons 86 and 87, is presented in Figure 4.227 and Figure 4.228, respectively. Thus far, two alterations associated with the MHS have been reported in exon 85 of the RYR1 gene. In one French MH pedigree, Monnier *et al.* (2005) reported the Ile3916Met alteration. In addition, Galli *et al.* (2006) identified an Arg3903Gln alteration in three MH pedigrees from Italy.

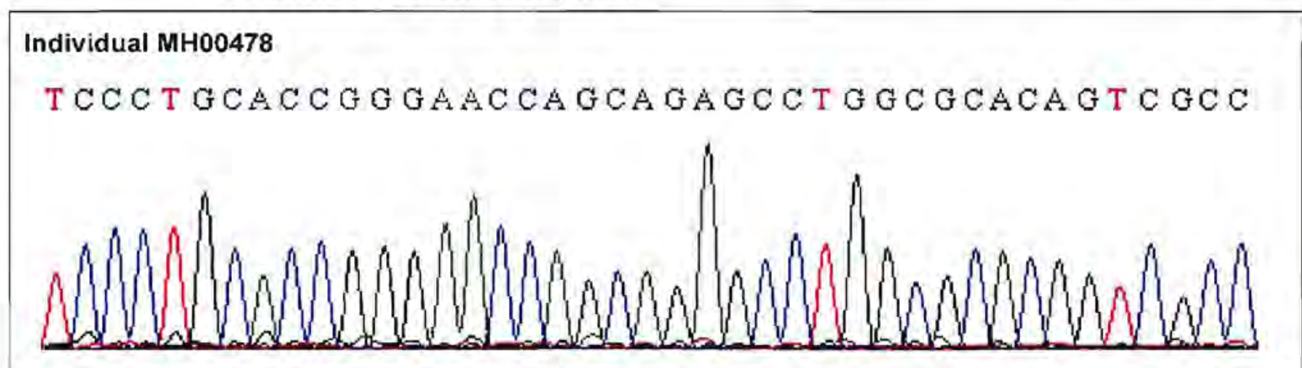
Figure 4.227: Representative electropherogram illustrating a portion of the amplified region of exon 86



A = adenine; C = cytosine; G = guanine; T = thymine.

To date, two alterations associated with the MHS have been reported in exon 85 of the RYR1 gene in populations from Western Europe, i.e. the Ile3916Met (Monnier *et al.*, 2005) and Arg3903Gln (Galli *et al.*, 2006). Neither of these alterations was detected in any of the South African MH probands analysed in the study presented here. In addition, novel alterations were not detected in either of the exons. Although the alterations were not observed in any South African MH individual included in the study presented here, analysis of this region using a larger number of samples may identify alterations associated with MHS, as discussed in Section 4.10.2 (page 275). Identification of two MH-associated mutations indicates that this region may harbour causative alterations in other MHS individuals. As the Caucasian South African population is of Western European descent, the possibility of individuals not included in this analysis harbouring one of the aforementioned alterations cannot be excluded.

Figure 4.228: Representative electropherogram illustrating a portion of the amplified region of exon 87

