

CHAPTER FOUR

Results and Discussion

The RYR1 is one of the largest genes that has been described for humans. It encompasses 106 exons, which consist of 15,117 nucleotides (Zorzato *et al.*, 1990). Due to its large size, routine screening for mutations resulting in the MH phenotype has been difficult. To date, mutation screening in MH patients has been limited to the three hotspots of the RYR1 gene. Mutations located in these three mutation hotspots of the RYR1 gene are continually being reported in MH individuals worldwide. However, recent studies have detected causative alterations outside these mutational hotspots (Monnier *et al.*, 2002; Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Wu *et al.*, 2006), indicating that the RYR1 gene contains other critical domains that may result in the MH phenotype if mutated. Therefore, exons of the RYR1 gene were screened in the study presented here, in order to determine the localisation and distribution of mutations in 15 South African MH probands. Previous MH studies have contributed to either Phase one (Phase 1) or Phase two (Phase 2) of the ongoing MH research programme at the Centre for Genome Research. The study presented here represents Phase three (Phase 3) of the extended MH research programme.

The initial aim of the Phase 1 study was to screen MH probands for 17 causative mutations in the RYR1 gene using automated sequencing strategies and/or RFLP analysis. In Phase 2 of the MH research programme, a sequencing strategy was used to identify an additional 21 reported mutations and to identify any novel alterations that may be present in the specific amplified region. In addition to the RYR1 mutations, MH probands were also screened via RFLP for the Arg1086His alteration of the CACNA1S gene in Phase 1 and Phase 2, as alterations in the RYR1-binding domain of the CACNA1S gene have been reported to account for approximately 1% of MH patients (Stewart *et al.*, 2001).

All individuals included in Phase 1, 2 and 3 of this ongoing research programme are Caucasian and were collected in South Africa. They are descendants of Dutch and other European settlers who immigrated to South Africa (Saunders, 1983). As most Caucasian

South African individuals have European ancestry (Saunders, 1983), results generated from the MH research programme will have important implications for all Caucasian MH individuals in the South African population.

A summary of the results obtained for the MH individuals analysed in Phase 3 is listed in Table 4.17 (page 387). The results obtained for each exonic region of the RYR1 gene that was screened are described and discussed separately in the subsequent sections of this chapter. PCR and sequencing were conducted as described in Sections 3.5 and 3.7 (page 92 and 94) respectively, unless stated otherwise. Changes made to these protocols are indicated in the relevant paragraphs.

4.1 ISOLATION OF GENOMIC DNA

Specific DNA isolation kits were used to isolate gDNA because of their many advantages. The kits are rapid, provide a simple method of purification, the isolated DNA has a high yield and purity and can consequently be used in a variety of techniques. DNA obtained from certain individuals included in this study was extracted previously (Havenga, 2000; Neumann, 2002; Dalton, 2004). These isolations were performed using either the Promega Wizard[®] Genomic DNA purification kit or the QIAgen FlexiGene[®] DNA kit. Samples collected as part of the continuous programme were isolated during the current phase of the study using the QIAgen FlexiGene[®] DNA kit. DNA was isolated from 1 - 3 mL whole blood collected in tubes containing EDTA that were subsequently stored at -70°C. The concentration of DNA was determined using the spectrophotometer as described in Section 3.4 (page 91), and the purity of DNA was determined by calculating the ratio of readings at 260 nm and 280 nm (A_{260}/A_{280}). An A_{260}/A_{280} ratio between 1.7 and 1.9 indicated that the DNA was sufficiently pure to be used in downstream applications. Both DNA isolation kits provided similar yields and purity. DNA obtained from the Promega Wizard[®] Genomic DNA isolation kit had a yield of 151 - 1,009 ng. μL^{-1} and A_{260}/A_{280} ratios between 1.78 and 2.01. DNA isolated using the QIAgen FlexiGene[®] DNA kit had a yield of 135 - 945 ng. μL^{-1} and A_{260}/A_{280} ratio of 1.76 - 1.83. DNA was stored at -20°C until required. Working dilutions of DNA were prepared by dilution with sterile ddH₂O to a final concentration of 50 ng. μL^{-1} and were stored at 4°C.

4.2 POLYMERASE CHAIN REACTION

The PCR reaction facilitates exponential amplification of a specific target sequence (Voet and Voet, 1999), as discussed in Section 3.5 (page 92). A PCR reaction was considered successful if the following criteria were met:

- a. The amplified product was of the desired size.
- b. Amplification was not detected in the negative control, thus indicating that the PCR reaction was not contaminated with foreign DNA.
- c. The expected amplification result was generated for the positive control, which was included with each batch of PCR reactions.

Due to the close proximity of certain exons of the RYR1 gene, for example exons 10 and 11, particular sets of exons were simultaneously amplified in a single PCR reaction. In this manner, all 106 exons of the RYR1 were amplified in 75 PCR reactions in order to analyse the entire coding sequence of this gene. The primers that were used for amplification and sequencing of all 106 exons are listed in Table 3.2 (page 86). Successful amplification was achieved for all exonic regions amplified in this study.

4.2.1 Primer design

A prerequisite for successful PCR is optimal primer design. The primer sequence determines the length of the product, the T_m and PCR yield (Wu *et al.*, 1991). Primer sets used in Phase 3 of the MH research programme were designed via the use of the IDT Oligo Analyser, as discussed in Section 3.2.1 (page 84). Optimal primer design in the study presented here resulted in little or no non-specific amplification and/or primer-dimer formation. A total of 75 primers sets were designed, as listed in Table 3.2 (page 86) in the intronic sequences flanking exons of the RYR1 gene in order to amplify the entire coding region of each exon as well as limited regions of the intron sequence.

4.2.2 PCR optimisation

PCR protocols were optimised for all 75 primer sets to ensure high specificity during amplification of certain regions in the RYR1 gene. In previous studies, difficulties were encountered in certain exonic regions, as secondary amplification was observed in the sequenced product (Dalton, 2004). These artefacts were generated as the GC-rich sequence possessed strong secondary structures that resisted denaturation and prevented specific primer annealing, which resulted in secondary amplification. Most suppliers of *Taq* polymerase provide a unique optimised $MgCl_2$ -free buffer using different

enhancers or concentrations of additives, which can influence the outcome of PCR. The Go Taq[®] Flexi DNA polymerase supplied by Promega varies both in concentration and the type of reagents used in the 1 x buffer compared to Super-therm[®] polymerase, as described in Section 3.5 (page 92). In order to eliminate this type of artefact, Super-therm[®] polymerase was used to increase yield and specificity, as well as to overcome the difficulties encountered as a result of the high GC content of the amplified sequence. In certain exonic regions, as listed in Table 4.1, optimised conditions were obtained using 0.125 U Super-therm[®] polymerase per PCR reaction. Analysis using this protocol did not generate secondary amplification in any of the samples that were visible in the sequenced products. This method was optimised during Phase 2 of this study and has been used throughout the study reported here.

As the RYR1 gene consists of GC-rich sequences, which form secondary structures with high thermal and structural stability (Keith *et al.*, 2004), the amplification of DNA was not optimal for certain primer sets and it was more difficult to achieve specificity. Enhancers such as formamide (Sarker *et al.*, 1990) or dimethyl sulfoxide (DMSO) can be used to increase yield and specificity and to overcome difficulties with high GC content (Adams *et al.*, 1999). Formamide was used to achieve efficient denaturation, leading to successful amplification (Sarker *et al.*, 1990). In addition, for a certain exonic region, as listed in Table 4.1, DMSO was used to improve sequence accuracy by eliminating or minimising template secondary structures and thus allowing the DNA polymerase to replicate the template more easily (Adams *et al.*, 1999). However, as formamide and DMSO improve amplification efficiency for one primer pair but decrease it for another, the additive was not used for all primer sets. For this reason, the addition of formamide or DMSO was determined experimentally for each primer set.

A region of the RYR1 gene, encompassing exon 91, has a high GC content (75%) and formed secondary structures, which led to the poor amplification of the 966 bp fragment. In order to analyse for the presence of secondary structures, the multiple fold (Mfold) program (Zuker, 2003) was used to predict DNA folding occurring in exon 91. The program predicts secondary structures using nearest-neighbour thermodynamic values and determines folding at 37°C. Exon 91 of the RYR1 was predicted to contain higher levels of secondary structure than other regions amplified, with a Gibbs free energy (ΔG) of 10.5 kilocalorie per mole (kcal.mol^{-1}). Amplification of this region could not be achieved using the standard PCR protocol with Promega GoTaq[®] Flexi DNA polymerase or Super-therm[®] polymerase as discussed in Section 3.5 (page 92). Therefore, FastStart[®]

Taq DNA polymerase was used in order to amplify the GC-rich template due to the abundance of secondary structures. The FastStart® *Taq* DNA polymerase used is inactive below temperatures of 75°C and is activated by a 4 min incubation step at 95°C. In addition, a GC-RICH® solution (a PCR additive), which improves PCR performance, was used to amplify the GC-rich template by modifying the melting behaviour. The additive was used to equalise the contribution of GC and AT-base pairing to the stability of the DNA duplex, without altering the conformation or behaviour of the DNA (Rees *et al.*, 1993). Therefore inclusion of the GC-RICH® solution resulted in an improvement in amplification efficiency.

In previous investigations (Phase 1 and Phase 2), PCR protocols were optimised for specific primer sets, and these conditions were used without alteration in this study. However, in the case of a primer set that was designed in Phase 3, the reaction was optimised at the following levels:

- The annealing temperature, T_a .
- Type of DNA polymerase used: Go *Taq*® Flexi DNA polymerase or Super-therm® polymerase.
- When required the addition of enhancing agents, namely formamide and DMSO were determined experimentally for each reaction.

Each primer set was optimised in different conditions, as discussed above. The optimised conditions used for PCR amplification of all 106 exons of the RYR1 gene are listed in Table 4.1.

Table 4.1: Optimised conditions for PCR reactions

Exonic region	Enzyme ^a	T_a (°C)	Additives ^b	Exonic region	Enzyme ^a	T_a (°C)	Additives ^b
1	Go <i>Taq</i> ® Flexi	63	---	58	Go <i>Taq</i> ® Flexi	70	DMSO
2	Super-therm®	58	formamide	59,60	Go <i>Taq</i> ® Flexi	70	---
3	Go <i>Taq</i> ® Flexi	70	DMSO	61	Go <i>Taq</i> ® Flexi	63	---
4,5	Go <i>Taq</i> ® Flexi	67	DMSO	62,63	Super-therm®	65	---
6,7	Super-therm®	66	formamide	64	Go <i>Taq</i> ® Flexi	61	---
8,9	Go <i>Taq</i> ® Flexi	65	---	65	Go <i>Taq</i> ® Flexi	64	DMSO
10,11	Go <i>Taq</i> ® Flexi	67	DMSO	66	Super-therm®	65	---
12	Go <i>Taq</i> ® Flexi	59	formamide	67	Go <i>Taq</i> ® Flexi	68	---
13	Go <i>Taq</i> ® Flexi	65	DMSO	68,69	Go <i>Taq</i> ® Flexi	65	formamide
14,15,16	Super-therm®	62	---	70	Go <i>Taq</i> ® Flexi	68	---
17,18	Super-therm®	65	DMSO	71	Go <i>Taq</i> ® Flexi	68	---
19	Go <i>Taq</i> ® Flexi	63	---	72	Go <i>Taq</i> ® Flexi	67	DMSO
20	Go <i>Taq</i> ® Flexi	61	formamide	73	Super-therm®	59	---

Table 4.1: Continued...

Exonic region	Enzyme ^a	T _a (°C)	Additives ^b	Exonic region	Enzyme ^a	T _a (°C)	Additives ^b
21,22	Go Taq [®] Flexi	61	---	74,75,76	Go Taq [®] Flexi	68	---
23	Go Taq [®] Flexi	64	---	77,78	Go Taq [®] Flexi	64	DMSO
24	Super-therm [®]	69	---	79,80,81	Super-therm [®]	63	DMSO
25	Go Taq [®] Flexi	58	---	82	Go Taq [®] Flexi	64	---
26,27	Go Taq [®] Flexi	64	---	83	Go Taq [®] Flexi	65	---
28	Go Taq [®] Flexi	64	formamide	84	Go Taq [®] Flexi	63	DMSO
29	Go Taq [®] Flexi	65	---	85,86,87	Super-therm [®]	63	DMSO
30	Go Taq [®] Flexi	61	---	88	Go Taq [®] Flexi	65	---
31	Go Taq [®] Flexi	64	---	89	Go Taq [®] Flexi	70	---
32,33	Go Taq [®] Flexi	65	---	90	Go Taq [®] Flexi	63	formamide
34	Super-therm [®]	63	DMSO	91	FastStart Taq [®] 1	56.3	GC-RICH ^{®2}
35	Go Taq [®] Flexi	63	---	92	Go Taq [®] Flexi	65	---
36,37	Super-therm [®]	69	formamide	93	Super-therm [®]	62	---
38	Super-therm [®]	62	DMSO	94	Go Taq [®] Flexi	60	---
39	Go Taq [®] Flexi	68	---	95	Go Taq [®] Flexi	63	---
40	Go Taq [®] Flexi	66	---	96	Go Taq [®] Flexi	68	---
41,42	Go Taq [®] Flexi	66	---	97	Go Taq [®] Flexi	65	---
43	Go Taq [®] Flexi	65	---	98,99	Go Taq [®] Flexi	65	DMSO
44,45	Super-therm [®]	62	---	100	Go Taq [®] Flexi	68	formamide
46	Super-therm [®]	64	formamide	101	Super-therm [®]	62	---
47	Go Taq [®] Flexi	65	formamide	102	Go Taq [®] Flexi	63	DMSO
48,49	Go Taq [®] Flexi	67	---	103	Go Taq [®] Flexi	54	---
50,51,52	Super-therm [®]	70	---	104,105	Go Taq [®] Flexi	63	DMSO
53,54	Go Taq [®] Flexi	65	DMSO	106	Super-therm [®]	58	DMSO
55,56,57	Super-therm [®]	69	---	---	---	---	---

^a = 0.125 U of Super-therm[®], 0.5 U of Go Taq[®] Flexi was used in the PCR reaction or 0.5 U FastStart Taq[®], ^b = 1% of formamide or 5% DMSO was used in the PCR reaction. A concentration of 1.5 mM of MgCl₂ was used in all PCR reactions. T_a = annealing temperature; Go Taq[®] Flexi = Go Taq[®] Flexi DNA polymerase; Super-therm[®] = Super-therm[®] polymerase, FastStart Taq[®] = FastStart Taq[®] DNA polymerase; DMSO = dimethyl sulfoxide; °C = degree Celsius; --- = indicates additive not used.

4.2.3 Artefacts observed in PCR amplified samples

Various artefacts were generated in the PCR reaction, even though precautions were taken to prevent the occurrence of artefacts in PCR amplified samples. The observed artefacts are described and discussed in subsequent sections of this chapter. Ideally the PCR reaction should have high specificity, yield and fidelity (Moretti *et al.*, 1998). However, adjusting conditions for maximum specificity may not be compatible with high yield. Similarly, optimising for PCR fidelity may result in reduced efficiency (Moretti *et al.*, 1998).

¹ FastStart[®] Taq DNA polymerase is a registered trademark of Roche Diagnostics GmbH, Mannheim, Germany.

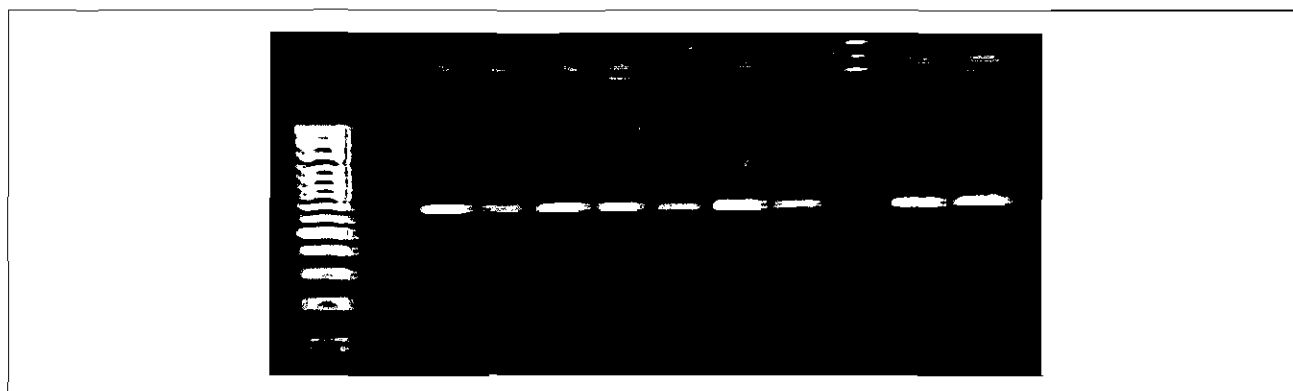
² GC-RICH[®] solution is a registered trademark of Roche Diagnostics GmbH, Mannheim, Germany.

Therefore, the PCR reaction was optimised by determining an effective balance between these three parameters. In all cases, where PCR artefacts were observed, they did not interfere with the PCR reaction, and successful amplification, as well as successful sequencing, was achieved.

4.2.3.1 Amplification efficiency

The amplification efficiency for all samples amplified within a particular run was not always equal. Variation in amplification efficiency occurred between samples, as depicted in Figure 4.1. It is likely that differences in the purity of the genomic DNA resulted in the variation in amplification efficiency between samples. In order to obtain equal amplification efficiencies for all samples, the DNA for samples that had low amplification efficiencies could be re-extracted. However, the variation in amplification efficiency did not affect the outcome of the sequencing experiment, as the same concentration of PCR product was used as template DNA for all samples in the sequencing reaction.

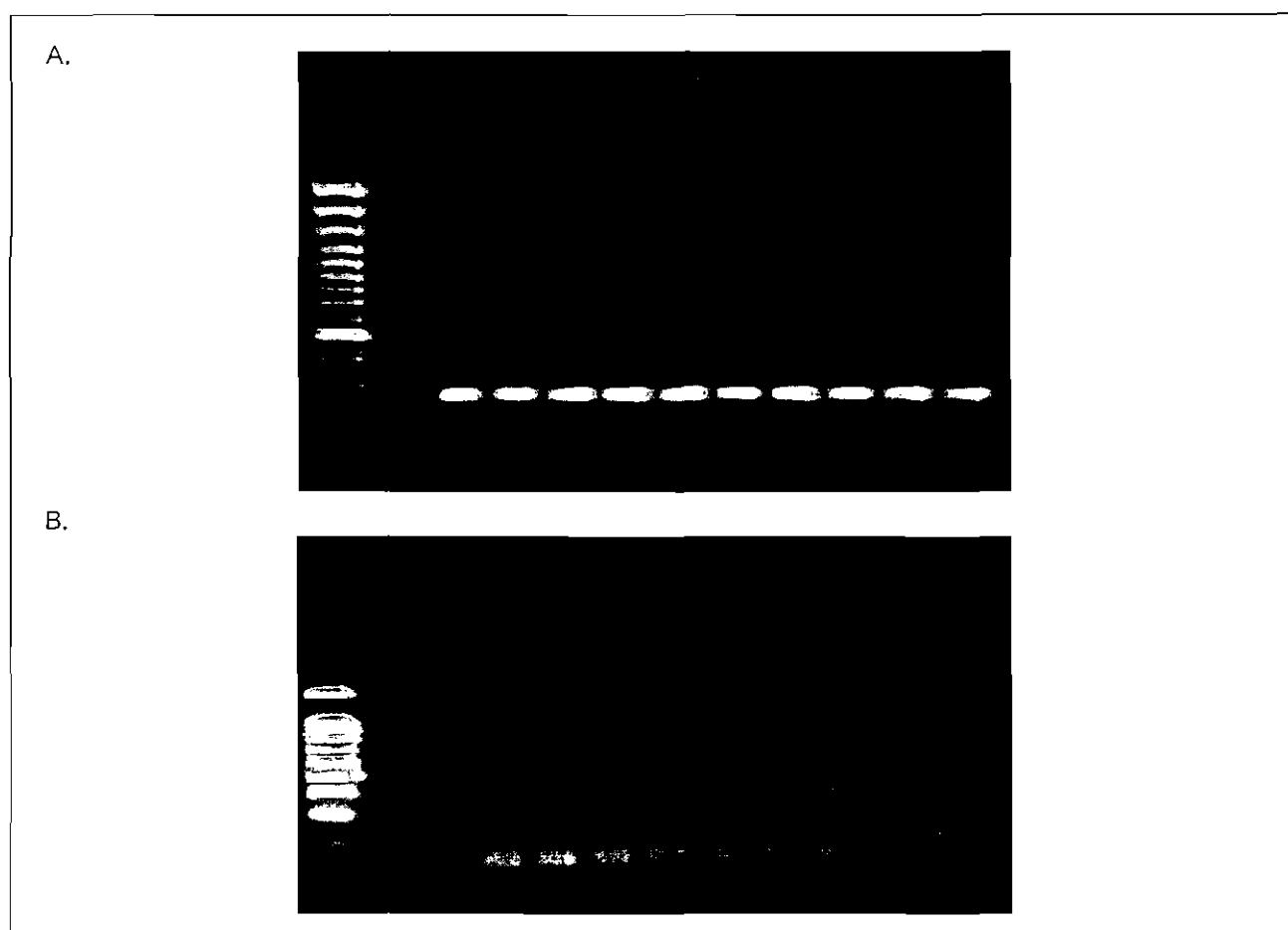
Figure 4.1: Photographic representation of the variation in amplification efficiency observed between amplified PCR products encompassing exons 104 and 105



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm⁻¹ for 30 min.

The amplification efficiency not only varied between samples but also between exonic regions amplified in the study presented here. As illustrated in Figure 4.2, the amplification efficiency of the amplified exonic region was lower for the amplification of exon 46 than for exon 3. Therefore, PCR amplification reactions for different exonic regions may have dissimilar efficiencies. The efficiency of a PCR reaction for the amplification of specific exonic regions depends on a variety of conditions including the genomic DNA, the primer sequence, cycling conditions, buffer conditions and PCR enzyme (Saiki *et al.*, 1988; Furrer *et al.*, 1990).

Figure 4.2: Photographic representation of the variation in amplification efficiency observed between exonic regions for amplified PCR products encompassing exons 3 and 46, respectively



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min.

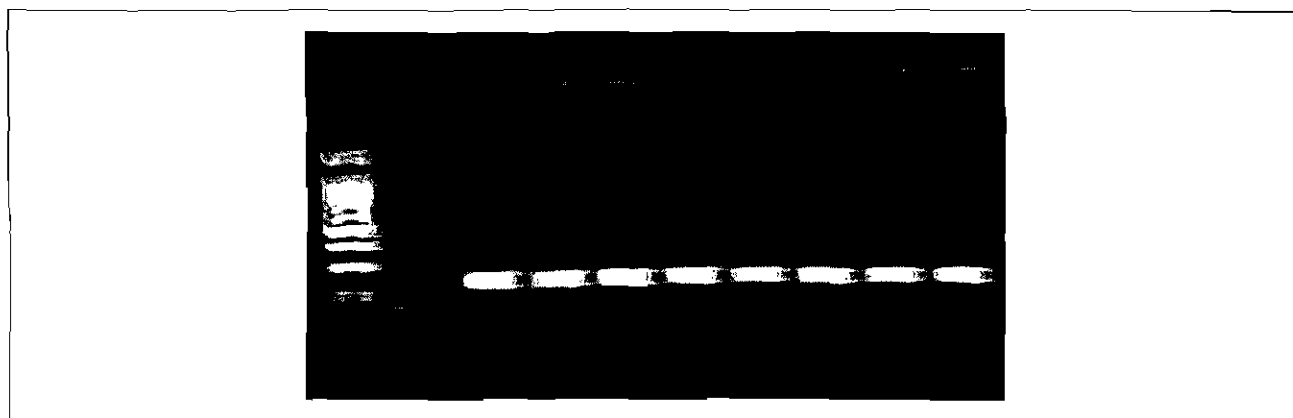
In the study presented here, the amplification efficiency may have been affected by the GC content of the region that was amplified. Arezi *et al.* (2003) compared amplification efficiencies of several different PCR enzymes using real-time quantitative PCR. The authors indicated that GC content significantly influenced amplification efficiency and could be improved by using different DNA polymerases that have, for example, proofreading activity. Therefore, the amplification efficiency of the region encompassing exon 46 may be improved by using a different DNA polymerase, which would have to be determined experimentally. However, as the lower amplification efficiency of the amplified product encompassing exon 46 did not interfere with the sequence result, the PCR protocol indicated in Table 4.1 (page 161) was used without change.

4.2.3.2 Background smear

In certain exonic regions, a faint background smear was observed. Background smears, as illustrated in Figure 4.3, generally occur during the amplification process and are due to

residual non-amplified genomic DNA that fragments as it moves through the pores of the agarose gel (Sambrook and Russell, 2001). The visualisation of the background smears depends on the resolving power of the detection system. Agarose gels have a lower resolving power than for example polyacrylamide gels, but have a greater range of separation (Sambrook and Russell, 2001). Background smears may be present in all amplified samples but may not always be detected.

Figure 4.3: Photographic representation of the background smear observed for amplified PCR products encompassing exon 43

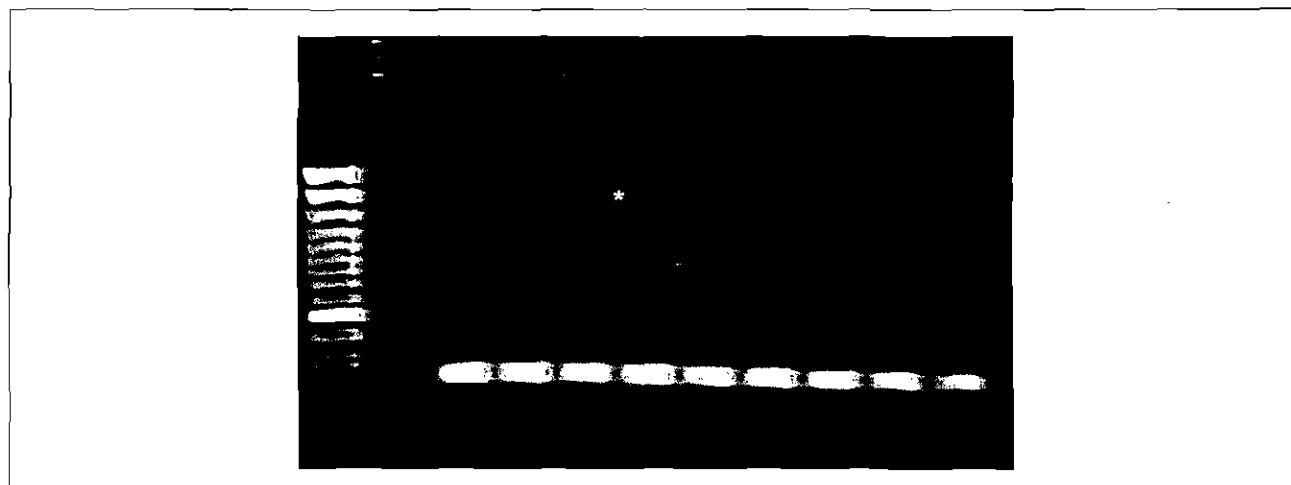


Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min.

4.2.3.3 Secondary amplification

Secondary amplification was observed for certain samples obtained from probands included in the study presented here, as indicated by the white asterisk (*) in Figure 4.4. It is unlikely that the observed secondary amplification occurred due to low annealing temperature, as this would have affected all the amplified samples. The presence of a nucleotide alteration (substitution, deletion or insertion) could create a secondary binding site for either the forward or reverse primer (as depicted in Figure 4.32), which would result in the occurrence of secondary amplification. In order to prevent secondary amplification in the sample obtained from individual MH00278 (indicated by the white asterisk), primers would have to be re-designed so as not to recognise the region that includes the nucleotide alteration. However, as the ratio of the correctly amplified product had a higher yield compared to the secondary amplified product, the presence of secondary amplified products did not interfere with the PCR reaction and all samples were successfully sequenced.

Figure 4.4: Photographic representation of secondary amplification observed for amplified PCR products encompassing exon 12

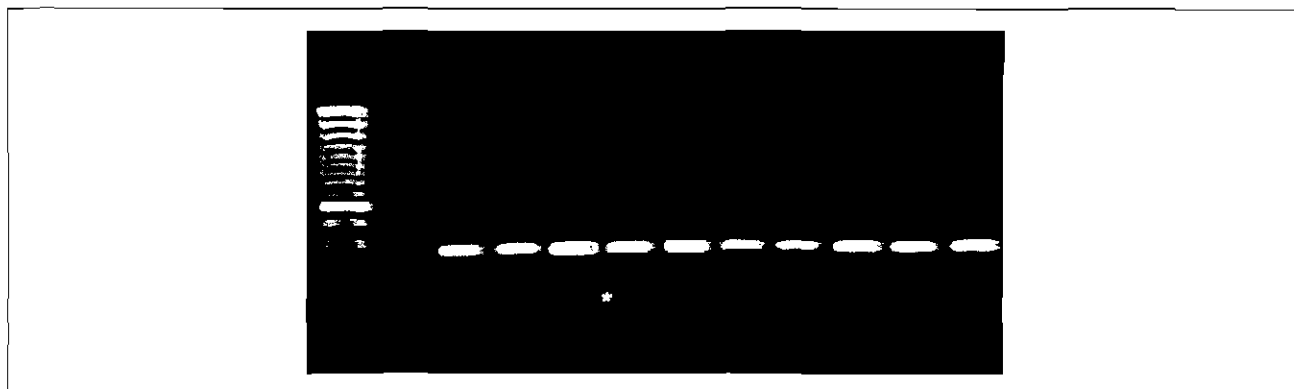


Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. The white asterisk (*) indicates the artefact observed due to secondary amplification.

4.2.3.4 Primer-dimers

For certain exonic regions, primer-dimers were observed, as indicated by the white asterisk (*) in Figure 4.5. Primer-dimers were only evident for certain exonic regions analysed, as their occurrence is dependent on the efficiency of the PCR reaction. The efficiency can vary significantly and depends on the nature of the target sequence, the primer sequence and the reaction conditions (Saiki *et al.*, 1988; Furrer *et al.*, 1990). Therefore, specific PCR amplification reactions for a certain exonic region have different efficiencies. In addition, primer-dimers were only observed for certain samples, which could be attributed to differences in the quality of the template DNA between samples. Primer-dimer formation can be reduced by the use of hot-start PCR or touch-down PCR. In addition, a different enzyme formulation, such as AmpliTaq Gold™, could be used to limit primer-dimer formation (Brownie *et al.*, 1997). However, in all cases where primer-dimers were observed in the study presented here, they did not interfere with the PCR reaction and successful amplification was achieved using PCR protocols listed in Table 4.1 (page 161).

Figure 4.5: Photographic representation of primer-dimers observed for amplified PCR products encompassing exon 23



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. The white asterisk (*) indicates the artefact observed due to primer-dimers.

4.3 AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis is a simple and highly effective method for separating and identifying the approximate size of a DNA fragment by comparing it to another fragment of known size. The molecular weight of each fragment was estimated by comparing its mobility to that of a 100 bp DNA molecular weight marker. Agarose gel electrophoresis was used to confirm successful amplification and thus served as a quality control measure prior to the subsequent downstream analysis of sequence.

For all amplified exonic regions a 2% (w/v) agarose gel was used in order to visualise the separation of DNA fragments that range in size from 0.1 to 2 kb, as described in Section 3.6 (page 94). A voltage of 10 V.cm^{-1} was used to provide optimum resolution of the desired DNA fragments. However, due to the photographic process used, some resolution in the depicted figures was unavoidably lost when it was captured as an electronic file, as illustrated in Figure 4.1 (page 163). Therefore, the resolution of the original gel on the UV transilluminator was higher than the picture of the gel captured as an electronic file.

4.3.1 Artefacts observed on agarose gels

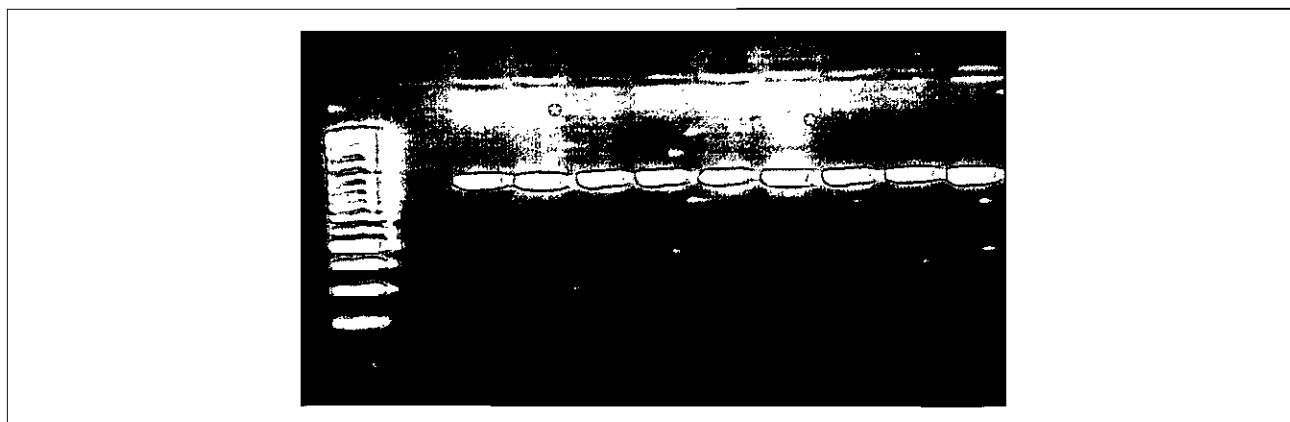
Despite taking precautions to prevent the presence of agarose artefacts, they were nevertheless observed in some of the agarose gels. Artefacts observed on agarose gels are described and discussed in subsequent sections of this chapter. As the presence of artefacts in agarose gels did not affect the confirmation of successful amplification, all

amplified samples visualised on agarose gels depicted in subsequent sections of this chapter were successfully sequenced.

4.3.1.1 Artefacts in the gel matrix

Artefacts in the gel matrix, as indicated by the white asterisks (*) in Figure 4.6, were observed throughout the agarose gel. An artefact in the gel matrix may have occurred due to the presence of lint on the towel used to clean the electrophoresis tray or the UV transilluminator, which would not be visible until the UV light is illuminated. In order to prevent the presence of external contaminants, a lint-free towel should be used to clean the electrophoresis tray.

Figure 4.6: Photographic representation of an artefact in the gel matrix observed for amplified PCR products encompassing exons 6 and 7



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. White asterisks (*) indicate examples of the presence of an artefact observed in the gel matrix.

4.3.1.2 Distortion of molecular weight marker

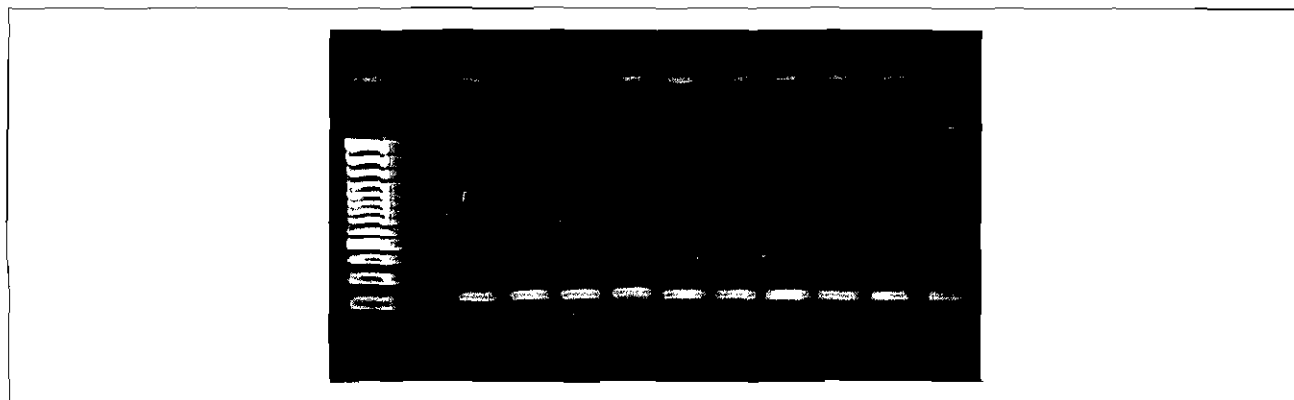
Distortion of the molecular weight marker was observed on various agarose gels. In the study presented here, three different 100 bp molecular weight markers were used consequentially, namely Promega^{®1} 100 bp DNA ladder (as depicted in Figure 4.2, page 164), O'GeneRuler^{™2} 100 bp DNA ladder (as depicted in Figure 4.1, page 163) and O'GeneRuler[™] 100 bp DNA ladder plus (as depicted in Figure 4.4, page 166). The different molecular weight markers used in the study presented here, represent improved versions of previously used molecular weight markers, e.g. the O'GeneRuler[™] 100 bp DNA ladder plus is a molecular weight marker that has a wider size range of fragments and was

¹ Promega[®] is a registered trademark of Promega Corporation, Madison, WI, USA.

² O'GeneRuler[™] is a registered trademark of Fermentas International Inc., Burlington, Ontario, Canada.

developed from chromatography-purified individual DNA fragments, which resulted in sharper fragments of equal intensity. The molecular weight markers (indicated as MM in lane one of the agarose gel) exhibited DNA fragments above 500 bp that were curved. In addition, fragment trailing and smearing of the DNA fragments of the molecular weight marker were observed, as visualised in Figure 4.7.

Figure 4.7: Photographic representation of molecular weight marker distortion observed for amplified PCR products encompassing exon 58



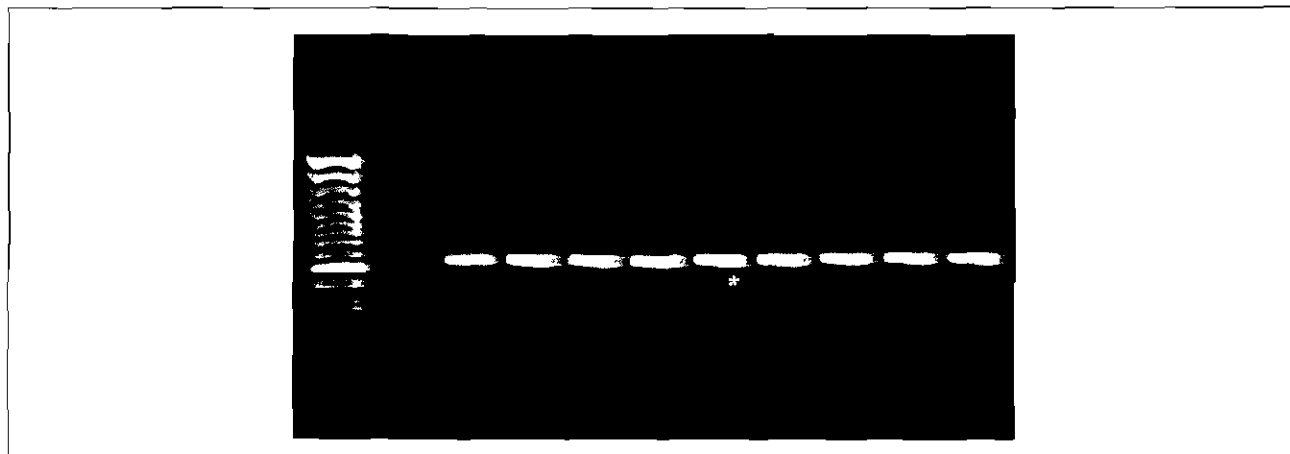
Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min.

Molecules of different molecular sizes experience different frictional forces and separation depends on both charge and molecular mass. Distortion of larger DNA fragments was observed and may have occurred due to overloading of the molecular weight marker (Sambrook and Russell, 2001). Therefore, the larger fragments of the molecular weight marker were curved, as they experienced a greater frictional drag because of their larger molecular mass. In order to prevent this occurrence a lower concentration of molecular weight marker should be used.

4.3.1.3 Distortion of sample fragment

Distortion of the amplified fragments occurred for certain fragments. In Figure 4.8, as indicated by the white asterisk (*), the fragment appears broader on the one side. Distortion of fragments may have occurred because the gel comb was not level during gel casting or may be due to residual agarose being present in the wells, following the removal of the comb. In addition, the samples may appear distorted due to sample overloading, as discussed in Section 4.3.1.2 (page 168). In order to prevent distortion of PCR fragments in an agarose gel, the gel casting tray and the electrophoresis tank used to generate the agarose gel should be level. In addition, residual agarose should be removed from the well and a lower concentration of sample should be loaded.

Figure 4.8: Photographic representation of sample fragment distortion observed for amplified PCR products encompassing exon 39

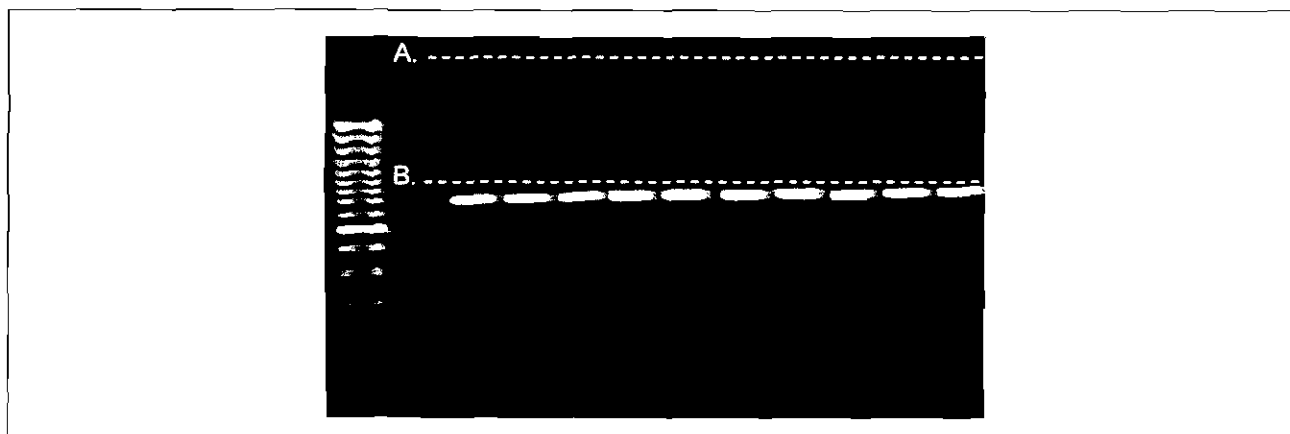


Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. The white asterisk (*) indicates the artefact observed due to fragment distortion.

4.3.1.4 Slanted fragments

For certain agarose gels, the lanes of the amplified fragments appear slanted, as indicated by line A and line B, which are both parallel to the wells depicted in Figure 4.9. Slanted fragments may have occurred because of the comb being placed in the gel at an angle or because the gel casting unit not being level. In order to prevent this artefact, the alignment of the comb and the level of the platform should be verified prior to each electrophoresis run being performed.

Figure 4.9: Photographic representation of slanted fragments observed for amplified PCR products encompassing exons 26 and 27

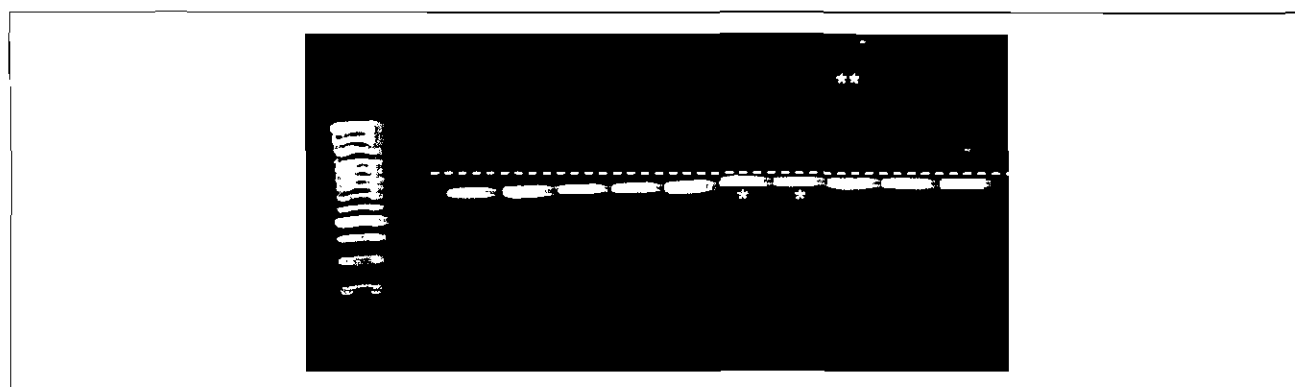


Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. As discussed in Sections 4.2 and 4.3, slanted lanes, background smears and MM distortion were observed. Line A and line B are both parallel to the wells indicated in the Figure.

4.3.1.5 Barrier in agarose gel

In some gels a fragment, or a few fragments, seemed to have migrated more slowly than the rest of the fragments on the same gel. The shadow depicted in Figure 4.10 by the two white asterisks (**) could represent a barrier in the gel and may explain the non-linear migration of the PCR products in the two wells indicated by the white asterisk (*). This may have occurred during the preparation of the gel. The presence of barriers in the agarose gel could be prevented by ensuring that the gel mixture is heated sufficiently to ensure equal temperature distribution and homogeneous polymerisation.