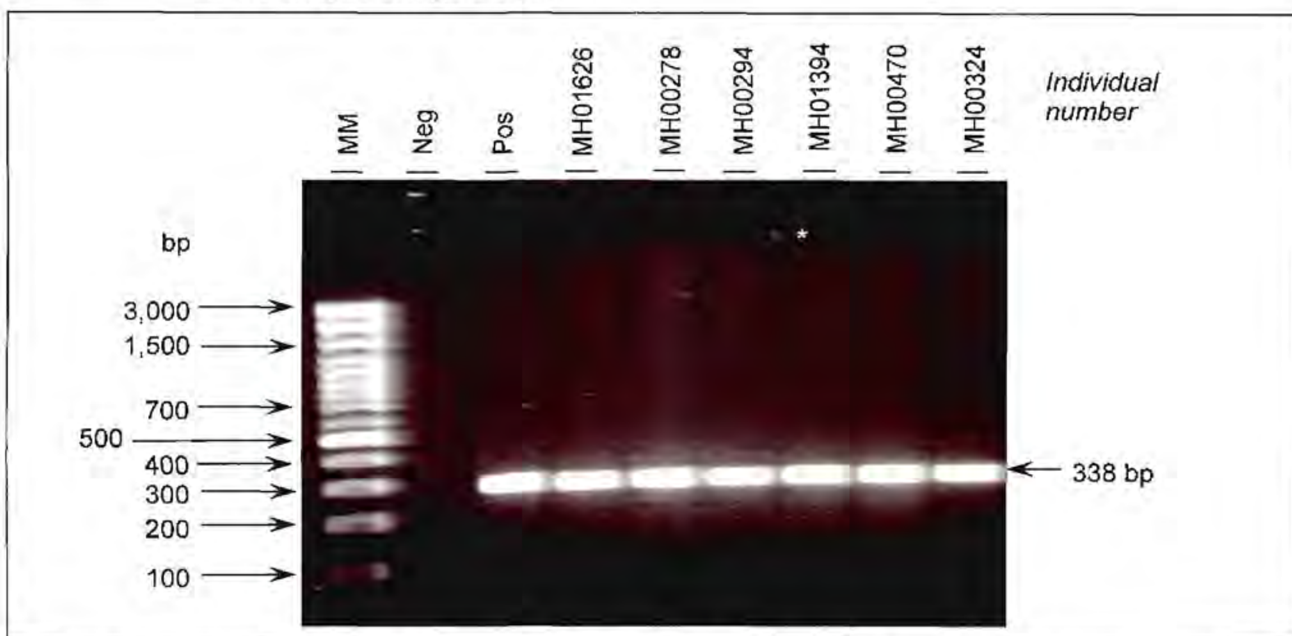


A = adenine; C = cytosine; G = guanine; T = thymine.

4.10.44 Exon 88 of the RYR1 gene

A 338 bp product encompassing exon 88 was amplified via PCR. The region currently does not harbour any reported alterations associated with the MH phenotype. Amplification conditions, as discussed in Section 4.2 (page 159), were used. A photographic representation of the amplicon encompassing the amplified exon is illustrated in Figure 4.229.

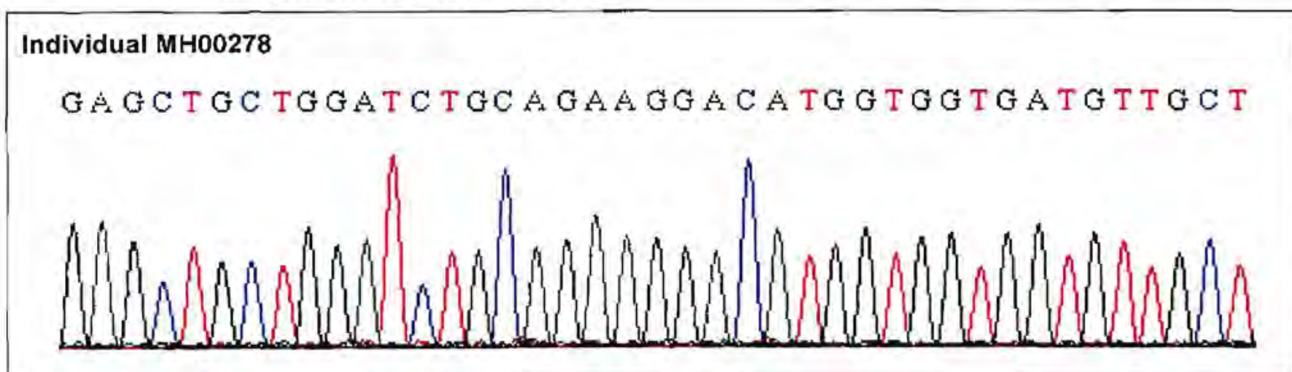
Figure 4.229: Photographic representation of amplified PCR products encompassing exon 88



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. An artefact in the gel matrix, as illustrated by the white asterisk (*), non-specific amplification, background smear and MM overloading were detected in the agarose gel, as discussed in Sections 4.2 and 4.3. In addition, the fragments do not appear linear, as discussed in Section 4.3.