Figure 4.10: Photographic representation of a barrier observed for amplified PCR products encompassing exons 6 and 7



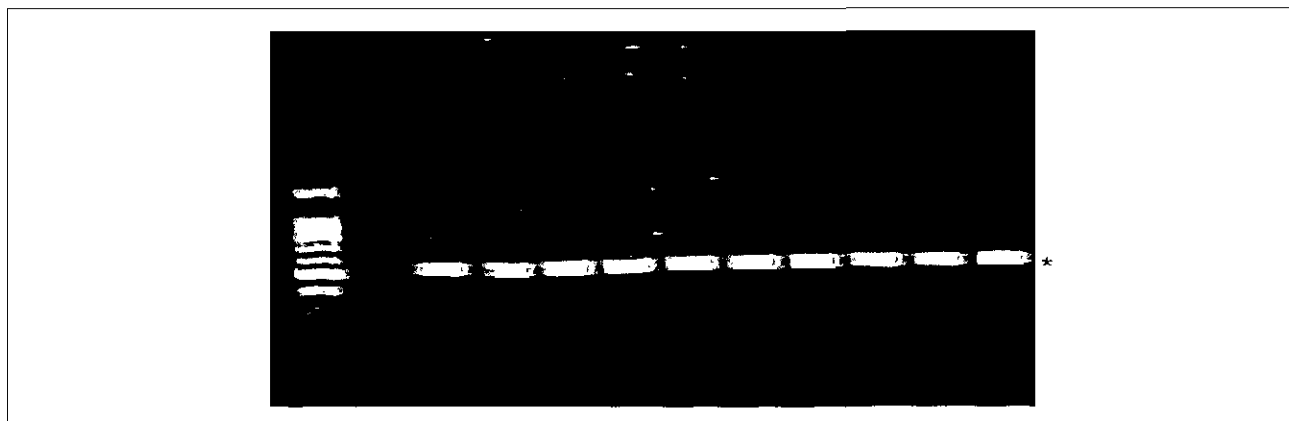
Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. The two white asterisks (**) indicate the artefact observed due to a barrier in the gel matrix; the asterisk (*) indicates non-linear migration in two wells due to the presence of the barrier.

4.3.1.6 Ethidium bromide migration front

The EtBr migration front was observed in some of the agarose gels, as indicated by the asterisk (*) in Figure 4.11. During electrophoresis EtBr migrates towards the cathode because of its net positive charge (Luedtke *et al.*, 2005), resulting in the observed EtBr migration front. In contrast, due to the negative charge imparted by their phosphate backbone at neutral pH, nucleic acids will migrate towards the anode (Sambrook and Russell, 2001).

In order to prevent the occurrence of the EtBr migration front, the agarose gel could be stained with EtBr following electrophoresis. However, this method has several disadvantages, including longer processing time and the handling of larger volumes of biologically hazardous material. Therefore, as the EtBr migration front did not interfere with the conformation of PCR amplification, EtBr was added directly to the gel mixture prior to polymerisation, as discussed in Section 3.6 (page 94).

Figure 4.11: Photographic representation of the ethidium bromide front observed for amplified PCR products encompassing exon 70



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. The asterisk (*) indicates the position of the EtBr migration front.

4.4 PCR PURIFICATION

PCR purification of samples was achieved by using either the QIAquick® PCR purification kit or the Zymo Research DCC-5™ kit for direct purification of PCR products. The concentration of the PCR product was determined using the spectrophotometer as described in Section 3.4 (page 91), and the purity of the PCR product was determined according to the A_{260}/A_{280} ratio. For both kits, the concentration ranged from $5.6 - 25.5 \text{ ng.}\mu\text{L}^{-1}$ and the A_{260}/A_{280} ratios were between 1.44 and 1.90. Both PCR purification kits provided similar yields and purity. However, the Zymo Research DCC-5™ kit provided a more economical PCR purification kit.

4.5 CHAIN TERMINATION SEQUENCING

All samples that amplified successfully were subsequently sequenced via the dideoxy chain termination method using the ABI PRISM® Big Dye™ Terminator version 3.1 Ready Reaction Cycle Sequencing Kit. Sequencing was conducted for all amplified regions according to the standard protocol outlined in Section 3.7 (page 94), unless stated otherwise. Although the amplified region was GC-rich, which could lead to prematurely terminated molecules, sequencing was successful for all samples analysed, as optimised PCR conditions and PCR purification protocols were used, which prevented this occurrence. For all mutation regions, a concentration of 3.2 pmol of primer was used. In order to sequence the entire exon of a specific RYR1 region, either the forward or reverse primer was selected. As the PCR products were approximately 200 – 1,000 bp in length, 20 ng of template was used for sequencing. This is in accordance with the recommended amount of template to be used in a sequencing reaction, as listed in Table 3.5 (page 96).

Following purification, the PCR product was diluted with ddH₂O when the concentration exceeded 20 ng. A higher volume of template was used in the sequencing reaction if the concentration was lower than 20 ng. Sequences were considered successful if the following was present:

- a. High template purity as indicated by high peak amplitude of the sequence
- b. Low amplitude of background peaks.

High quality sequence data thus indicated that the PCR template was pure, that the template quality was within the appropriate range and the optimum concentration of primer was used.

Previously reported alterations are referred to by the amino acid change that is present, i.e. Cys35Arg in representative electropherograms of nucleotide sequence, as depicted in Figure 4.15 (page 177). International convention dictates that nucleotide alterations not be labelled with reference to the position of an amino acid change in the translated protein. However, in this text the genetic alteration is referred to by the amino acid change, as this is the convention in the international MH literature.

Generally, heterozygous positions contain fluorescence peaks with equivalent heights, as indicated by the arrow in Figure 4.31 (page 192). However, in certain cases the peak heights of the two different nucleotide bases were not identical, as indicated in Figure 4.24 (page 185). Peak heights may vary considerably within a sequence and the variation in peak heights may have occurred due to the AmpliTaq[®] enzyme discriminating against ddNTPs in a manner that is dependent on the sequence context (Korch and Drabkin, 1999). Therefore, samples were sequenced using the forward and reverse primers if an alteration was observed, in order to verify the presence of the heterozygous alteration.

4.5.1 Background peaks generated during sequencing

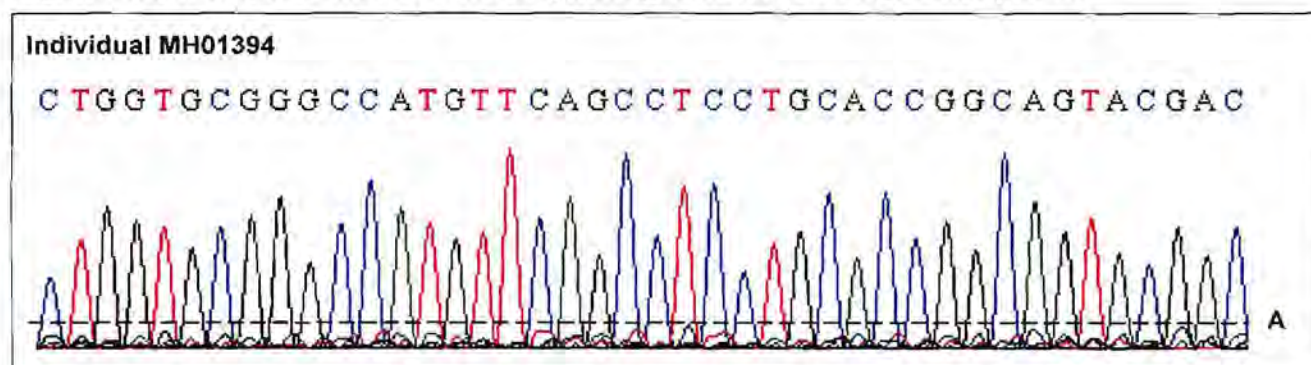
Background peaks are characterised as undefined peaks under the sequence peaks of interest. In sequence results obtained for certain exonic regions, background peaks were visible below the dotted line in Figure 4.12. A small amount of background noise is generally present in sequencing data but does not interfere with the result if the sequences have high signal strength. In all cases, the background peaks occurred throughout the sequence. Numerous factors may lead to the presence of low-level background peaks, including template quality; multiple primers or templates (residual primer and dNTPs in the PCR product) and template artefacts. In the study presented

here, it is most likely that template quality resulted in the presence of background peaks, as discussed in the subsequent section of this chapter.

4.5.2 Template quality

The quality of the template can depend on a number of factors. Inhibitory contaminants, namely salts or phenol, can affect the sequence data quality and can partially or completely inhibit the sequencing reaction. Careful precipitation of the DNA will remove contaminating substances. DNA may be degraded due to the presence of nucleases, repeated freeze-thawing and UV light exposure, which will also influence the quality of sequence obtained. Primer contamination could result in background peaks due to the sequence products that are generated by the primers and dNTP contamination, which in turn can result in an increase in the overall level of ambiguous base calls. Template artefacts can occur due to the sequence of the template. GC-rich templates can form secondary structures and are more difficult to denature, which can result in the presence of background peaks. As regions of the RYR1 gene are GC-rich, it is most likely that the formation of secondary structures resulted in background peaks in some of the sequenced samples. However, as the signal-to-noise ratio was high, the presence of background peaks did not interfere with the sequence result and the sequence protocol described in Section 3.7 (page 94) was used.

Figure 4.12: Representative electropherogram with background peaks



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

4.6 SEQUENCE PRECIPITATION

Sequence precipitation was conducted prior to electrophoresis, in order to remove unincorporated dye terminators from the sequencing reaction. Two different methods were employed in order to precipitate the sequencing reaction. Initially, the ethanol/sodium acetate method of precipitation was used to purify the samples, as discussed in

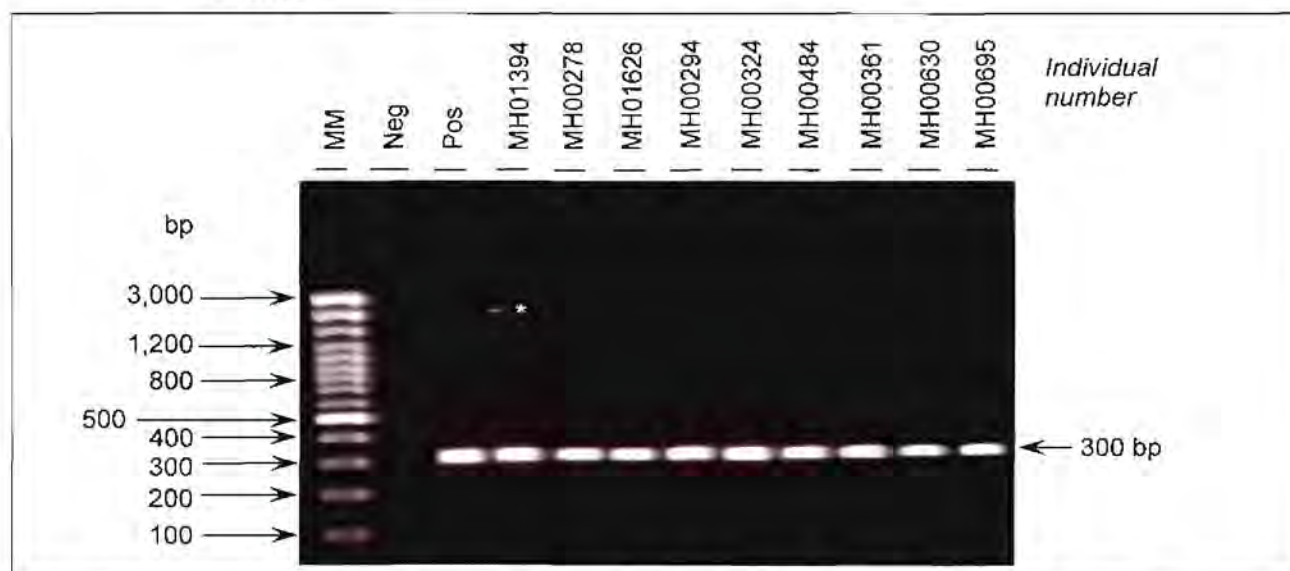
Section 3.7 (page 94). However, in order to obtain a higher uniform signal intensity, the ethanol/sodium acetate method was replaced by the Centri-sep™ 96 well clean-up kit. The Centri-sep™ 96 well filter plate removes excess dye terminators via a cross-linked preservative-free gel. Following precipitation, the sequencing products were re-suspended in deionised formamide and were subsequently electrophoresed. Sequence products were run on either a SpectruMedix™ SCE2410 genetic analysis system sequencer or on an Applied Biosystems® 3130xl genetic analyser. Sequence precipitation via the Centri-sep™ 96 well clean-up kit and electrophoresis of the sequenced products were not performed by the author, but were analysed on contract by a second party. Raw sequence data obtained following electrophoresis was analysed by the author, using the BioEdit Sequence Alignment Editor version 5.0.9 software (Hall, 1999), as discussed in Section 3.7 (page 94).

4.7 HOTSPOT ONE OF THE RYR1 GENE

The first hotspot of the RYR1 gene includes exons 2 to 17. Analysis of these exons led to the detection of several synonymous SNPs and the verification of the Arg614Cys alteration in one MH proband from South Africa. As discussed in Section 2.11.3.3.1 (page 47), a variety of alterations in hotspot one can cause aberrations in the RyR1 channel function, such as hyper-activation (Tong *et al.*, 1997) and/or hyper-sensitisation (Yang *et al.*, 2003) to various physiological and pharmacological antagonists, which result in Ca²⁺ dysregulation. Therefore, the entire coding region and certain regions of the intronic sequence of hotspot one were analysed in 15 South African MH probands for alterations that may result in the MH phenotype. The study presented denotes the first analysis of all exons that reside within hotspot one in 15 South African MH probands.

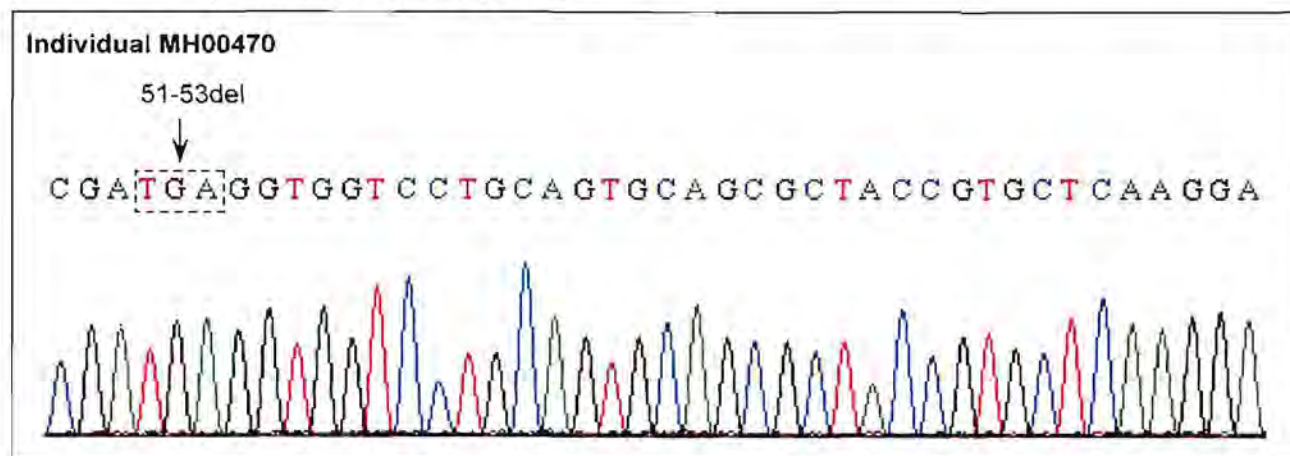
4.7.1 Exon 2 of the RYR1 gene

Exon 2 of the RYR1 gene harbours three reported mutations i.e. Cys35Arg (Lynch *et al.*, 1997), Arg44His (Halsall and Robinson, 2004) and an Asp17del that has been observed in a single MH proband from Japan (Ibarra *et al.*, 2006). A region encompassing exon 2 was successfully amplified in 15 South African probands and the PCR was optimised using the conditions listed in Table 4.1 (page 161). The results are illustrated in Figure 4.13. Exon 2 is located within the N-terminal domain of the RyR1 protein in the first mutational hotspot.

Figure 4.13: Photographic representation of amplified PCR products encompassing exon 2

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, background smear and MM overloading were observed.

Sequencing was conducted using the reverse primer (RYRex2R), therefore sequence results are depicted as the reverse complement. A representative electropherogram obtained for individual MH00470 illustrating the nucleotide position of the Asp17del is indicated in Figure 4.14.

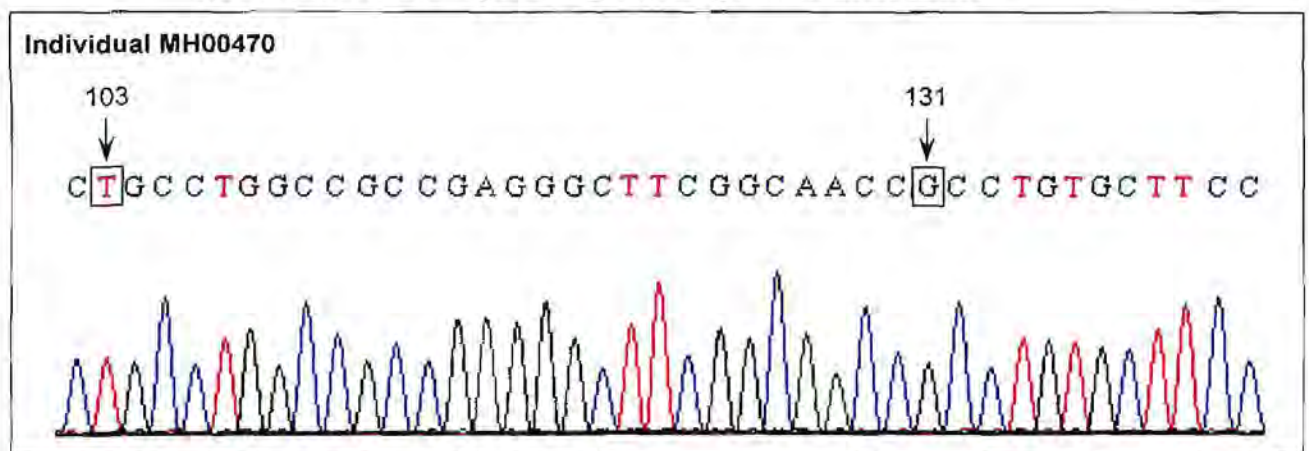
Figure 4.14: Representative electropherogram of exon 2 indicating the nucleotide position of the Asp17del

A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Asp17del at nucleotides 51-53.

The Cys35Arg or Arg44His alterations were not identified in any of the 15 probands analysed in the study presented here and the nucleotide positions of the previously reported alterations are illustrated in Figure 4.15. Thus far, the Asp17del has only been

described in a single MH family, therefore it could be specific to that particular family. The Cys35Arg and Arg44His alterations have been observed in two families each and all four families are of European descent (Lynch *et al.*, 1997; Halsall and Robinson, 2004; Monnier *et al.*, 2005; Galli *et al.*, 2006). The Cys35Arg alteration was not observed in 100 MH families from Germany, 66 MH families from Switzerland and 297 families with MH from the UK (Robinson *et al.*, 2003b). All 15 South African MH probands analysed in the study presented here are Caucasian and are likely to be immigrants of mainly Western European origin, i.e. Dutch, French, German and British (Saunders, 1983).

Figure 4.15: Representative electropherogram of exon 2 indicating the nucleotide positions of the Cys35Arg and Arg44His alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Cys35Arg at nucleotide 103 and Arg44His at nucleotide 131.

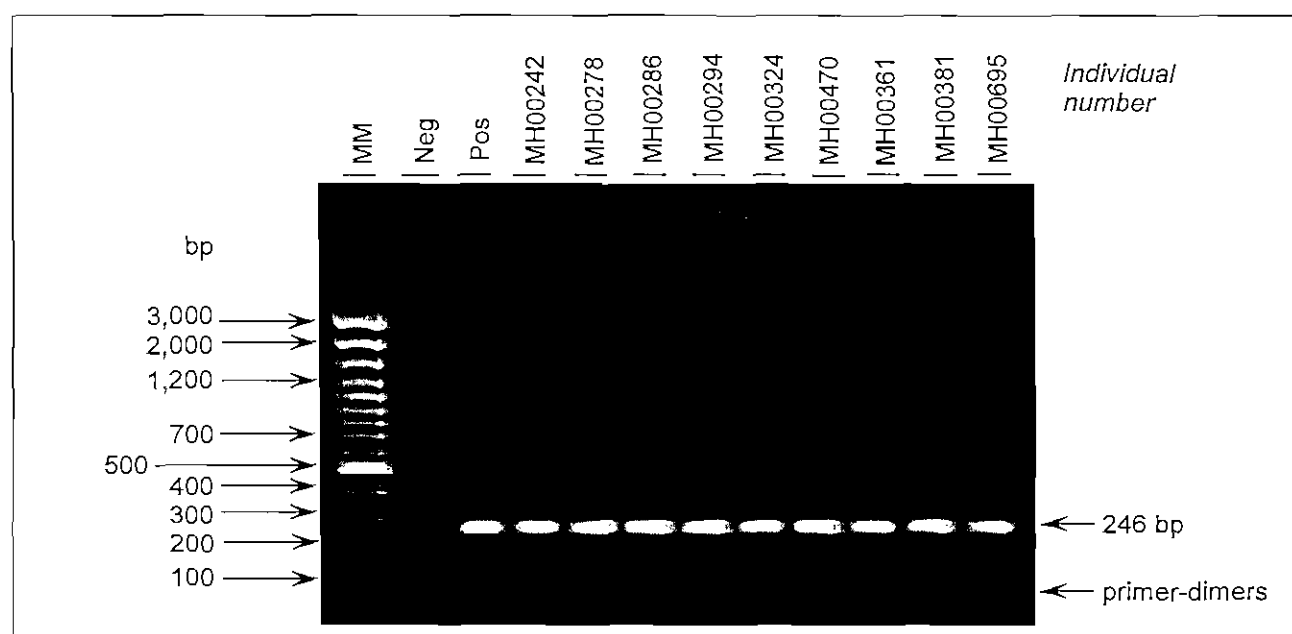
In view of the geographic origins of Caucasian individuals of the South African population (Saunders, 1983), detection of the previously identified Cys25Arg and Arg44His mutations would be expected in the South African MH population. However, the alterations have thus far only been reported in one or two families, which may indicate that they are specific to the above-mentioned families in which they were observed. The alterations may also have arisen as a result of founder effects, indicating that the families may have had a common ancestor. In order to verify this possibility, haplotype analysis using chromosome 19q markers would have to be conducted to determine if the above-mentioned reported families are related. The Cys35Arg alteration is currently being used in the genetic diagnosis of MH in the European population and the causative status of this alteration has been determined via functional analysis (Tong *et al.*, 1997), as listed in Appendix A (page 443). As discussed in Section 5.6 (page 413), alterations observed in the RYR1 gene can only be classified as disease-causing if they fulfil the criteria delineated by the EMHG. Thus far, the causative status of the Arg44His alteration has not been determined,

and would have to be analysed prior to the incorporation of this alteration into a genetic diagnostic test. Currently, the causative status of an alteration cannot be determined via testing concordance of the alteration with IVCT results due to the limitations of the IVCT. As discussed in Section 5.5.2 (page 407), the specificity and sensitivity of the IVCT is not 100% (Ørding *et al.*, 1997). To date, alterations are only considered causative of MH if they have been functionally characterised via recombinant *in vitro* expression on a defined genetic background or via assays of RYR1 function in *ex vivo* tissues (Tong *et al.*, 1997). The absence of reported and novel alterations in exon 2 indicates that this exon does not contribute to the phenotype responsible for MHS in this South African cohort.

4.7.2 Exon 3 of the RYR1 gene

Currently, exon 3 does not harbour any reported alterations associated with MHS. However, an Asp60Asn alteration (Wu *et al.*, 2006) and a Ser71Tyr mutation (Zhou *et al.*, 2005) have been identified in patients diagnosed with CCD. As this region of the RYR1 gene occurs in hotspot one and harbours reported CCD alterations, a 246 bp region encompassing exon 3 was amplified, as discussed in Section 4.2 (page 159). The results of the amplified PCR product are illustrated in Figure 4.16.

Figure 4.16: Photographic representation of amplified PCR products encompassing exon 3



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 amplification efficiency was not identical for all samples, and primer-dimers were observed as discussed in Section 4.2. The MM and fragments appear distorted, as discussed in Section 4.3.

The amplified region was subsequently sequenced in order to screen for novel alterations that may be associated with MHS. Sequencing was conducted using the forward primer (RYRex3F). A representative electropherogram obtained for individual MH00242, depicting the nucleotide positions of alterations Asp60Asn and Ser71Tyr, is indicated in Figure 4.17. None of the 15 individuals analysed in the study presented here harboured any novel or reported alterations within the amplified region.

Figure 4.17: Representative electropherogram of exon 3 indicating the nucleotide positions of the Asp60Asn and Ser71Tyr alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Asp60Asn at nucleotide 178 and Ser71Tyr at nucleotide 212.

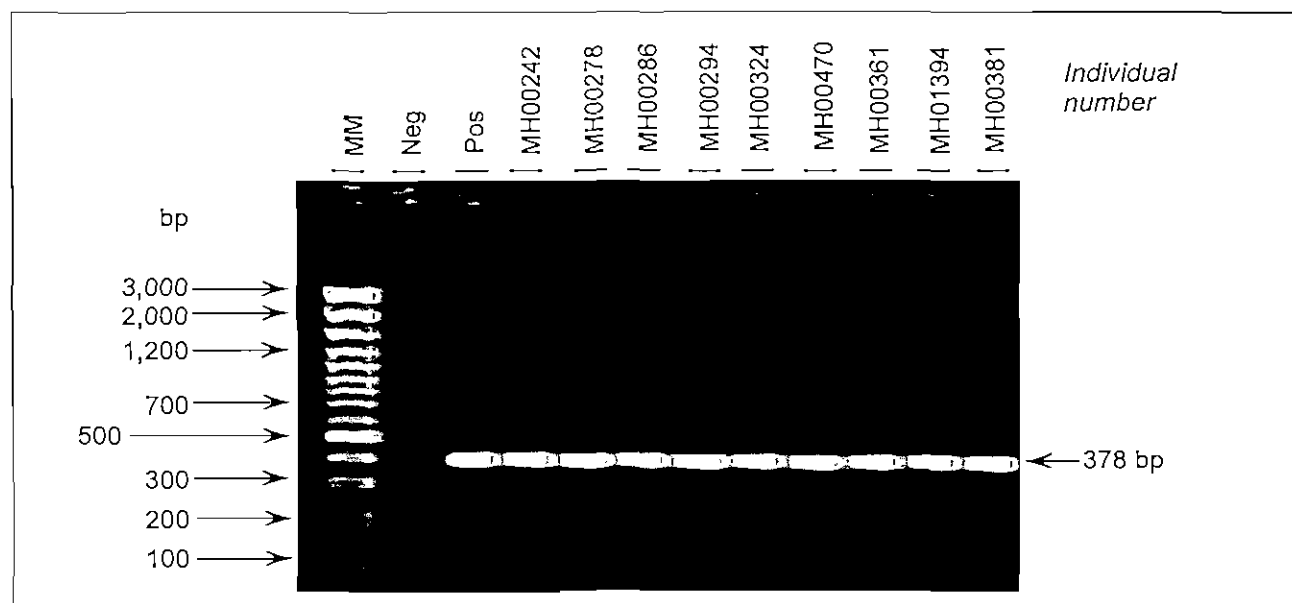
The N-terminal region of the RyR1 channel plays an important role in E-C coupling and optimises the interaction between RyR1 and DHPR (Perez *et al.*, 2003a). However, as only alterations that have been associated with CCD have been observed in exon 3, it may indicate that alterations in this region of the RyR1 protein could result in the promotion of SR Ca^{2+} leakage and store depletion, which is a unique characteristic of CCD mutations. Avila and Dirksen (2001) conducted experiments on dyspedic myotubes expressing CCD-causing mutations and observed that mutations located in hotspot one promote Ca^{2+} leakage through the release channel. However, further studies would have to be conducted in order to determine the functional effects of specific regions of the RyR1. Determining the molecular mechanism of RyR channel function has been hampered by the enormous size of the protein complex (McCarthy and Mackrill, 2004). As discussed in Section 2.9.6 (page 31), cryo-electron microscopy coupled with computer-assisted image reconstruction has been employed to map the three-dimensional structure of purified RyR complexes, as well as the sites of interaction of certain accessory proteins. To date, many of the functional characteristics of the RyR1 have not been determined. The questions that remain unresolved are the nature of protein-protein interactions within the RyR1, regulation of RyR expression during development, targeting

to the junctional-face membrane of the SR and synthesis and folding of the channel protein (Dulhunty and Pouliquin, 2003). Increased knowledge of the structure and function of the RyR1 channel and their association with disorders such as CCD and MH would lead to improved pharmacological and genetic treatment strategies.

4.7.3 Exons 4 and 5 of the RYR1 gene

Alterations associated with the MH phenotype have thus far not been reported to occur in exons 4 and 5. However, an Arg109Trp alteration in exon 4 has been identified in a patient diagnosed with CCD (Zhou *et al.*, 2005), as described in Section 3.7.4 (page 99). A 378 bp product was successfully amplified for all 15 samples obtained from South African MH probands, as described in Section 4.2 (page 159). The results of the amplified PCR product encompassing exons 4 and 5 are depicted in Figure 4.18.

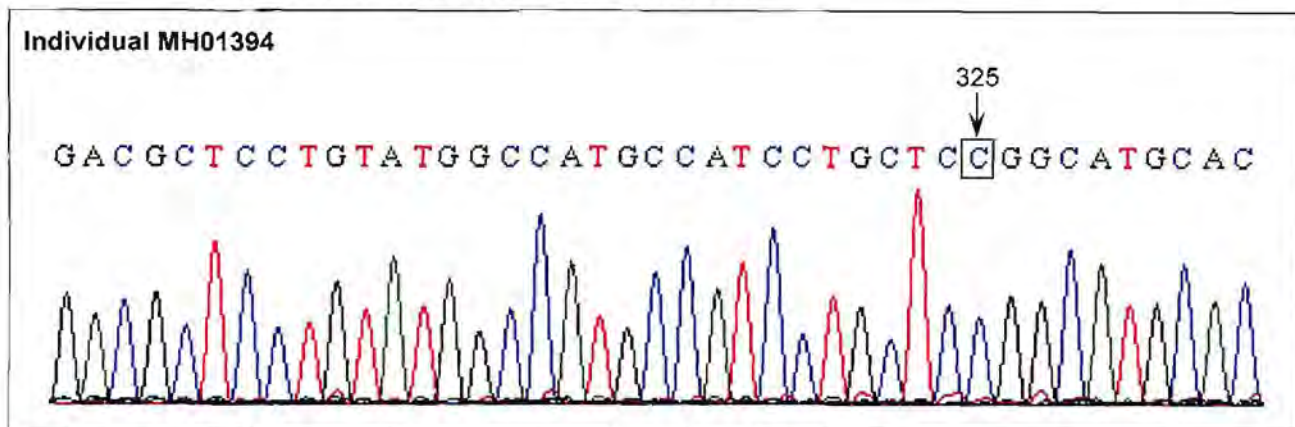
Figure 4.18: Photographic representation of amplified PCR products encompassing exons 4 and 5



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. Background smear and MM overloading were detected, in addition to fragments appearing slanted, as discussed in Sections 4.2 and 4.3.

Successful sequencing of all 15 samples obtained via PCR was achieved using the standard protocol outlined in Section 3.7 (page 94). Sequencing was conducted using the reverse primer (RYRex4R), thus the sequence results are illustrated as the reverse complement. A representative electropherogram obtained for individual MH01394, depicting the nucleotide position of the previously reported Arg109Trp alteration, is illustrated in Figure 4.19. In addition, a representative electropherogram illustrating a portion of the amplified region of exon 5 is indicated in Figure 4.20.

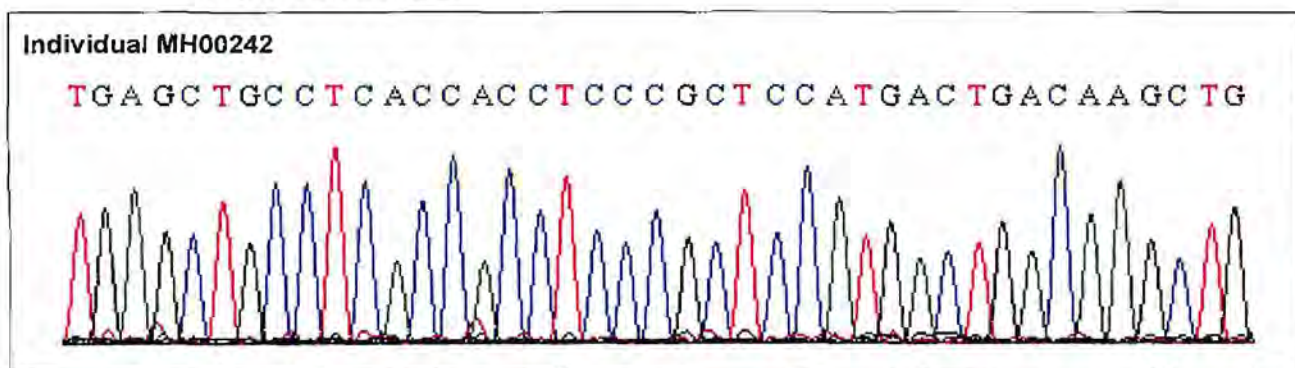
Figure 4.19: Representative electropherogram of exon 4 indicating the nucleotide position of the Arg109Trp alteration



A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Arg109Trp at nucleotide 325.

Novel alterations were not detected in any of the 15 MHS probands analysed in the study presented here. In addition, the Arg109Trp alteration that has previously been detected in a patient diagnosed with CCD (Zhou *et al.*, 2005) was not identified in any of the 15 South African MHS probands analysed. As discussed in Section 4.7.2 (page 178), alterations in this region of the RyR1 protein may only result in increased Ca^{2+} leakage in the SR.

Figure 4.20: Representative electropherogram illustrating a portion of the amplified region of exon 5

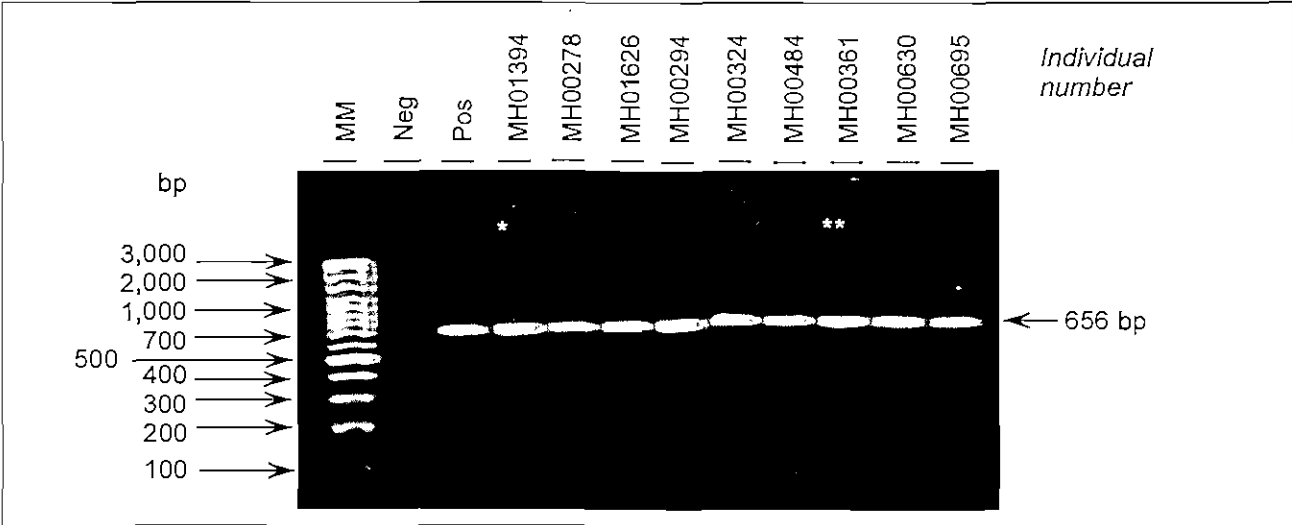


A = adenine; C = cytosine; G = guanine; T = thymine.

4.7.4 Exons 6 and 7 of the RYR1 gene

PCR was conducted in order to amplify a 656 bp region encompassing exons 6 and 7, as discussed in Section 4.2 (page 159). As described in Section 3.6 (page 94), the product was subsequently electrophoresed, and the results are presented in Figure 4.21.

Figure 4.21: Photographic representation of amplified PCR products encompassing exons 6 and 7



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 (*), background smear, MM distortion and a barrier in the gel matrix, as indicated by two white asterisks (**), were detected, as outlined in Sections 4.2 and 4.3.

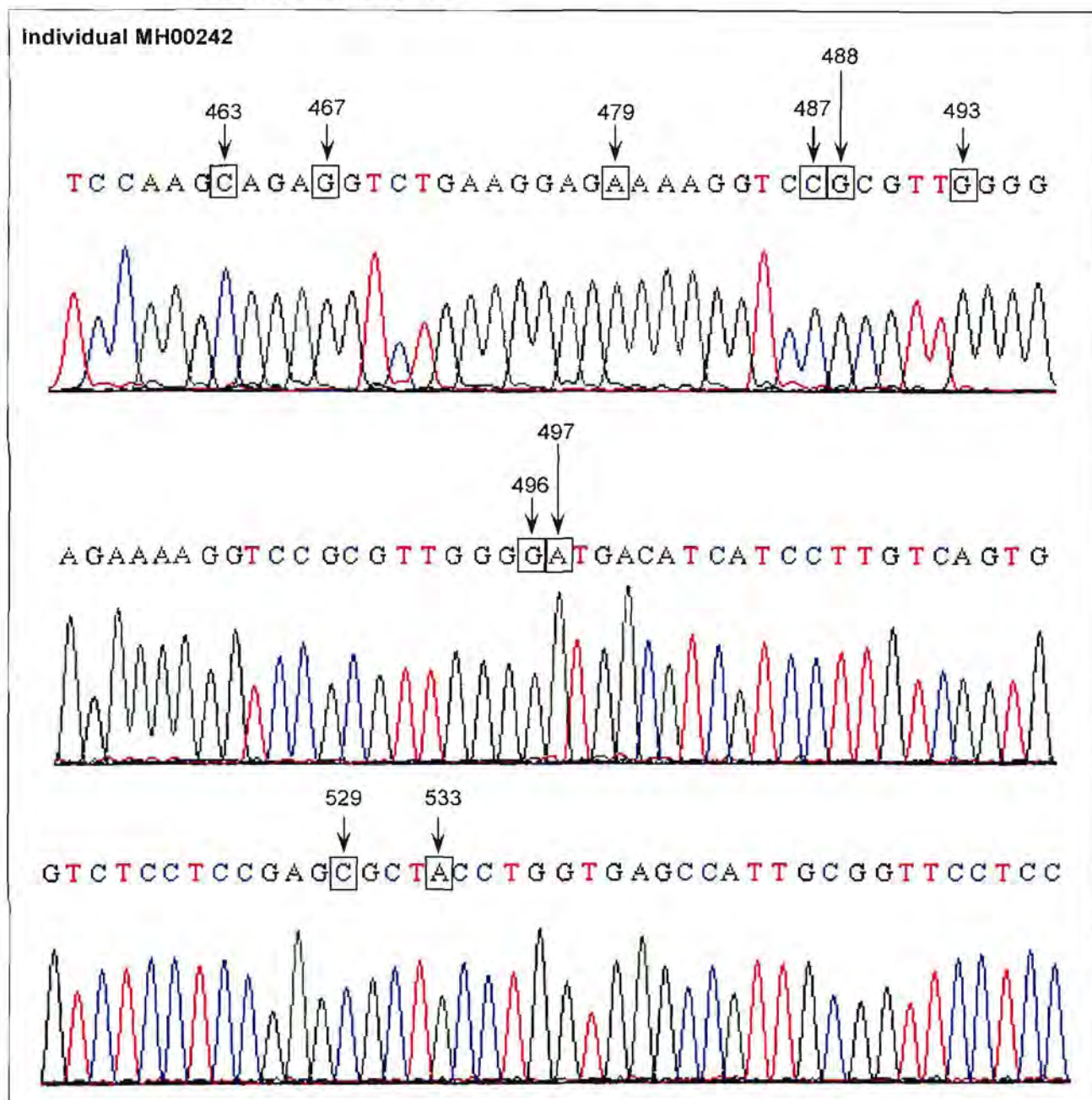
Exons 6 and 7 of the RYR1 gene were sequenced, as they occur in the N-terminal mutation cluster and include functional protein domains. To date, alterations associated with the MH phenotype have not been reported for exon 7. As listed in Table 4.2, exon 6 harbours ten reported alterations that have previously been identified in patients diagnosed as MHS.

Table 4.2: Reported alterations in exon 6 of the RYR1 gene

Amino acid change	Nucleotide change	Reference	Amino acid change	Nucleotide change	Reference
Gln155Lys	C463A	Ibarra <i>et al.</i> , 2006	Gly165Arg	G493A	Monnier <i>et al.</i> , 2005
Arg156Lys	A467G	Galli <i>et al.</i> , 2006	Asp166Asn	G496A	Rueffert <i>et al.</i> , 2002
Glu160Gly	A479G	Halsall and Robinson, 2004	Asp166Gly	A497G	Ibarra <i>et al.</i> , 2006
Arg163Cys	C487T	Quane <i>et al.</i> , 1993	Arg177Cys	C529T	Monnier <i>et al.</i> , 2005
Arg163Leu	G488T	Halsall and Robinson, 2004	Tyr178Cys	A533T	Monnier <i>et al.</i> , 2005

The amplified region was subsequently sequenced, in order to screen for novel and reported alterations in this region of the RYR1 gene. Sequencing was conducted using the reverse primer (RYRex6R) and the sequence results are thus depicted as the reverse complement. A representative electropherogram obtained for individual MH00242, depicting the nucleotide positions of reported alterations observed in exon 6 is indicated in Figure 4.22.

Figure 4.22: Representative electropherogram of exon 6 indicating the nucleotide positions of the Gln155Lys, Arg156Lys, Glu160Gly, Arg163Leu, Arg163Cys, Gly165Arg, Asp166Asn, Asp166Gly, Arg177Cys and Tyr178Cys alterations



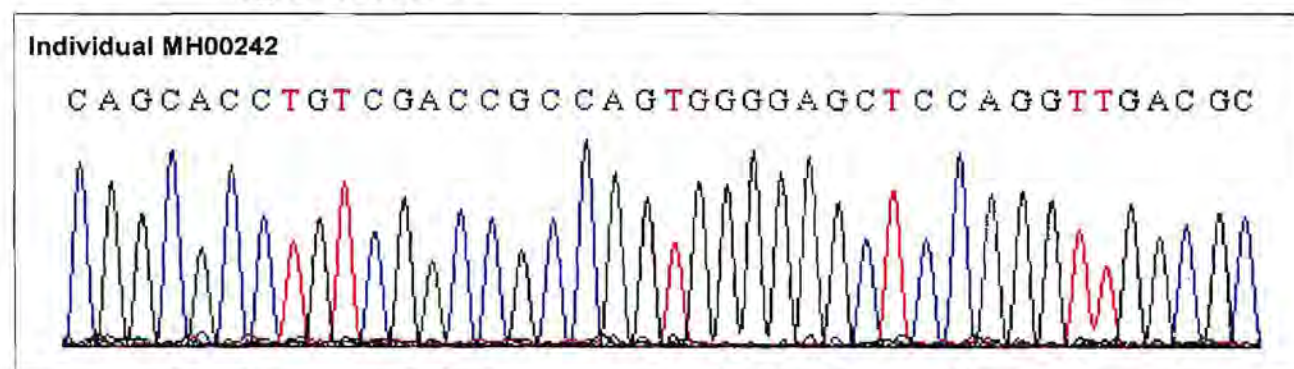
A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Gln155Lys at nucleotide 463; Arg156Lys at nucleotide 467; Glu160Gly at nucleotide 479; Arg163Cys at nucleotide 487; Arg163Leu at nucleotide 488; Gly165Arg at nucleotide 493; Asp166Asn at nucleotide 496; Asp166Gly at nucleotide 497; Arg177Cys at nucleotide 529 and Tyr178Cys at nucleotide 533.

A representative electropherogram obtained for individual MH00242, illustrating a portion of the amplified region of exon 7 is indicated in Figure 4.23. None of the 15 individuals analysed in this study harboured any of the reported alterations in this region of the RYR1 gene. In addition, novel alterations were not observed within the amplified region. Many of the reported alterations have thus far only been observed in single MH families, which

may indicate that they are specific to those families. However, Quane *et al.* (1993) identified the Arg163Cys alteration that has been reported in 2 - 3% of individuals with MH or CCD. Therefore, the Arg163Cys alteration exhibits phenotypic heterogeneity and can predispose individuals to either MH or CCD. However, the mechanism by which a single mutation results in two different clinical phenotypes remains unclear. Screening of the entire coding region of RYR1 has previously identified several compound heterozygous mutations in several probands (Ibarra *et al.*, 2006). In addition, Monnier *et al.* (2002) identified two different genetic traits in an MH family. The authors identified a patient harbouring a RYR1 mutation and a CACNA1S alteration. Therefore, phenotypic heterogeneity could occur due to the presence of more than one alteration. However, further studies would have to be conducted in order to verify this finding, as discussed in Section 5.2.2.1 (page 399).

The Arg163Cys alteration has since been identified in several other populations, including families from North America (Sambuughin *et al.*, 2001a), Italy (Barone *et al.*, 1999), New Zealand (Pollock *et al.*, 2002) and Denmark (Fagerlund *et al.*, 1994). However, the alteration has not been identified in MH families from Sweden (Fagerlund *et al.*, 1997). As the Arg163Cys alteration has been identified in several different populations and in several individuals, it is unlikely to be family-specific. The Arg163Cys alteration has been functionally analysed, as listed in Appendix A (page 443), and fulfils the criteria of a causative alteration, as discussed in Section 5.6 (page 413). The alteration, however, does not play a role in MHS in the cohort of South African MH probands analysed in the study presented here.