A representative result generated via automated sequencing is presented in Figure 4.230. Sequencing was conducted using the reverse primer (RYRex88R). Therefore, sequences are depicted as the reverse complement. None of the 15 MHS probands analysed in the study presented here harboured any novel alterations in this region. In addition, the region did not harbour any polymorphisms. Novel alterations in this region of the RYR1 gene may not have been present in the South African population. This may either be due to the small number of individuals included in the study presented here, as discussed in Section 4.10.2 (page 275), or to exon 88 or the RYR1 gene not playing a role in the MH phenotype.

Figure 4.230: Representative electropherogram illustrating a portion of the amplified region of exon 88

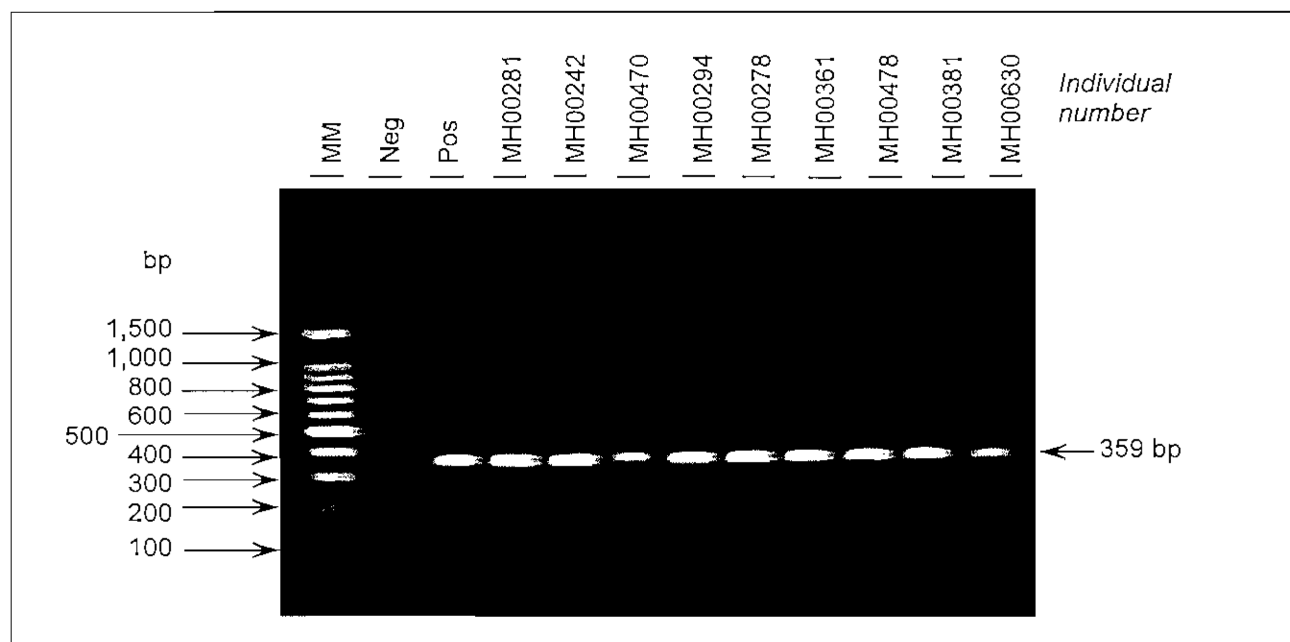


A = adenine; C= cytosine; G = guanine; T = thymine.

4.10.45 Exon 89 of the RYR1 gene

PCR was used to amplify a 359 bp product of exon 89, in order to identify novel alterations that may occur in this region of the RYR1 gene. PCR conditions were optimised using conditions listed in Table 4.1 (page 161). Due to the high T_m of both primers, the two-step fast PCR protocol could be used as discussed in Section 3.5 (page 92). Results depicting the amplified region of exon 89 are illustrated in Figure 4.231.