Figure 4.23: Representative electropherogram illustrating a portion of the amplified region of exon 7



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

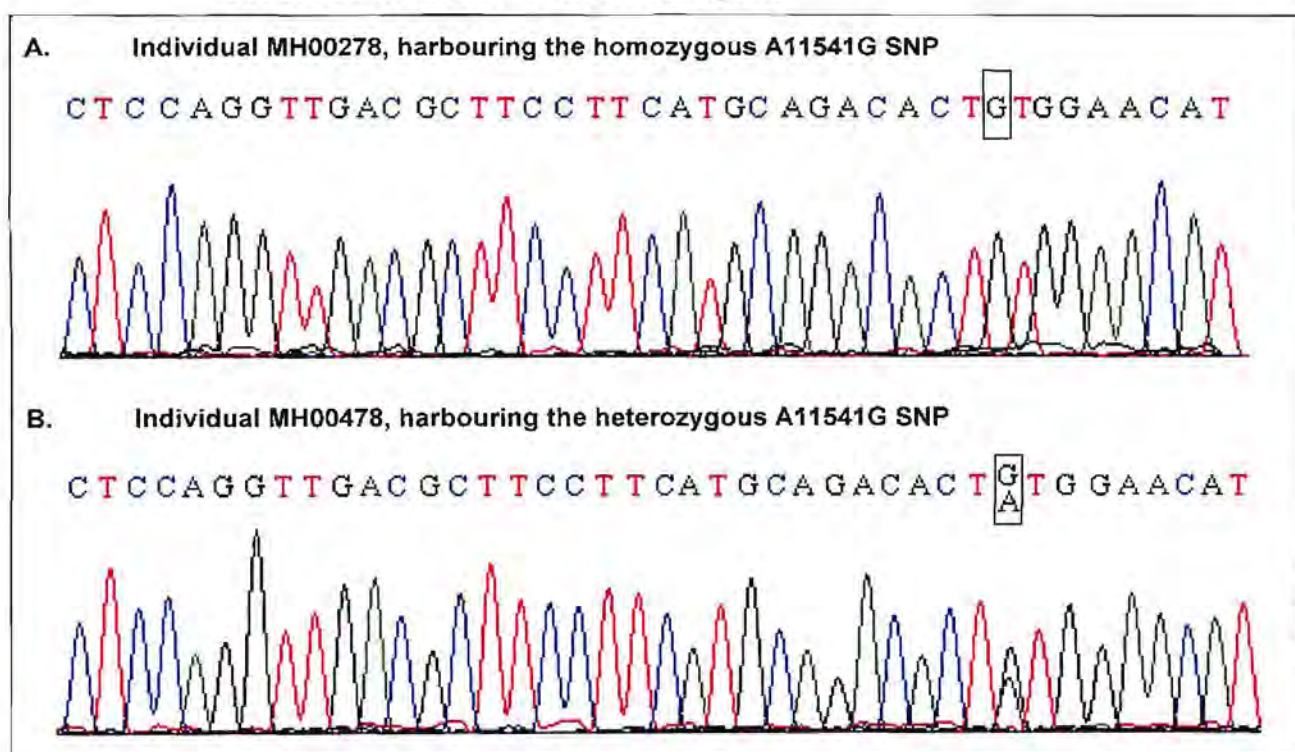
4.7.4.1 Synonymous substitution in the amplified region of exons 6 and 7 of the RYR1 gene

An A11541G SNP was identified in exon 7 of the RYR1 gene in ten of the South African probands analysed in the study presented here. The remaining individuals did not harbour the SNP. Six probands were homozygous for the A11541G SNP and four probands harboured the heterozygous A11541G SNP.

4.7.4.1.1 SNP A11541G

A11541G is a synonymous substitution and is classified as a SNP of the RYR1 gene in GenBank® (International Human Genome Sequencing Consortium, 2004; with accession number rs12985668). The SNP does not result in a change in the amino acid Leu. The genotype and allele frequencies per population for the SNP have not been determined worldwide. Figure 4.24 illustrates the sequence generated for the homozygous and heterozygous A11541G SNP, respectively.

Figure 4.24: Representative electropherograms indicating the A11541G SNP observed in exon 7 of the RYR1 gene



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

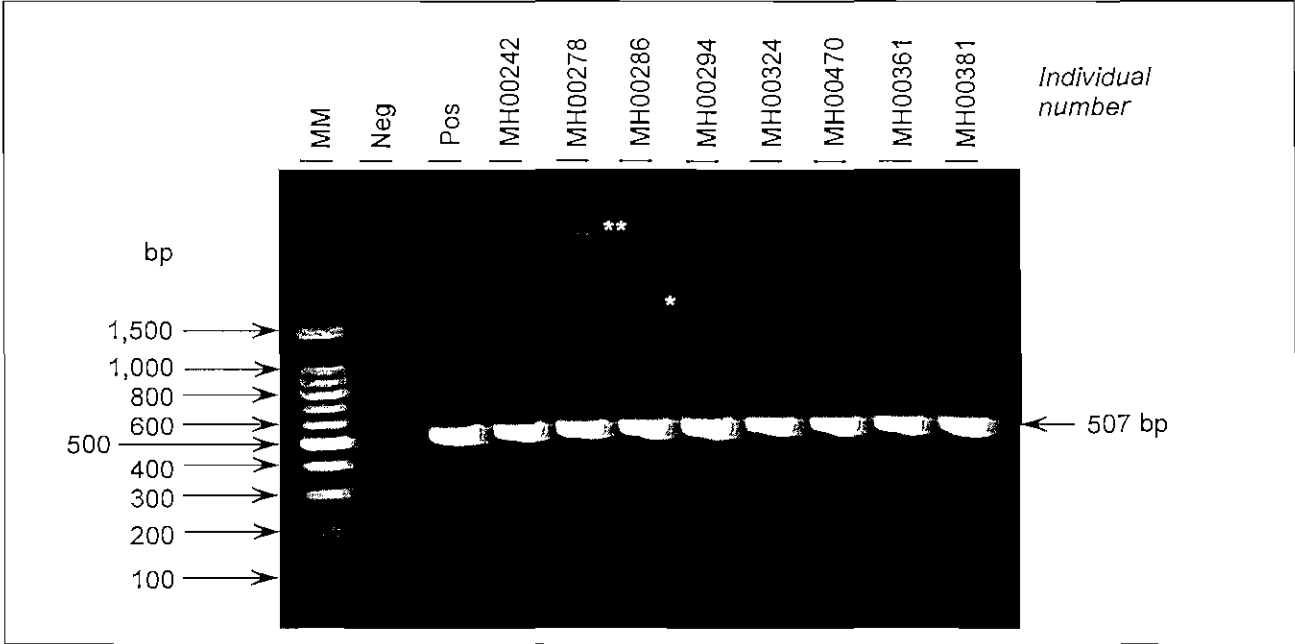
A11541G is classified as a SNP as it is a single base change at a single position and occurs with a prevalence of 1% or more in the population, worldwide, which does vary

between different geographical and ethnic groups. The A11541G SNP was identified as synonymous, as it did not result in a change in the amino acid sequence of the protein (Cargill *et al.*, 1999). As discussed in Section 2.11.3.4 (page 65), SNPs observed in the RYR1 gene may not play a primary role in the development of the MH phenotype, however, they may play an important secondary role. Susceptibility to MH may be due to epistasis, in which several different mutations and SNPs contribute to a threshold being reached. The contribution of all of these determinants would have to be analysed further in order to resolve each of their effects on the MH phenotype, as discussed in Section 5.4 (page 406).

4.7.5 Exons 8 and 9 of the RYR1 gene

Exon 8 harbours two reported MH alterations, i.e. Asp227Val (Monnier *et al.*, 2005) and Val218Ile (Ibarra *et al.*, 2006) and exon 9 harbours one reported alteration, Gly248Arg (Gillard *et al.*, 1992). Both exons occur in hotspot one and have not previously been analysed for alterations in the South African MHS population. Amplification of the 507 bp region for all 15 MHS probands was achieved using the optimised PCR conditions listed in Table 4.1 (page 161). The results of the amplified PCR product encompassing both exons 8 and 9 are depicted in Figure 4.25.

Figure 4.25: Photographic representation of amplified PCR products encompassing exons 8 and 9



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. A single unidentified artefact, as indicated by two white asterisks (**), was detected, which did not affect the outcome of the PCR reaction. In addition, an artefact in the gel matrix, as indicated by the white asterisk (*), background smear, distortion of fragments and MM were observed, as discussed in Sections 4.2 and 4.3.

Following PCR amplification, the PCR product was purified and sequenced in order to investigate for the presence of reported and novel alterations that may be present in the amplified region. Sequencing was conducted using the forward primer (RYRex8F), according to the protocol outlined in Section 3.7 (page 94). A representative electropherogram obtained for individual MH00242 illustrating the nucleotide positions of previously reported alterations that occur in exon 8, is presented in Figure 4.26.

Figure 4.26: Representative electropherogram of exon 8 indicating the nucleotide positions of the Gly215Glu, Val218Ile and Asp227Val alterations



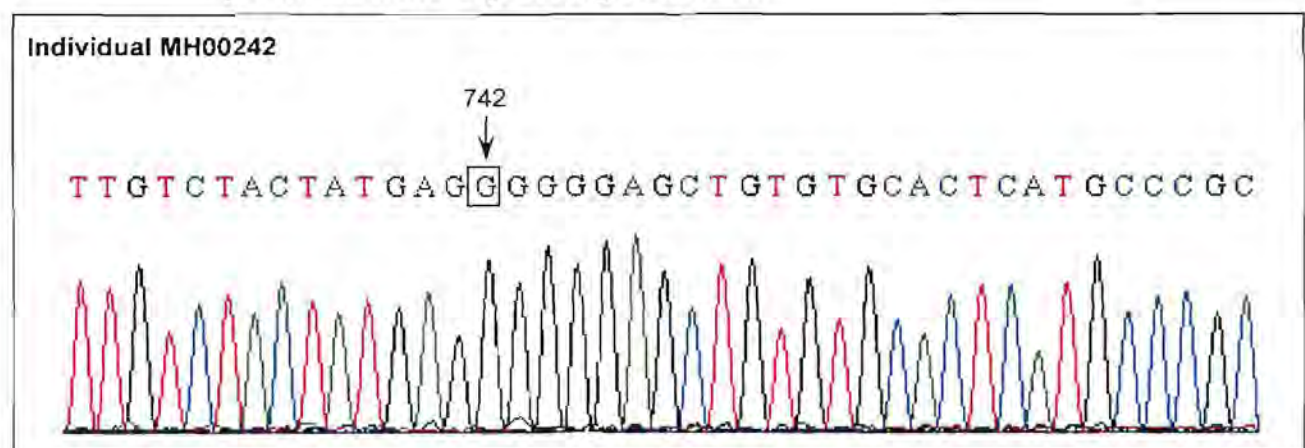
A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Gly215Glu at nucleotide 644; Val218Ile at nucleotide 652 and Asp227Val at nucleotide 680.

A representative electropherogram obtained for individual MH00242, depicting the nucleotide position of the Gly248Arg alteration that has previously been reported to occur in exon 9, is presented in Figure 4.27. Analysis of all 15 samples obtained from MHS probands indicated that none of these individuals harboured any alterations associated with the MH phenotype in exons 8 or 9.

Three MH alterations have previously been identified in this region of the RYR1 gene. Two of the reported MH alterations, Asp227Val and Val218Ile, have thus far only been observed in single families, which may indicate that they are unique to the families described by Monnier *et al.* (2005) and Ibarra *et al.* (2006). The Gly248Arg alteration has been described in several different populations including North American (Sambuughin *et al.*, 2001b; Sei *et al.*, 2004), Canadian (Gillard *et al.*, 1992) and European (Halsall and Robinson, 2004). It is thus evident that this alteration is not population-specific. In addition, the alteration functionally alters the RyR1 protein, as listed in Appendix A (page 443). The Gly248Arg alteration does not play a role in the development of MH in this cohort of South African MH probands. Further analysis of this region of the RYR1 gene would have to be

conducted in a larger group of South African MH individuals, in order to determine if the Gly248Arg alteration plays a role in the development of MHS in this population.

Figure 4.27: Representative electropherogram of exon 9 indicating the nucleotide position of the Gly248Arg alteration

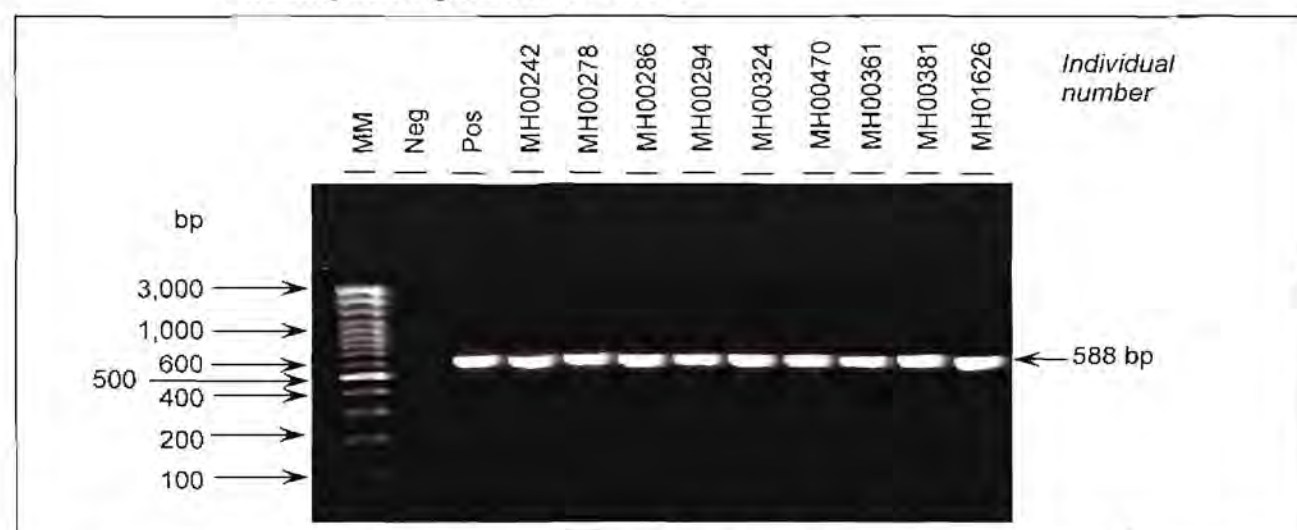


A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Gly248Arg at nucleotide 742.

4.7.6 Exons 10 and 11 of the RYR1 gene

A region of 588 bp was amplified as discussed in Section 4.2 (page 159) and the results are depicted in Figure 4.28. The region encompassing exons 10 and 11 was subsequently sequenced in order to identify reported alterations and to detect novel alterations that may occur in the amplified region.

Figure 4.28: Photographic representation of amplified PCR products encompassing exons 10 and 11



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 as well as fragment and MM distortion were observed, as discussed in Sections 4.2 and 4.3.

The region encompassing exons 10 and 11 harbours three reported alterations, i.e. Arg316Leu (Ibarra *et al.*, 2006), Arg328Trp (Loke *et al.*, 2003) and Gly341Arg (Quane *et al.*, 1994a), as described in Section 3.7.7 (page 102). Sequencing was conducted using the reverse primer (RYRex10R) as indicated in Table 3.2 (page 86). Thus, the sequence results are depicted as the reverse complement. Figure 4.29 is a representative electropherogram obtained for individual MH00242 depicting the nucleotide position of the previously reported Arg316Leu alteration observed in exon 10.

Figure 4.29: Representative electropherogram of exon 10 indicating the nucleotide position of the Arg316Leu alteration

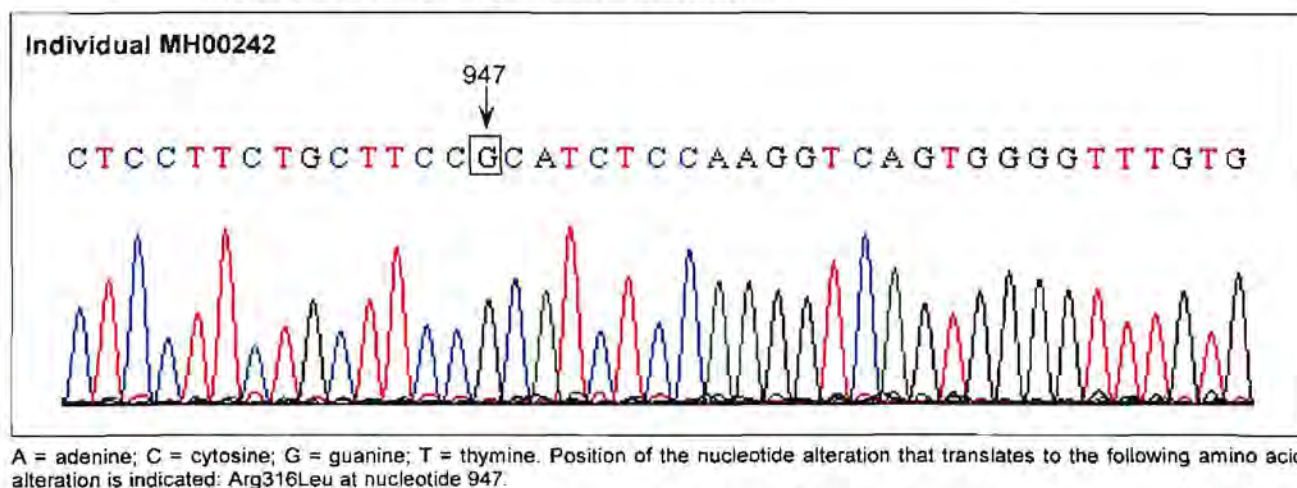
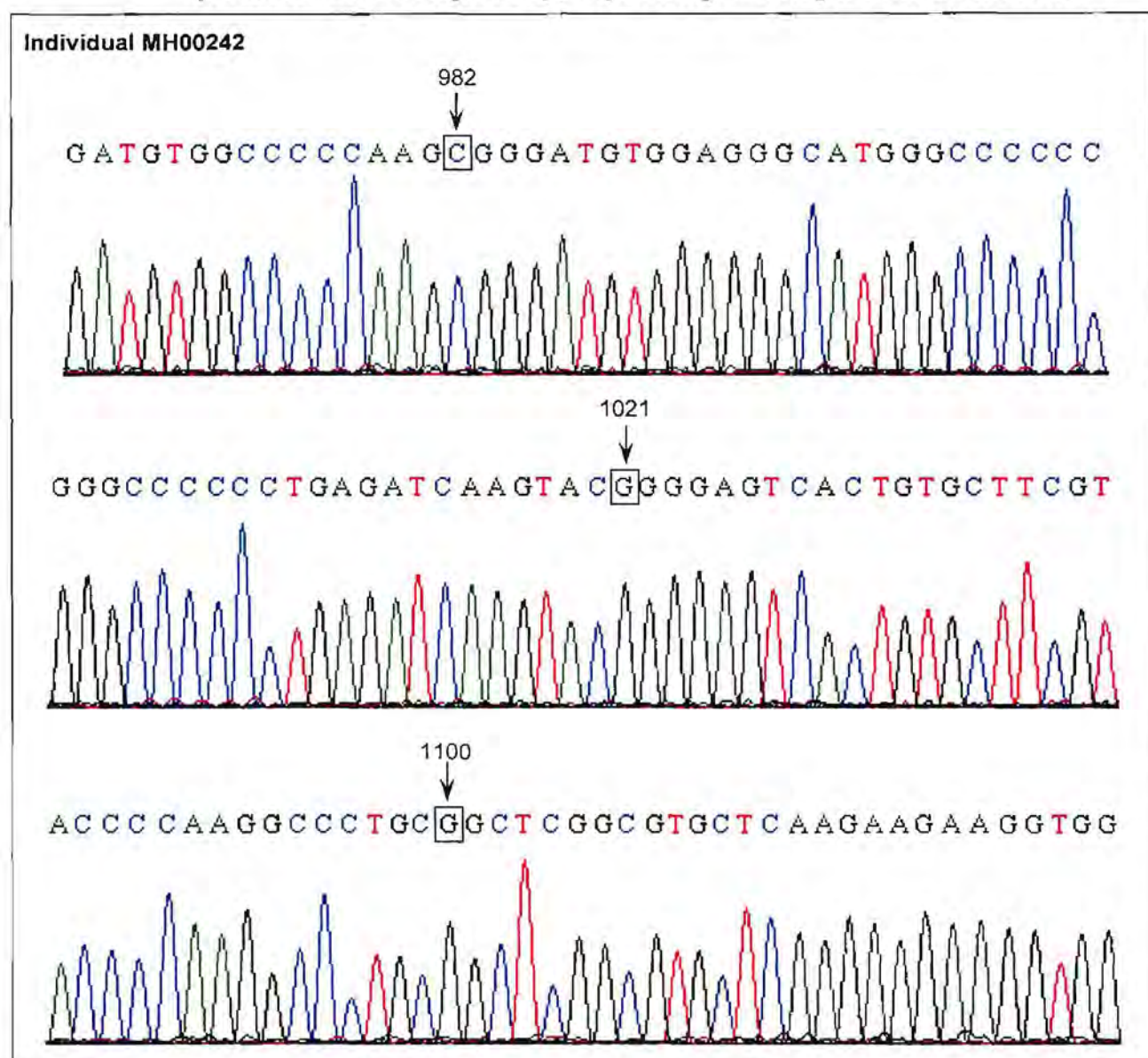


Figure 4.30 is a representative electropherogram obtained for individual MH00242, depicting the nucleotide positions of previously reported alterations observed in exon 11. None of the 15 probands analysed in the study presented here harboured any novel or reported alterations. Thus far, only two family-specific alterations have been reported in this region, namely Arg316Leu (Ibarra *et al.*, 2006) and Arg328Trp (Loke *et al.*, 2003). Due to the family-specific nature of many causative alterations in the RYR1 gene, they may not be observed in other families with MH.

However, Quane *et al.* (1994a) identified a Gly341Arg alteration that has been reported in 10% of Caucasian MHS individuals. The alteration is currently being used in the genetic diagnosis of MHS in European countries (Urwyler *et al.*, 2001) and in North America (Sei *et al.*, 2004). In addition, the functional significance of this alteration has been determined in *ex vivo* tissues, as listed in Appendix A (page 443). Therefore, the Gly341Arg alteration is causative of MH, as it fulfils the criteria provided by the EMHG, as discussed in Section 5.6 (page 413).

Figure 4.30: Representative electropherogram of exon 11 indicating the nucleotide positions of the Arg328Trp, Gly341Arg and Arg367Gln alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Arg328Trp at nucleotide 982; Gly341Arg at nucleotide 1021 and Arg367Gln at nucleotide 1100.

Adeokun *et al.* (1997) identified the alteration in seven British MH probands, Monsieurs *et al.* (1998) detected the Gly341Arg alteration in 13 Belgian individuals and Barone *et al.* (1999) observed the alteration in two Italian MH probands. This alteration is common in individuals from certain European populations (Irish, English and French) but is rare in other populations such as those originating from Northern Europe (McCarthy *et al.*, 2000) and North America (Stewart *et al.*, 1998). In addition, the alteration was not observed in MH populations from Germany, Switzerland and Austria (Brandt *et al.*, 1999). Therefore, the alteration is highly dependent on the geographic distribution of gene pools and appears to be restricted to certain European populations. It is plausible that the Gly341Arg

alteration plays a role in the development of MH in the South African MH population in view of the mainly Western European origin of the Caucasian South African population (Saunders, 1983). However, the alteration does not contribute to the MH phenotype in the South African MH cohort analysed in the study presented here.