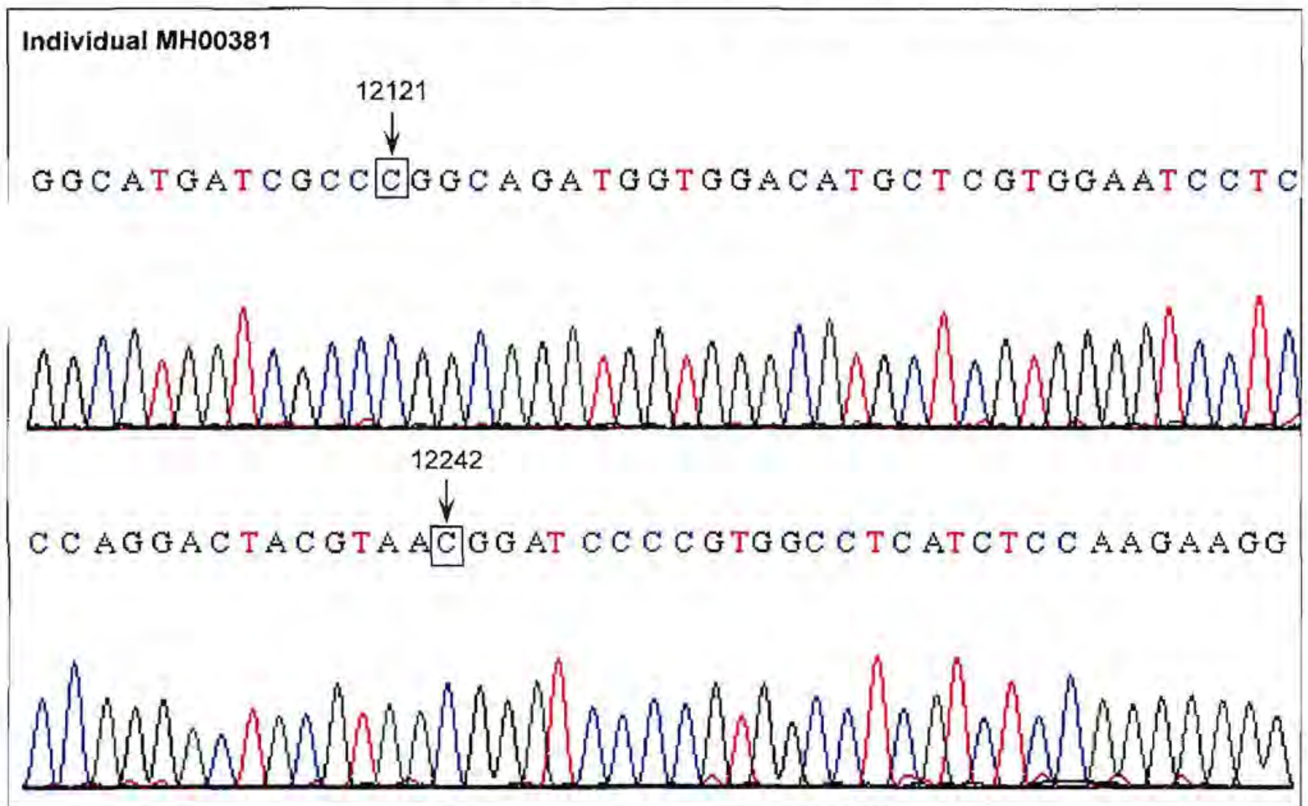
Figure 4.231: Photographic representation of amplified PCR products encompassing exon 89



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Variation in amplification efficiency and fragment distortion was noted, as discussed in Sections 4.2 and 4.3.

Thus far, two alterations, Arg4041Trp and Thr4801Met, have been reported in single MH pedigrees from Italy and Japan, respectively (Galli *et al.*, 2006; Ibarra *et al.*, 2006). Exon 89 resides at the border of the third mutational hotspot, which consists of exons 90 to 104. As many alterations may be overlooked by only screening the three hotspots, exon 89 was analysed to determine if any mutations occur in any of the South African MH probands within this region. Following PCR amplification, the PCR product was purified and sequenced in order to investigate for the presence of novel and reported alterations or polymorphisms that may occur in this region of the RYR1 gene. Sequencing was conducted using the forward primer (RYRex89F). A representative electropherogram obtained for individual MH00381, indicating the nucleotide positions of the Arg4041Trp and Thr4081Met alterations for exon 89, is presented in Figure 4.232.

Figure 4.232: Representative electropherogram of exon 89 indicating the nucleotide positions of the Arg4041Trp and Thr4081Met alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Arg4041Trp at nucleotide 12121 and Thr4081Met at nucleotide 12242.

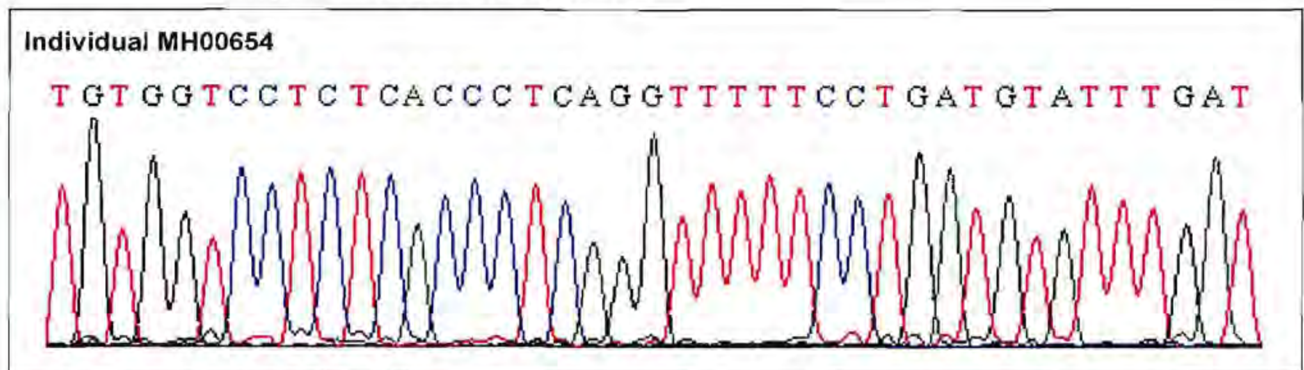
Novel and reported alterations or polymorphisms were not detected in 15 MH probands analysed in Phase 3 for exon 89. Thus far, two alterations, namely Arg4041Trp and Thr4801Met, have been reported in single MH pedigrees (Galli *et al.*, 2006; Ibarra *et al.*, 2006). The alterations could represent private mutations, only present in the aforementioned family. However, as the alterations have only been identified recently, their frequencies have not yet been determined, as discussed in Section 4.10.2 (page 275).

4.10.46 Exon 105 of the RYR1 gene

Exon 105 does not currently harbour any alterations associated with MHS and is observed outside hotspot three. Exon 104 and exon 105 were simultaneously amplified via PCR, as discussed in Section 4.9.14 (page 271) and the results of the amplified PCR product encompassing exons 104 and 105 are depicted in Figure 4.97 (page 272). The amplified region for all 15 MH probands was subsequently sequenced in order to investigate the presence of novel alterations in this region. Sequencing was conducted using the standard protocol. The forward primer (RYRex104F) was used in the sequencing reaction. A

representative electropherogram obtained for individual MH00654, illustrating a portion of the amplified region for exon 105, is depicted in Figure 4.233.

Figure 4.233: Representative electropherogram illustrating a portion of the amplified region of exon 105