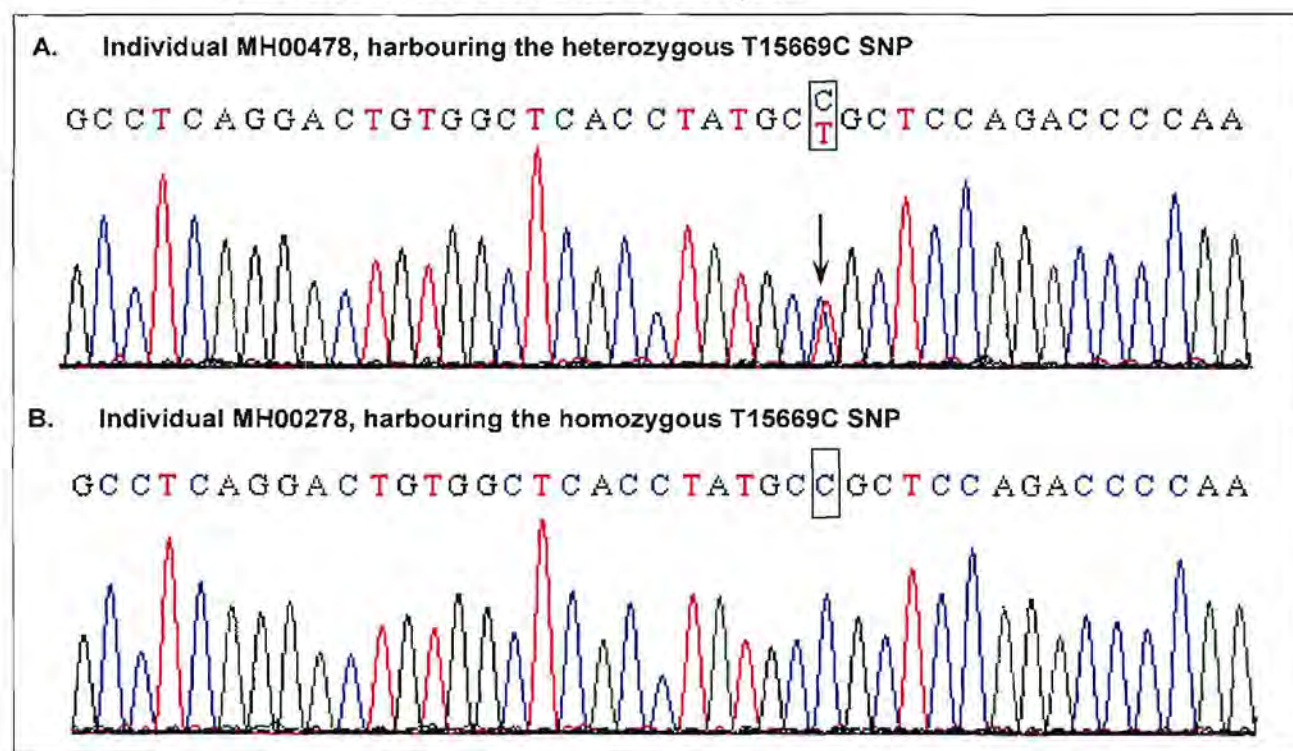
4.7.6.1 Synonymous substitution in the amplified region of exons 10 and 11 of the RYR1 gene

A T15669C SNP was identified in exon 11 of the RYR1 gene. The SNP was identified in all 15 South African MHS probands analysed. Six probands were heterozygous for this SNP and the remaining probands were homozygous for the SNP. As discussed in Section 4.7.4.1 (page 185), synonymous SNPs may affect the expression and function of the RYR1 gene and may contribute to the MH phenotype via epistasis.

4.7.6.1.1 SNP T15669C

T15669C is a synonymous substitution that has been classified as a SNP of the RYR1 gene in Genbank® (International Human Genome Sequencing Consortium, 2004; with accession number rs10406027) and does not result in a change in the amino acid Ala. Figure 4.31 depicts the sequence generated for both the heterozygous and the homozygous T15669C SNP, respectively. The genotype and allele frequencies for the SNP have been determined in the North American population (International Human Genome Sequencing Consortium, 2004; with accession number rs10406027). The frequency of the C/C genotype and the C/T genotype has been identified as 0.82 and 0.18 in North American individuals, respectively (International Human Genome Sequencing Consortium, 2004; with accession number rs10406027). The allele frequencies were 0.09 for the T allele in North American individuals and 0.91 for the C allele (International Human Genome Sequencing Consortium, 2004; with accession number rs10406027).

Figure 4.31: Representative electropherograms indicating the T15669C SNP observed in exon 11 of the RYR1 gene

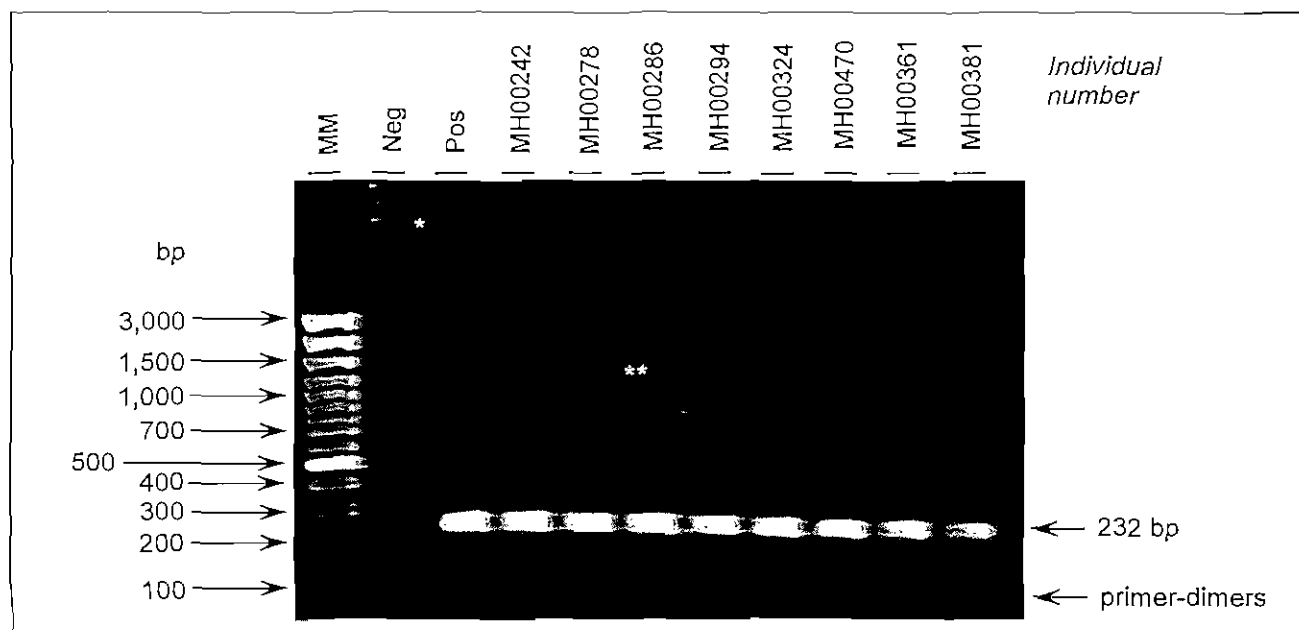


A = adenine; C = cytosine; G = guanine; T = thymine. Arrow indicates fluorescence peaks with equivalent heights.

4.7.7 Exon 12 of the RYR1 gene

Exon 12 is located in the N-terminal region of the RYR1 gene, which represents the first mutational hotspot. This region plays an important role in E-C coupling, as discussed in Section 2.11.3.3.1 (page 47). Thus far, five alterations have been reported to occur in this region of the RyR1 protein, of which three alterations, namely Arg401Cys (Davis *et al.*, 2002), Arg401His (Rueffert *et al.*, 2002), and Arg401Ser (Monnier *et al.*, 2005), have been reported in MH probands and two alterations, i.e. Met402Ile (Zhou *et al.*, 2005) and Ile403Met (Quane *et al.*, 1993), have been identified in CCD patients. Amplification of a 232 bp region was conducted, as discussed in Section 4.2 (page 159), and the results of the amplified PCR product encompassing exon 12 are illustrated in Figure 4.32.

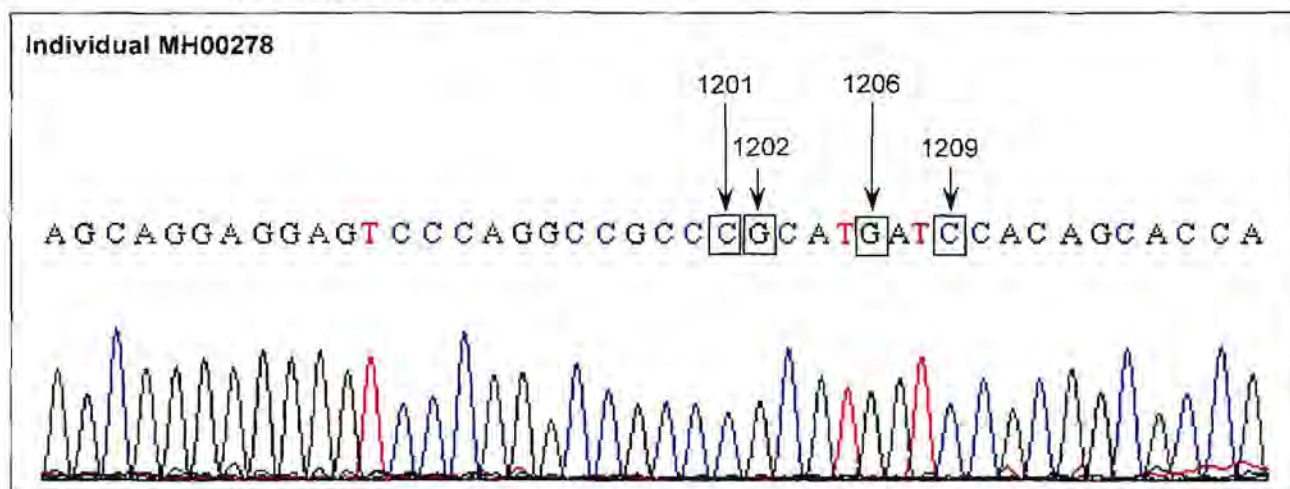
Figure 4.32: Photographic representation of amplified PCR products encompassing exon 12



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. Background smears, secondary amplification, as indicated by two white asterisks (**), an artefact in the gel matrix, as indicated by the white asterisk (*), variation in amplification efficiency, distortion of MM and fragments were detected, as discussed in Sections 4.2 and 4.3. In addition, as discussed in Section 4.3 the fragments appear slanted.

The amplified region was subsequently sequenced in order to screen for novel and reported alterations that may be present in the amplified region of exon 12. Sequencing was conducted using the reverse primer (RYRex12R) and a representative electropherogram obtained for individual MH00278, illustrating the nucleotide positions of the previously reported alterations, is indicated in Figure 4.33. Thus far, five alterations that have been reported in MH probands, occur in exon 12. They have been described in three different populations (New Zealand Maori, German and French) as well as several different pedigrees (Davis *et al.*, 2002; Rueffert *et al.*, 2002; Monnier *et al.*, 2005), indicating that they do not represent family-specific alterations. To date, functional analysis has only been conducted for the Ile403Met alteration, as listed in Appendix A (page 443). The causative status of the Arg401Cys, Arg401Ser, Arg401His and Met402Ile alterations remains to be determined.

Figure 4.33: Representative electropherogram of exon 12 indicating the nucleotide positions of the Arg401Cys, Arg401Ser, Arg401His, Met402Ile and Ile403Met alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Arg401Cys and Arg401Ser at nucleotide 1201; Arg401His at nucleotide 1202; Met402Ile at nucleotide 1206 and Ile403Met at nucleotide 1209.

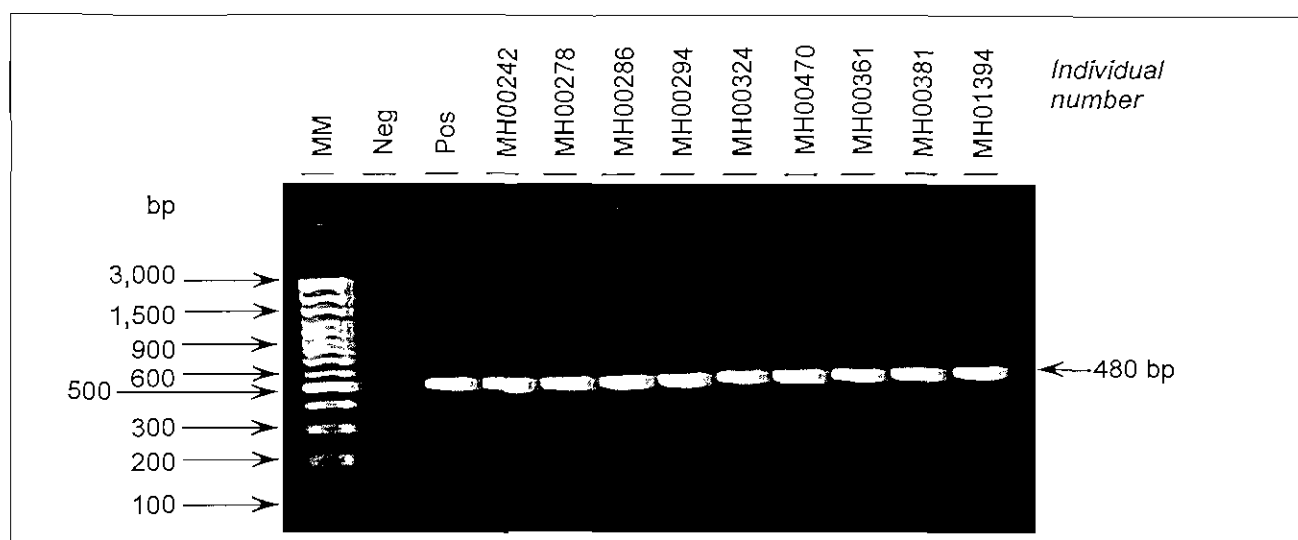
Three alterations (Arg401Cys, Arg401Ser and Arg401His) involve a change in the amino acid Arg, which may indicate that a positively charged amino acid plays an important regulatory role in the RyR1 protein at this position. However, the Arg-to-Cys alteration was only reported in the Maori population and the Arg-to-Ser/His alterations were reported in the European population. Therefore, nucleotide transitions within the RYR1 gene may be population-specific. None of the reported alterations or any novel alterations were observed in any of the 15 South African MH probands analysed. It is unlikely that the Arg401Cys alteration would be observed in any of the South African MH probands, as most of these individuals are immigrants of Western European origin and thus far the alteration has only been observed in the Maori population. However, as the Arg401His and Arg401Ser alterations have been reported in European populations, they may play a role in susceptibility to MH in the South African population. Both the Met402Ile and Ile403Met alterations were not detected in any of the South African MH probands analysed in the study presented here. The alterations have both only been observed in patients clinically diagnosed with CCD and not MH. Therefore, it would be expected that they would not be observed in any of the South African probands analysed, as none of these individuals has been diagnosed with CCD.

4.7.8 Exon 13 of the RYR1 gene

Analysis of a 480 bp region was conducted in order to detect both reported and novel alterations or polymorphisms that may occur in exon 13 of the RYR1 gene. Successful

amplification of a 480 bp region encompassing exon 13 was obtained for all 15 samples as discussed in Section 4.2 (page 159) and the results of the amplified PCR product encompassing exon 13 are illustrated in Figure 4.34. Exon 13 is located in the first mutational hotspot of the RYR1 gene and thus far, only one alteration has been described in a single MH proband, whereas two alterations have been reported in CCD patients. Gillard *et al.* (1992) reported an Arg471Cys alteration in one MHS family. Both the Ser427Leu and Gln474His alterations have been reported in CCD probands, respectively (Wu *et al.*, 2006).

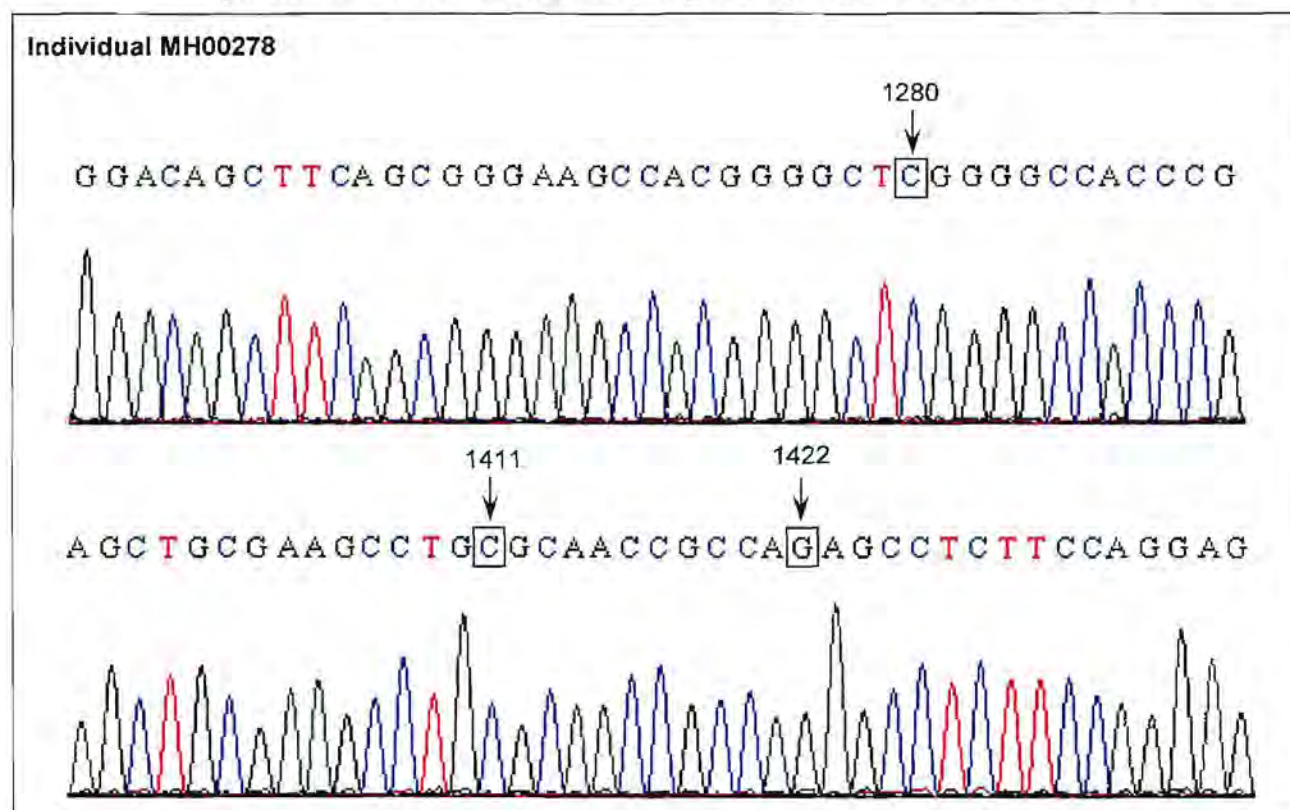
Figure 4.34: Photographic representation of amplified PCR products encompassing exon 13



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. Slanted fragments, secondary amplification, background smears, fragment and MM distortion were observed, as discussed in Sections 4.2 and 4.3.

In order to detect both reported and novel alterations that may reside in exon 13, the amplified region was sequenced using the forward primer (RYRex13F). A representative electropherogram obtained for individual MH00278, illustrating the nucleotide positions of the previously reported alterations, is indicated in Figure 4.35.

Figure 4.35: Representative electropherogram of exon 13 indicating the nucleotide positions of the Ser427Leu, Arg471Cys and Gln474His alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Ser427Leu at nucleotide 1280; Arg471Cys at nucleotide 1411 and Gln474His at nucleotide 1422.

None of the 15 probands analysed in the study presented here harboured any reported or novel alterations. Thus far, only one alteration, Arg471Cys (Gillard *et al.*, 1992) has been reported to occur in this region of the RYR1 gene. The alteration was observed in a single MH family. Therefore it is likely that the Arg471Cys alteration is specific to that particular family. Both the Ser427Leu and Gln474His alterations (Wu *et al.*, 2006) have only been reported in CCD probands. The families included in this investigation do not have a history of CCD, therefore the absence of these alterations would be expected in the individuals under investigation, as both alterations are only associated with CCD and not MH.

4.7.8.1 Synonymous substitutions in the amplified region of exon 13 of the RYR1 gene

Analysis of 15 MHS probands from South Africa resulted in the identification of SNPs within the amplified region of exon 13. A heterozygous C19691T SNP was identified in only one proband, MH00242, and the G19989A SNP was observed in 11 probands, of which five were heterozygous and six were homozygous for the SNP. Both synonymous substitutions occur in the intron sequence and are defined as SNPs of the RYR1 gene in

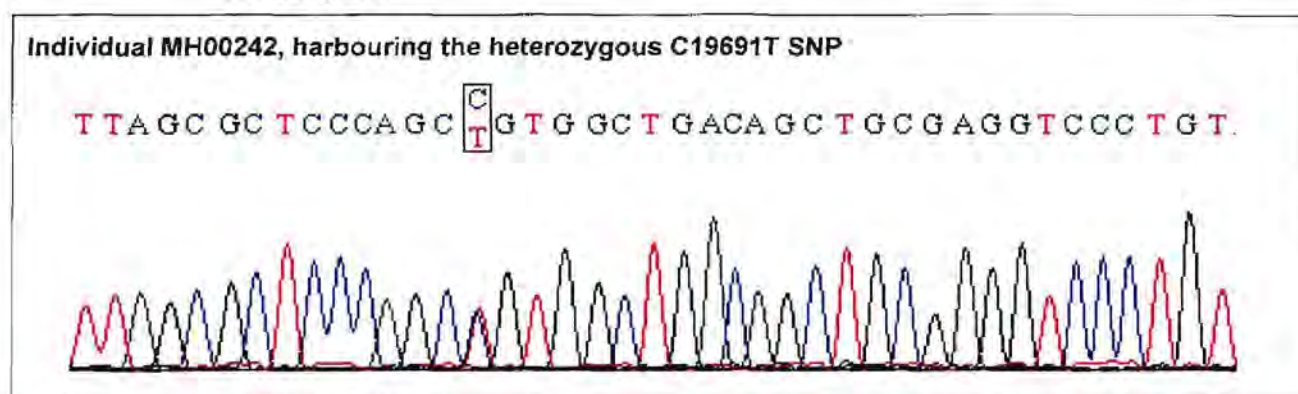
Genbank® (International Human Genome Sequencing Consortium, 2004; with accession numbers rs35839245 and rs12460768, respectively).