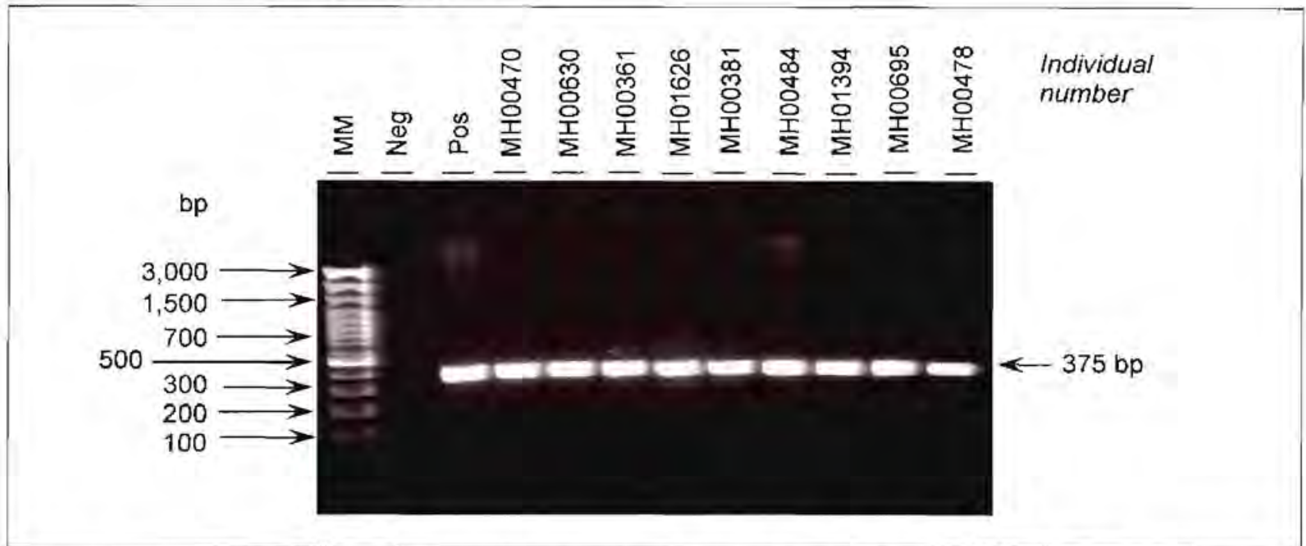
A = adenine; C = cytosine; G = guanine; T = thymine.

All 15 individuals included in the study presented here were analysed for alterations in exon 105 of the RYR1 gene. The study presented here indicates the first analysis of this exon in South African MH probands. None of the individuals analysed harboured any novel alterations in exon 105. As exon 105 has only recently been analysed, the presence of alterations in this exon in all populations has not yet been determined, as discussed in Section 4.10.2 (page 275).

4.10.47 Exon 106 of the RYR1 gene

Thus far, exon 106 has not been reported to harbour any alterations that result in the MH phenotype and only a single SNP has been reported to occur in this region. Exon 106 resides outside the mutation hotspots in close proximity to hotspot three. In order to identify novel alterations and/or polymorphisms that may occur in exon 106, a 375 bp region was amplified, as discussed in Section 4.2 (page 159) and subsequently sequenced. Thereafter, the PCR product was purified (Figure 4.234) and sequenced according to the standard sequencing protocol.

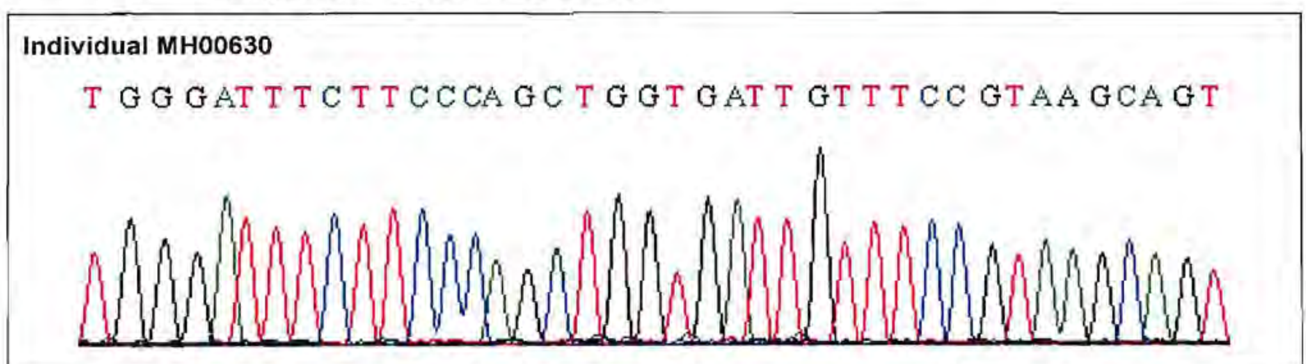
Figure 4.234: Photographic representation of amplified PCR products encompassing exon 106



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Variation in amplification efficiency, background smear and MM distortion were observed, as discussed in Sections 4.2 and 4.3.