In general, a high level of sequence conservation occurs in the non-coding DNA, which may indicate that regions of the intron sequence play an important role in gene regulation (Hare and Palumbi, 2003) since these regions harbour regulatory elements. Cargill *et al.* (1999) indicated that synonymous SNPs in non-coding DNA that occur in the immediate vicinity of a gene may contain sequences regulating gene expression and splicing. Smith *et al.* (2002) indicated that non-coding substitution rates are affected by GC content, which may indicate that the process of mutagenesis or repair is affected by GC content. Therefore, although both SNPs were detected in the intron sequence between exons, they may play a role in development of the MH phenotype. Synonymous and non-synonymous SNPs in coding and non-coding DNA of the RYR1 gene, in addition to environmental factors, may in combination result in the MH phenotype. However, further analysis would have to be conducted in order to determine the role that each SNP plays in the development of the disorder, as discussed in Section 4.7.4.1 (page 185).

4.7.8.1.1 SNP C19691T

The C19691T SNP was observed in the intron sequence between exons 12 and 13. The genotype and allele frequencies per population for the C19691T SNP have not been determined worldwide. Figure 4.36 depicts the sequence generated for the heterozygous C19691T observed in individual MH00242.

Figure 4.36: Representative electropherogram indicating the C19691T SNP observed in the intron sequence between exons 12 and 13 of the RYR1 gene

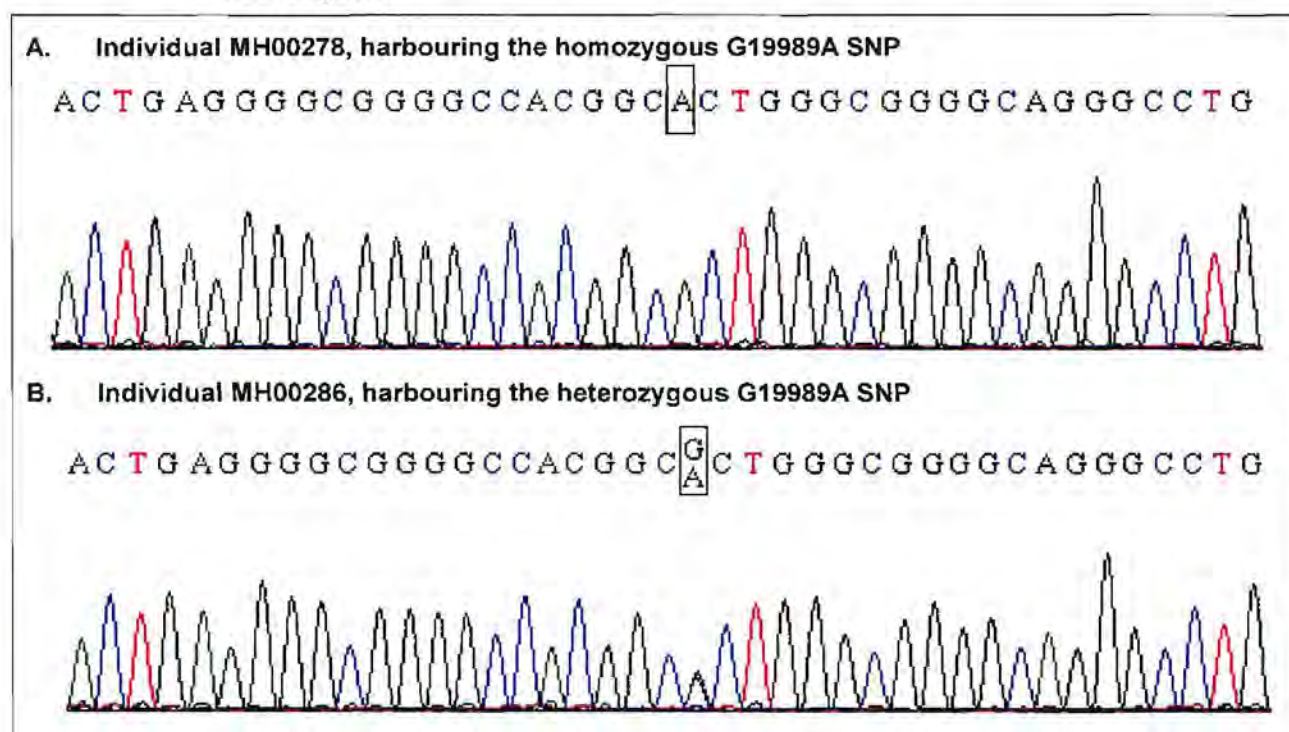


A = adenine; C = cytosine; G = guanine; T = thymine.

4.7.8.1.2 SNP G19989A

The G19989A SNP was detected in the intron sequence between exons 13 and 14. The genotype and allele frequency for the G19989A SNP have not been determined worldwide. Figure 4.37 illustrates the sequence generated for the homozygous and heterozygous G19989A SNP, respectively.

Figure 4.37: Representative electropherograms indicating the G19989A SNP observed in the intron sequence between exons 13 and 14 of the RYR1 gene



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

4.7.9 Exons 14, 15 and 16 of the RYR1 gene

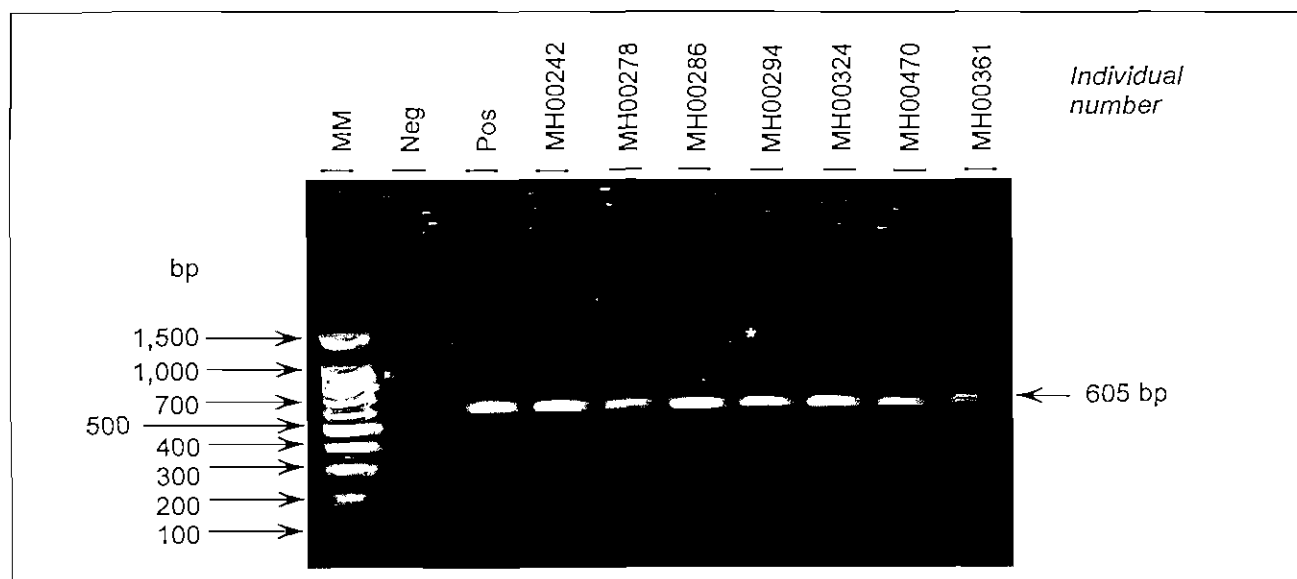
Thus far, alterations that may be associated with MH or CCD have not been reported in exon 16. However, three alterations have been reported in exon 14. In addition, three alterations have been reported in exon 15, as listed in Table 4.3. As several alterations associated with MHS have been reported to occur in this region and because all three exons reside in hotspot one of the RYR1 gene, the entire region encompassing exons 14, 15 and 16 was simultaneously amplified and sequenced.

Table 4.3: Reported alterations in exons 14 and 15 of the RYR1 gene

Exon	Amino acid change	Nucleotide change	Reference	Amino acid change	Nucleotide change	Reference
14	Glu512Lys ¹	G1534A	Wu <i>et al.</i> , 2006	Tyr522Cys	A1565G	Yeh <i>et al.</i> , 2005
	Thr522Ser	A1565C	Quane <i>et al.</i> , 1994a	---	---	---
15	Arg533His	G1598A	Brandt <i>et al.</i> , 1999	Arg552Trp	C1654T	Keating <i>et al.</i> , 1997
	Arg533Cys	C1597T	Tammaro <i>et al.</i> , 2003	---	---	---

— = indicates no information given; ¹ = indicates alteration observed in a patient clinically diagnosed with CCD.

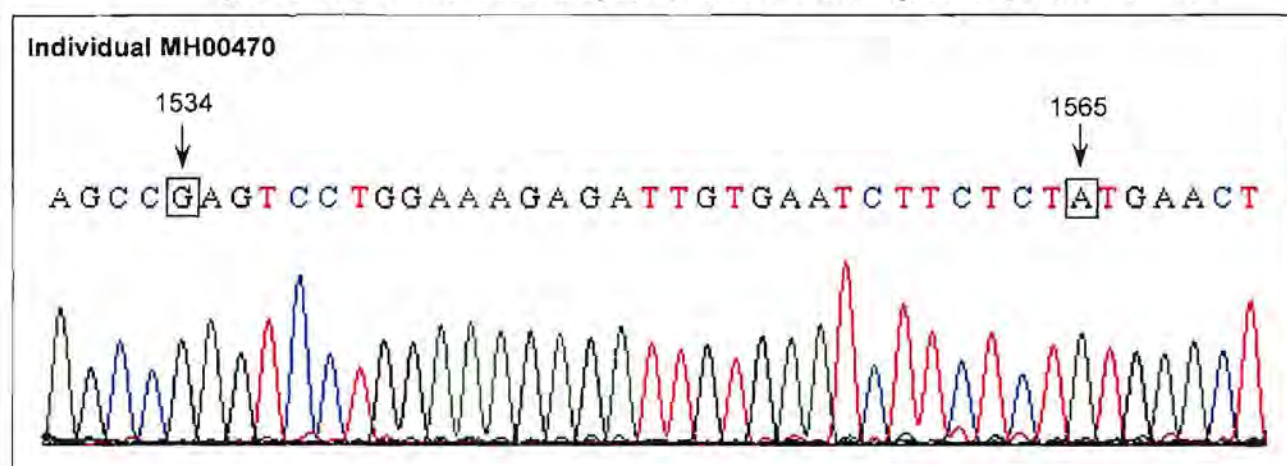
Amplification of the 605 bp region for all samples obtained from 15 South African MHS probands was achieved as discussed in Section 4.2 (page 159). The PCR products were subsequently electrophoresed and the results of the amplified PCR product encompassing exons 14, 15 and 16 are depicted in Figure 4.38.

Figure 4.38: Photographic representation of amplified PCR products encompassing exons 14, 15 and 16

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 (*), background smears, variation in amplification efficiency of samples, distortion of samples and MM were observed, as discussed in Sections 4.2 and 4.3.

The amplified region for all 15 MH probands was subsequently sequenced in order to investigate for the presence of novel or reported alterations in this region. Sequencing was conducted using the standard protocol as discussed in Section 3.7 (page 94). The forward primer (RYRex14F) was used in the sequencing reaction. A representative electropherogram obtained for individual MH00470, depicting the nucleotide positions of previously reported alterations observed in exon 14, is illustrated in Figure 4.39.

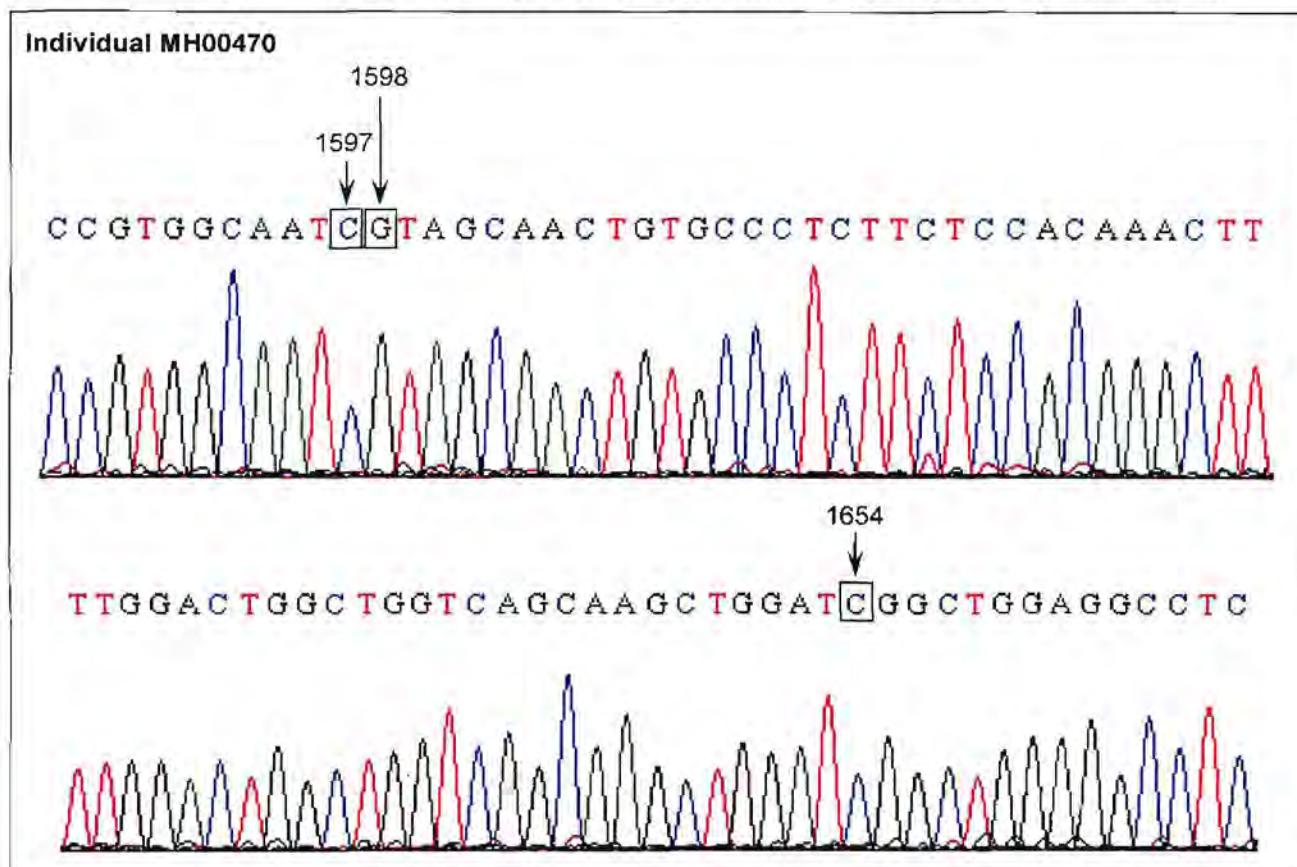
Figure 4.39: Representative electropherogram of exon 14 indicating the nucleotide positions of the Glu512Lys, Tyr522Ser and Tyr522Cys alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Glu512Lys at nucleotide 1534; Tyr522Ser and Tyr522Cys at nucleotide 1565.

A representative electropherogram obtained for individual MH00470 depicting the nucleotide positions of the alterations reported to reside in exon 15 is illustrated in Figure 4.40. None of the 15 South African MH probands harboured any novel or reported alterations within the amplified region. Thus far, several alterations in numerous individuals from different populations have been reported to occur in exons 14 and 15. The alterations, Tyr522Cys, Glu512Lys, Arg533His and Arg533Cys, have all been reported to occur in single pedigrees (Quane *et al.*, 1994a; Brandt *et al.*, 1999; Tammaro *et al.*, 2003; Wu *et al.*, 2006), which may indicate that they all represent family-specific alterations.

Figure 4.40: Representative electropherogram of exon 15 indicating the nucleotide positions of the Arg533His, Arg533Cys and Arg552Trp alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Arg533His at nucleotide 1597; Arg533Cys at nucleotide 1598 and Arg552Trp at nucleotide 1654.

A representative electropherogram obtained for individual MH00470, illustrating a portion of the amplified region for exon 16, is depicted in Figure 4.41. The Tyr522Ser alteration has been identified in families from Europe (Quane *et al.*, 1994a; Monnier *et al.*, 2005) and Taiwan (Yeh *et al.*, 2005). In addition, the Arg552Trp alteration has been reported in families from Europe (Keating *et al.*, 1997) and Taiwan (Yeh *et al.*, 2005). The presence of these alterations in two very different populations may be coincidental and these observations could suggest that these alterations arose from two independent founding events for the Tyr522Ser and Arg552Trp alterations. Although the Tyr522Ser and Arg552Trp alterations were not observed in the cohort analysed in the study presented here, they may play a role in the development of MH in other South African families, which may to date have been undetected, and are thus not included in this study.

Figure 4.41: Representative electropherogram illustrating a portion of the amplified region of exon 16



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

4.7.9.1 Synonymous substitutions in the amplified region of exons 14, 15 and 16 of the RYR1 gene

Two SNPs were identified within the amplified region of exons 14, 15 and 16. A G22443A SNP was identified in exon 15 and a G22476C SNP was detected in the intron sequence between exon 15 and exon 16. Both synonymous substitutions are indicated as SNPs of the RYR1 gene in Genbank® (International Human Genome Sequencing Consortium, 2004; with accession numbers rs2288888 and rs2288889, respectively). As discussed in Sections 4.7.4.1 (page 185) and 4.7.8.1 (page 196), SNPs observed in exonic and intronic sequence may play a role in the development of MHS via epistasis. Linkage disequilibrium (LD) between the G22443A and G22476C SNPs has been described in Genbank® in populations from Europe, America, West Africa and Asia (International Human Genome Sequencing Consortium, 2004; with accession numbers rs2288888 and rs2288889, respectively). The presence of LD between the G22443A and G22476C SNPs observed in populations from Europe, America, West Africa and Asia occurred due to the physical distance between the SNPs (Ott, 2000).

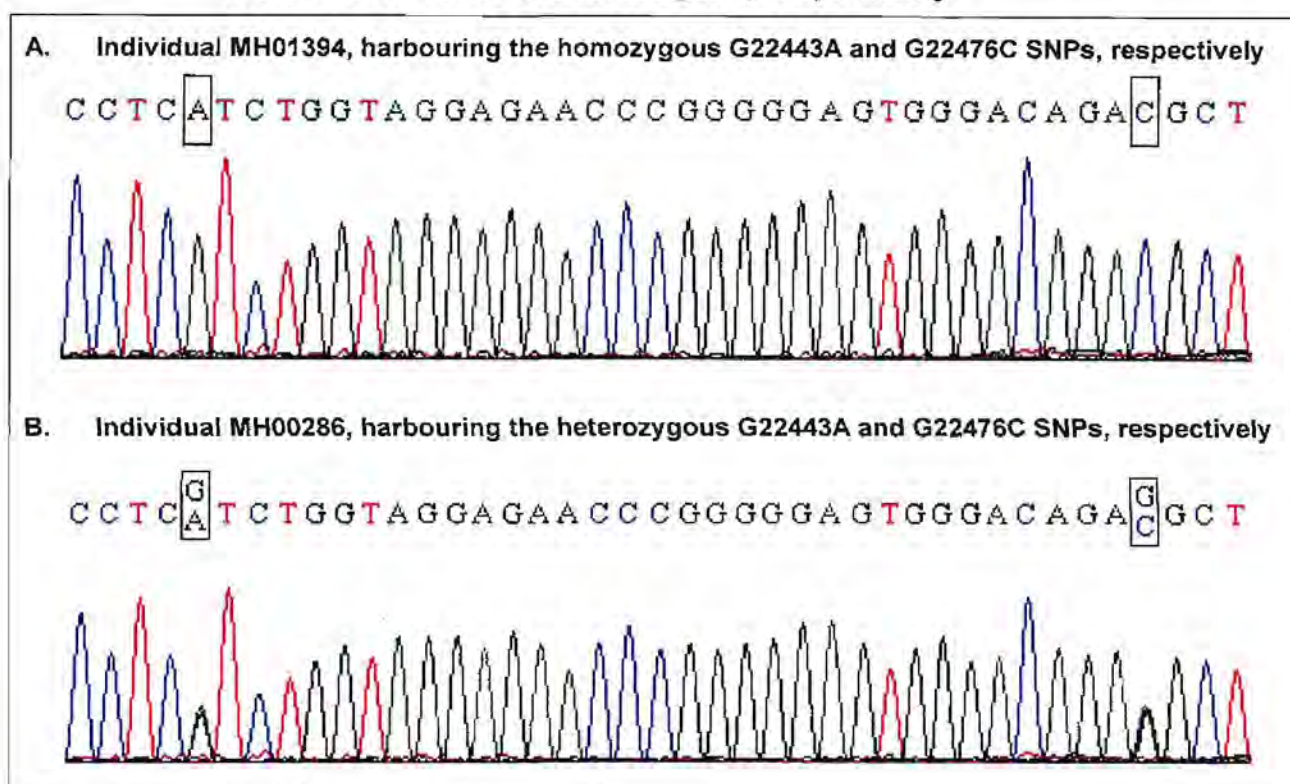
Studies have provided evidence of LD blocks (or haploblocks) with regions of limited numbers of haplotypes which may have occurred due to limited ancestral recombination in these regions (Sawyer *et al.*, 2005). De La Vega *et al.* (2005) indicated that the patterns of LD blocks vary across the genome and differ among populations originating from different continents. The authors indicated that for example on chromosome 22, the LD blocks in Caucasians extend an average of 36 kb, whereas the LD blocks in African-Americans are generally smaller, averaging approximately 29 kb. LD block patterns can provide insight into human evolutionary history as the extent of the LD block pattern is determined, in part, by population history (Norborg and Tavaré, 2002). However, the SNP density

required for LD studies will vary based on the specifics of both the sample population and the genomic region being studied (De La Vega *et al.*, 2005). Therefore, the demographic history of the South African population could be determined by analysing the patterns of LD blocks for SNPs of the RYR1 gene observed in the study presented here, in combination with LD blocks for SNPs that may be identified in other regions of chromosome 19. In this manner, it would be possible to determine if the South African Caucasian population has recently expanded, which would indicate that MH patients in this population are most likely affected by recent mutations. In contrast, if the South African Caucasian population displays higher levels of LD it can be inferred that the population is of constant size (Laan and Pääbo, 1997) and therefore may harbour older mutations in association with the disorder.

4.7.9.1.1 SNP G22443A

Analysis of samples obtained from 15 MHS probands from South Africa identified six probands that were heterozygous for the G22443A SNP and four probands that were homozygous for the SNP. The remaining individuals did not harbour the SNP. The genotype and allele frequencies worldwide for the G22443A SNP have been determined and vary between the different populations. In most cases, the A/A and A/G genotypes are the most common. The frequency of the SNP in the European population was 0.48 for the A/A genotype, 0.43 for the A/G genotype and 0.09 for the G/G genotype. In the Asian population, the genotype frequency was as follows: 0.31 for A/A, 0.55 for A/G and 0.14 for G/G. Lastly, the population from Sub-Saharan Africa had the following genotype frequencies: 0.37 for A/A, 0.49 for A/G and 0.14 for G/G (International Human Genome Sequencing Consortium, 2004; with accession number rs2288888). Figure 4.42 depicts the sequence generated for an individual homozygous for the G22443A and heterozygous for the G22443A SNP, respectively.