Sequencing was conducted using the forward primer (RYRex106F). A representative electropherogram obtained for individual MH00630, illustrating an excerpt of the amplified region for exon 106, is presented in Figure 4.235. None of the 15 individuals analysed in the study presented here harboured any novel alterations in exon 106. As discussed in Section 4.10.2 (page 275), additional alterations outside the mutational hotspots have been observed only recently. Therefore, analysis of this exon in all populations may identify additional alterations that result in susceptibility to MH.

Figure 4.235: Representative electropherogram illustrating a portion of the amplified region of exon 106



A = adenine; C = cytosine; G = guanine; T = thymine.

4.11 SUMMARY OF MUTATION ANALYSIS RESULTS

Table 4.17 is a summary of the results obtained in the study presented here, as discussed in the relevant sections of this chapter. In Phase 1 of the ongoing MH research programme, only two alterations were observed, i.e. Arg614Cys (in family MH105) and Val2168Met (in family MH110). In the Phase 2 study, the Thr4826Ile alteration was detected in a South African MH proband (MH113-14) and two other members of her family (MH113-2 and MH113-11). In addition, none of the South African individuals analysed in the Phase 1 or Phase 2 studies harboured the Arg1086His alteration of the CACNL1A3 gene. In Phase 3, an additional six RYR1 alterations were observed in nine probands, of which four harboured two alterations each, as listed in Table 4.17. Ibarra *et al.* (2006) reported six probands that harboured potentially causative compound heterozygous sequence variations and indicated that the individuals exhibited CICR enhancement. Monnier *et al.* (2002) reported four families with more than one allele and indicated that their CK concentrations were significantly higher than those of individuals with a single alteration. In addition, Wu *et al.* (2006) identified several individuals diagnosed with CCD that harboured two alterations. Therefore, several individuals diagnosed with MH or CCD harbour two alterations. The observation of compound heterozygous alterations in probands from the South African population in the study presented here raises the question of the relationship between the presence of two alterations in the RYR1 gene and the clinical phenotype, as discussed in Section 5.2.2.3 (page 401). Correlation between IVCT results, CICR or blood CK values and the genotype of the proband would indicate if the presence of two different alterations had a cumulative effect on the MHS trait in the South African MH population. Unfortunately, these data were not available and should be analysed further in order to determine the physiological significance of compound heterozygous alterations in individuals diagnosed as MHS in South Africa.

Table 4.17: Summary of alterations detected in the coding regions in 15 South African MH probands

No.	Family no. of proband[a]	Identification no. of proband[b]	Exon	Nucleotide change	Amino acid change	Published/ Novel alteration
1	MH101-6	MH00242	---	---	---	---
2	MH102-125	MH00278	---	---	---	---
3	MH103-4	MH00286	---	---	---	---
4	MH104-26	MH00294	102 38	G14803A G6178T	Gly4935Ser Gly2060Cys	Novel alteration Published alteration
5	MH105-38	MH00324	17	C1840T	Arg614Cys	Published alteration
6	MH113-14	MH00381	100	C14477T	Thr4826Ile	Published alteration
7	—	MH00630	---	---	---	---
8	MH111-1	MH00361	---	---	---	---
9	MH108-1	MH00470	43 38	G7007A G6178T	Arg2336His Gly2060Cys	Published alteration Published alteration
10	MH122-1	MH00478	38	G6178T	Gly2060Cys	Published alteration
11	MH114-1	MH00695	38 34	G6178T C5360T	Gly2060Cys Pro1787Leu	Published alteration Published alteration
12	MH115-1	MH01394	---	---	---	---
13	MH123-1	MH00484	---	---	---	---
14	MH125-1	MH01626	44 73	T7090G G10747C	Phe2364Val Glu3583Gln	Published alteration Novel alteration
15	—	MH00654	---	---	---	---

[a] = indicates family number of MH proband used in this study; [b] = indicates individual number of MH proband used in this study; (—) Indicates only individual number given to individuals; (—) indicates information not available; No. = number.

Previously, mutation screening worldwide has been limited to the mutation hotspots. This observation may indicate that only single alterations have been observed in several individuals and that the second alteration exists outside the RYR1 hotspots or on another chromosome. Genetic heterogeneity has been reported in MH. Alternative MHS loci have been proposed on chromosomes 17q11.2-q24 (Levitt *et al.*, 1992), 7q21.1 (Iles *et al.*, 1994), 3q13.1 (Sudbrak *et al.*, 1995), 1q32 (Monnier *et al.*, 1997; Robinson *et al.*, 1997); 5p (Robinson *et al.*, 1997) and 2q (Olckers *et al.*, 1999), as discussed in Section 2.11.4 (page 66). Epistasis may play a role in MH susceptibility, where two or more alterations at the same or different genetic susceptibility loci may interact and contribute either major or minor phenotypic effects, as discussed in Section 5.2.2 (page 399). As mentioned earlier, the results listed below (Table 4.17) are a summary of results obtained, as discussed in the relevant sections of this chapter.

Out of 15 MHS subjects characterised in this study, eight were negative for mutations in the 106 exons of the RYR1 gene. This indicates that alterations outside the coding region of the RYR1 gene or alterations other than the Arg1086His mutation observed in the

DHPR α_1-subunit or other currently unidentified genes are responsible for the MH phenotype in these probands. This is supported by the fact that Olckers (1997) identified one of the South African families included in the study presented here (MH102) as exhibiting linkage to chromosome 2q. Reported genetic heterogeneity alludes to the fact that an alternative locus may even be responsible for the MH phenotype in other South African individuals diagnosed with MH. This is supported by the fact that linkage to other loci on chromosomes 17q11.2-q24 and 2q (Olckers *et al.*, 1992; Vita *et al.*, 1995; Olckers *et al.*, 1999) was reported in MH families of South African origin. Alternatively, the MHS phenotype may be a result of several interacting proteins that are encoded by different chromosomes, as suggested by Robinson *et al.* (2000). It would therefore be important to investigate all possible alleles at different susceptibility loci that contribute minor phenotypic effects to MHS, as well as the entire RYR1 sequence, which has been suggested to play a primary role in this disorder (MacLennan *et al.*, 1990).

In conclusion, the data reported in the present study allow for a more accurate estimation of the frequency of RYR1 alterations in the South African MHS population. Eight different RYR1 alterations were observed, six of which were previously reported and two of which were novel. Therefore, the prevalence for RYR1 alterations in the study presented here was 47% (7/15). Screening of the entire coding region of the RYR1 gene has been conducted previously in other populations, as listed in Table 4.18. The prevalence of RYR1 mutations in the South African population is still much lower than in other populations analysed for alterations in all 106 exons, as discussed in Section 5.5.3 (page 409). In addition, the distribution and frequency of alterations in the RYR1 for South African probands was observed to be different compared to MH-susceptible individuals from other populations, as discussed in Section 5.5.3.1 (page 409).

Table 4.18: Summary of previous studies which screened the entire coding region of the RYR1 gene

		Results			
Population	**Technique**	**No. of individuals with alterations[1]**	**No. of individuals screened**	**Prevalence[2]**	**Reference**
Italian	DHPLC	43	50	86%	Galli *et al.*, 2006
N. American	DHPLC	21	30	70%	Sambuughin *et al.*, 2005
French	DHPLC	80	133	60%	Monnier *et al.*, 2005
Japanese	Sequencing[3]	33	56	57%	Ibarra *et al.*, 2006

[1] = indicates number of individuals that were observed to harbour alterations; [2] = indicates that the prevalence was calculated as the number of individuals with alterations divided by the number of individuals screened and is indicated as a percentage; [3] = indicates direct sequencing of the coding region of the RYR1 gene. DHPLC = denaturing high performance liquid chromatography; No. = number; N. American = North American.

Three of the alterations observed in the South African MH population were detected outside the mutational hotspots in exons 38, 34 and 73. Two of the alterations (in exons 34 and 73, respectively) were not in close proximity to any of the three hotspots. Studies have indicated that alterations observed in the region from exon 18 to exon 38 and the region from exon 56 to exon 84 have been reported either not to be detected or else to be very rare (Galli *et al.*, 2006). Therefore, comprehensive screening of the RYR1 gene is recommended for molecular investigations in MHS individuals, as many mutations may be located outside the RYR1 mutational hotspots. The contribution of the RYR1 gene to the MH phenotype of an individual will only be definitively determined by screening of the entire coding region of this gene.