Figure 4.42: Representative electropherograms indicating the G22443A and G22476C SNPs observed in exon 15 and the intron sequence between exons 15 and 16 of the RYR1 gene, respectively



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

4.7.9.1.2 SNP G22476C

Six South African MH probands were observed to be heterozygous for the G22476C SNP, four probands were homozygous for the SNP and the remaining probands did not harbour the SNP. The genotype frequencies per population have also been determined worldwide for the G22476C SNP and the most common genotypes are C/C and C/G. The average frequency of the SNP in three different populations (i.e. European, Asian and Sub-Saharan African) is as follows: 0.47 for C/C, 0.43 for C/G and 0.10 for G/G (International Human Genome Sequencing Consortium, 2004; with accession number rs2288889). Figure 4.42A depicts the sequence generated for an individual homozygous for the G22476C SNP and Figure 4.42B illustrates the sequence generated for an individual heterozygous for the G22476C SNP.

4.7.10 Exon 17 of the RYR1 gene

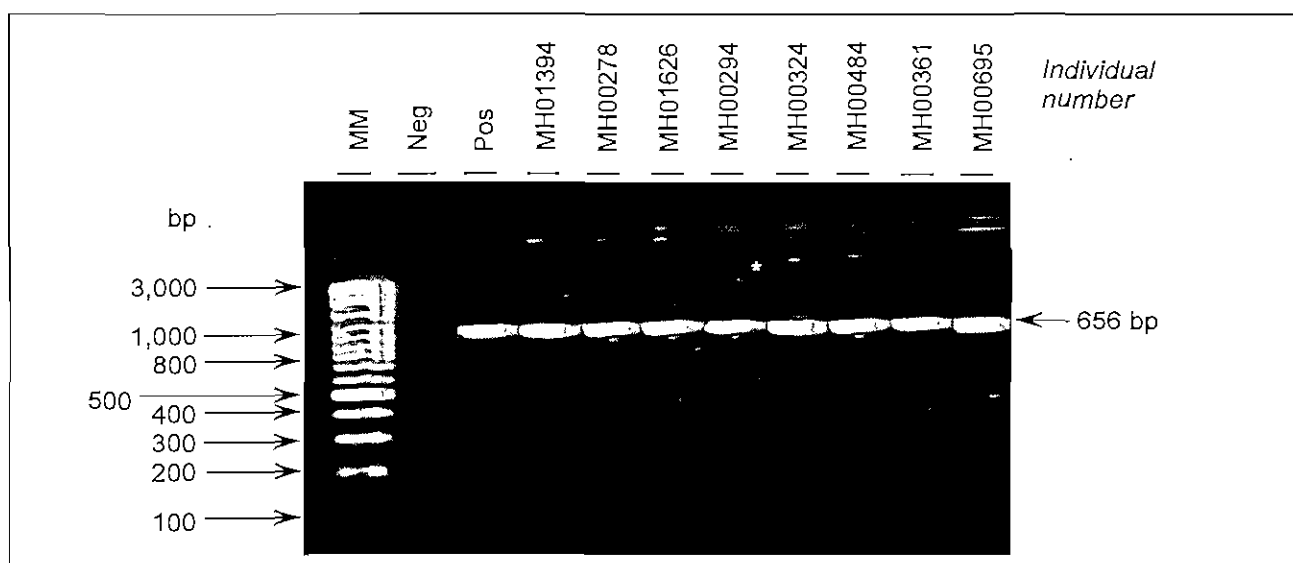
Exons 17 and 18 were simultaneously amplified via PCR. Exon 17 occurs in the first mutational hotspot of the RYR1 gene and harbours two reported alterations, namely

Arg614Cys (Gillard *et al.*, 1991) and Arg614Leu (Quane *et al.*, 1997). Exon 18, however, resides outside of hotspot one, and is discussed in Section 4.10.2 (page 275).

Exon 17 harbours the Arg614Cys alteration that has been reported in both human and porcine RyR1. Studies on animal models have indicated that this alteration changes the sensitivity of the RyR1 protein to divalent cations (Mickelson *et al.*, 1988), causes increased sensitivity to activating CaM concentrations (O'Driscoll *et al.*, 1996) and exhibits both a stronger Ca^{2+} activation and a weaker Ca^{2+} inactivation (Richter *et al.*, 1997). In addition, exon 17 forms part of a dantrolene binding sequence. Paul-Pletzer *et al.* (2002) indicated that the dantrolene binding site on the RyR1 protein is composed of amino acids 590 - 609, which demonstrates that this region of the RyR1 plays an important role in the regulation of channel function.

In Phase 1 of the MH research programme, several individuals were analysed for the Arg614Cys alteration via an RFLP strategy. In the study presented here (Phase 3), all 15 samples obtained from South African probands were analysed for reported or novel alterations that may occur in the amplified region. Amplification of the 1004 bp region for all 15 MHS probands was conducted as described in Section 4.2 (page 159) and the results of the amplified PCR product encompassing exons 17 and 18 are depicted in Figure 4.43.

Figure 4.43: Photographic representation of amplified PCR products encompassing exons 17 and 18



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 fragments and MM appeared distorted. Variation in amplification efficiency and background smear were observed, as discussed in Sections 4.2 and 4.3.

All 15 MH probands were subsequently sequenced in order to investigate the presence of novel or reported alterations that may occur in the amplified region. Sequencing was conducted using the standard protocol as discussed in Section 3.7 (page 94). The forward primer (RYRex17F) was used in the sequencing reaction. A representative electropherogram obtained for individual MH00242 depicting the nucleotide positions of the previously reported alterations observed in exon 17, is illustrated in Figure 4.44.

Figure 4.44: Representative electropherogram of exon 17 indicating the nucleotide positions of the Arg614Cys and Arg614Leu alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Arg614Cys at nucleotide 1840 and Arg614Leu at nucleotide 1841.

4.7.10.1 Synonymous substitutions in the amplified region of exons 17 and 18 of the RYR1 gene

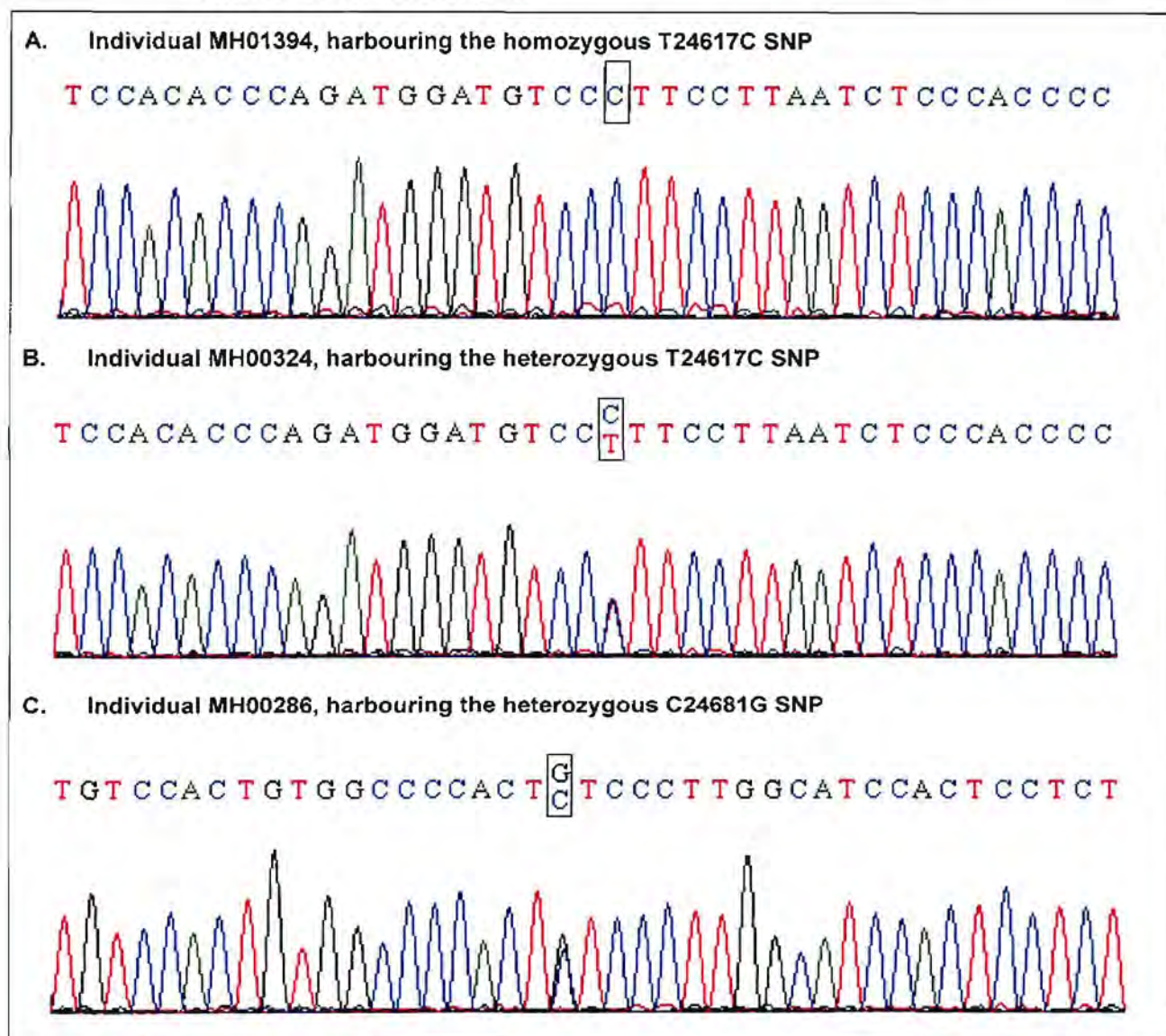
Two SNPs were identified within the intron sequence between exons 17 and 18. The first SNP occurs due to a T24617C transition, and was observed in 11 South African MH probands, as listed in Appendix C (page 451). The SNP was heterozygous in eight analysed probands and homozygous in three individuals. The synonymous substitution is indicated as a SNP of the RYR1 gene in Genbank® (International Human Genome Sequencing Consortium, 2004; with accession number rs2071085). In addition to the above-mentioned SNP, a novel heterozygous C24681G SNP was also observed within this region of the RYR1 gene, as listed in Appendix C (page 451).

4.7.10.1.1 SNP T24617C

The genotype and allele frequencies worldwide for the T24617C have been determined and vary between the different populations. In most cases the C/C and C/T genotypes are

the most common. The frequency of the SNP in the European population was 0.43 for the C/C genotype, 0.50 for the C/T genotype and 0.07 for the T/T genotype (International Human Genome Sequencing Consortium, 2004; with accession number rs2071085). In the Asian population, the genotype frequency was as follows: 0.36 for C/C, 0.51 for C/T and 0.13 for T/T (International Human Genome Sequencing Consortium, 2004; with accession number rs2071085). However, genotype data were not available for the population from Sub-Saharan Africa (International Human Genome Sequencing Consortium, 2004; with accession number rs2071085). Figure 4.45 depicts the sequence generated for the homozygous T24617C and the heterozygous T24617C SNP, respectively.

Figure 4.45: Representative electropherograms indicating the T24617C and C24681G SNPs observed in the intron sequence between exons 17 and 18 of the RYR1 gene



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

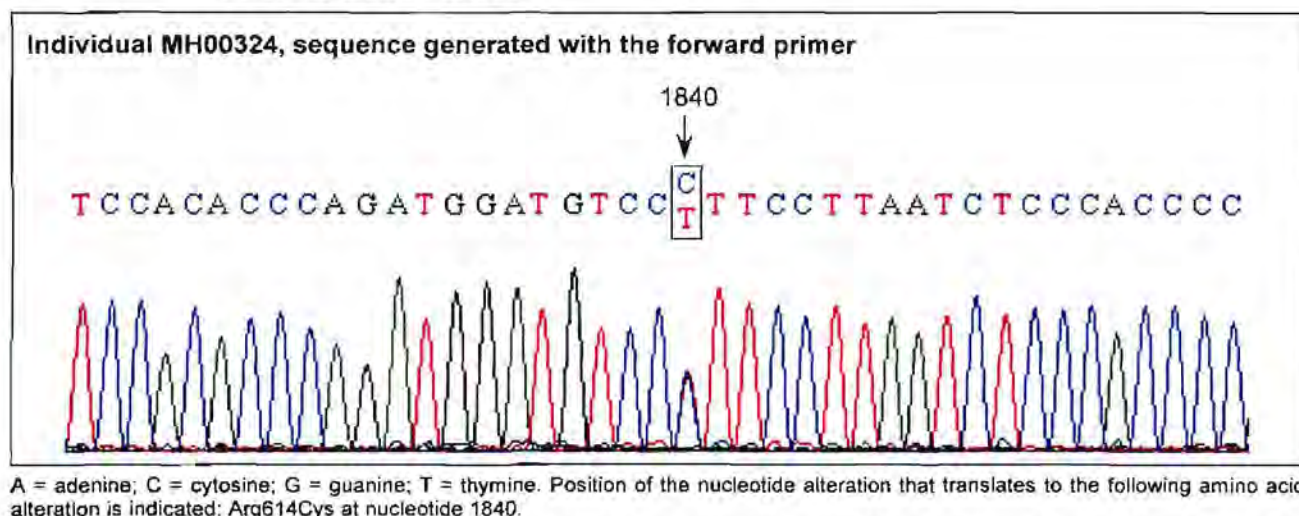
4.7.10.1.2 SNP C24681G

A novel SNP was identified in five South African MH individuals, as listed in Appendix C (page 451) and occurs due to a C24681G transition. Figure 4.45C depicts the sequence generated for the heterozygous C24681G SNP. Cargill *et al.* (1999) identified several SNPs that were confined to a specific population. Due to the small sample size, the observation of a novel SNP in the South African MH cohort does not necessarily imply that it is unique to this population. However, this observation may provide information about population substructure. A novel SNP that is only observed in the South African population may indicate that it occurred after a group of European settlers colonised South Africa during the seventeenth century (Saunders, 1983). However, if the SNP is observed in the European population, this may indicate that the group of European settlers that colonised South Africa harboured the SNP. Further analysis of the C24681G SNP in the European population would verify which of the above-mentioned statements are correct. As discussed in Section 4.7.4.1 (page 185), the SNPs observed in this region may play a role in susceptibility to MH due to epistasis.

4.7.10.2 The Arg614Cys alteration in the RYR1 gene

In Phase 1 of the ongoing MH research programme, 39 individuals from family MH105 were screened for the presence of the Arg614Cys mutation via RFLP (Olckers *et al.*, 1994; Havenga, 2000). Previously, family MH105 has been reported to demonstrate linkage to chromosome 19 (Olckers, 1993). The Arg614Cys alteration was first observed in the proband, MH105-38, and was later identified in an additional 12 individuals from this family (Olckers *et al.*, 1994; Havenga, 2000). The proband was sequenced in the study presented here, in order to verify the RFLP results. None of the other 14 probands analysed in Phase 3 harboured any novel or reported alterations in exon 17. As presented in Figure 4.46, at nucleotide position 1840, two peaks of similar intensity representing two different nucleotides, i.e. C and T, were observed in one sample analysed in this study. This correlates to two distinct codons that result in the expression of two different amino acids, namely Arg and Cys, respectively.

Figure 4.46: Representative electropherogram indicating the Arg614Cys alteration observed in exon 17



The Arg614Cys alteration exhibited phenotype-genotype discordance in the South African family. One individual, MH105-64, was diagnosed as MHS via the IVCT but did not inherit the alteration, whereas MH105-23 that was diagnosed as MHN was observed to harbour the alteration. With the exception of these two individuals, the Arg614Cys alteration was observed to segregate with the IVCT phenotype in this five-generation family (Olckers *et al.*, 1994; Havenga, 2000). Fagerlund *et al.* (1997) reported discordance between MHS and the Arg614Cys alteration in two Scandinavian MH families. In addition, discordance has been reported between the IVCT result and the Arg614Cys alteration in other populations (Deufel *et al.*, 1992; Fortunato *et al.*, 1999; Hopkins, 2000). Monnier *et al.* (2005) reported that the discordance rate among French individuals was 3.1%. However, inclusion of IVCT MHE data as a clinically at-risk group increased the frequency of discordance to 19.4%. The high rate of discordance is a concern for the development of diagnostic genetic testing. Discordance could be due to:

- a. The identified mutation not being causative.
- b. The presence of two independent genetic traits.
- c. Mutation penetrance, genetic background and environmental effects contributing to the discordance.
- d. False negative or false positive diagnosis via the IVCT (Robinson *et al.*, 2003a).

Functional analysis in the skeletal muscle of rabbits has indicated that the Arg614Cys alteration results in both increased sensitivity to activation by Ca^{2+} and an increased rate of Ca^{2+} release compared to that of SR vesicles obtained from unaffected muscle (Mickelson *et al.*, 1990). Therefore, the causative status of this mutation remains

unchallenged. Family MH105 demonstrated linkage only to chromosome 19 (Olckers, 1993) and in the study presented here, was not observed to harbour any additional RYR1 alterations, as indicated in Table 4.17 (page 387). Therefore, this may indicate that an additional independent genetic trait may be responsible for the MH phenotype in family MH105. However, this does not exclude the possibility of susceptibility to MH in this family occurring due to epistasis, in which SNPs contribute to the MH phenotype, which may account for, or modify the observed IVCT response, as discussed in Section 5.5.2.1 (page 408). In addition, the report of discordance in these individuals may indicate that the IVCT diagnosis was either a false positive in the MHS individual and a false negative in the MHN individual. It has been reported that IVCT contracture response varies with mutation genotype (Tong *et al.*, 1997). The Arg614Cys alteration has been associated with a weaker contracture (Tong *et al.*, 1997). Therefore, as the threshold used to indicate a positive result in the IVCT has an impact on the status of genetic linkage (Girard *et al.*, 2001; Robinson *et al.*, 2002), alterations associated with weaker contractures may exhibit a higher discordance rate than those associated with more severe contracture phenotypes.

4.8 HOTSPOT TWO OF THE RYR1 GENE

In the study presented here, analysis of exons 39 to 46 led to the detection of several SNPs and two alterations, Arg2336His and Phe2364Val, in hotspot two of the RYR1 gene. As discussed in Section 2.11.3.3.2 (page 52), the central domain of the RyR1 interacts with the N-terminal domain, which is stabilised in the presence of the drug dantrolene (George *et al.*, 2004). Alterations in the second hotspot may result in the disruption of inter-domain interactions that stabilise the closed state (McCarthy and Mackrill, 2004). As discussed in Section 2.9.4 (page 28), hotspot two harbours a DHPR II - III loop binding domain (Proenza *et al.*, 2000) and an FKBP12 interacting domain. As several alterations that may be associated with MH have been reported to occur in this region of the RYR1 gene and since this region of the gene harbours several functional domains, the entire coding region of hotspot two of the RYR1 gene was analysed. Prior to the study reported here, analysis of all exons in hotspot two of the RYR1 gene has never been conducted in the South African population.

4.8.1 Exon 39 of the RYR1 gene

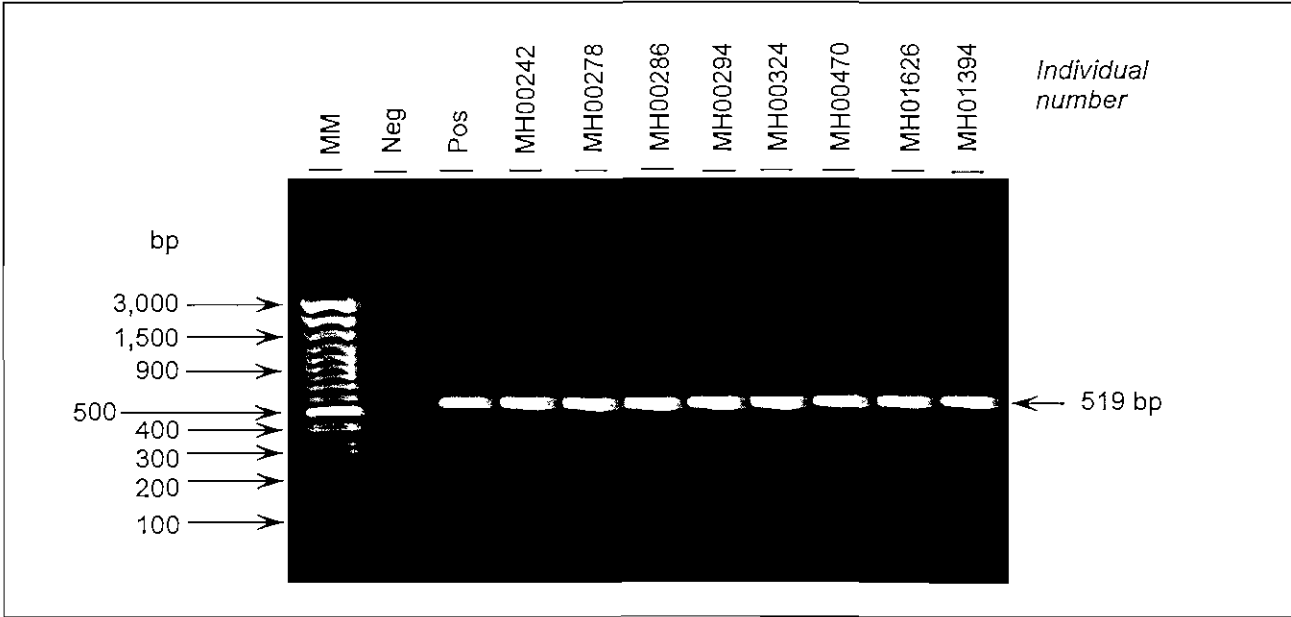
PCR was used to amplify a 519 bp product, which included exon 39. The central region of the RYR1 gene harbours eight reported alterations, as listed in Table 4.4. The mutations are clustered in the central portion of the gene, in the second mutation hotspot. As discussed in Sections 2.9.4 (page 28) and 2.11.3.3.2 (page 52), this region of the RYR1 gene was selected as it plays an important role in E-C coupling and harbours a phosphorylation site at Ser2843 (Suko *et al.*, 1993).

Table 4.4: Reported alterations in exon 39 of the RYR1 gene

Amino acid change	Nucleotide change	Reference	Amino acid change	Nucleotide change	Reference
Met2101Leu	A6302C	Tammaro <i>et al.</i> , 2003	Arg2163Pro	G6488C	Fortunato <i>et al.</i> , 2000
Val2117Leu	G6349C	Tammaro <i>et al.</i> , 2003	Arg2163His	G6488A	Manning <i>et al.</i> , 1998a
Asp2129Glu	C6387G	Rueffert <i>et al.</i> , 2001	Val2168Met	G6502A	Manning <i>et al.</i> , 1998a
Arg2163Cys	C6487T	Manning <i>et al.</i> , 1998a	Ile2182Phe	A6504T	Rueffert <i>et al.</i> , 2001

In Phase 1, three alterations, i.e. Arg2163Cys, Arg2163His and Val2168Met, were analysed in 38 individuals, using RFLP and automated sequencing strategies. An additional ten MH probands were screened for these alterations during Phase 2. In Phase 3, a new forward primer (RYRex39bF) was designed, as listed in Table 3.2 (page 86), in order to screen the entire exon in 15 South African MH probands. PCR conditions as described in Section 4.2 (page 159) were used. Figure 4.47 is a photographic representation of the amplicon encompassing exon 39.

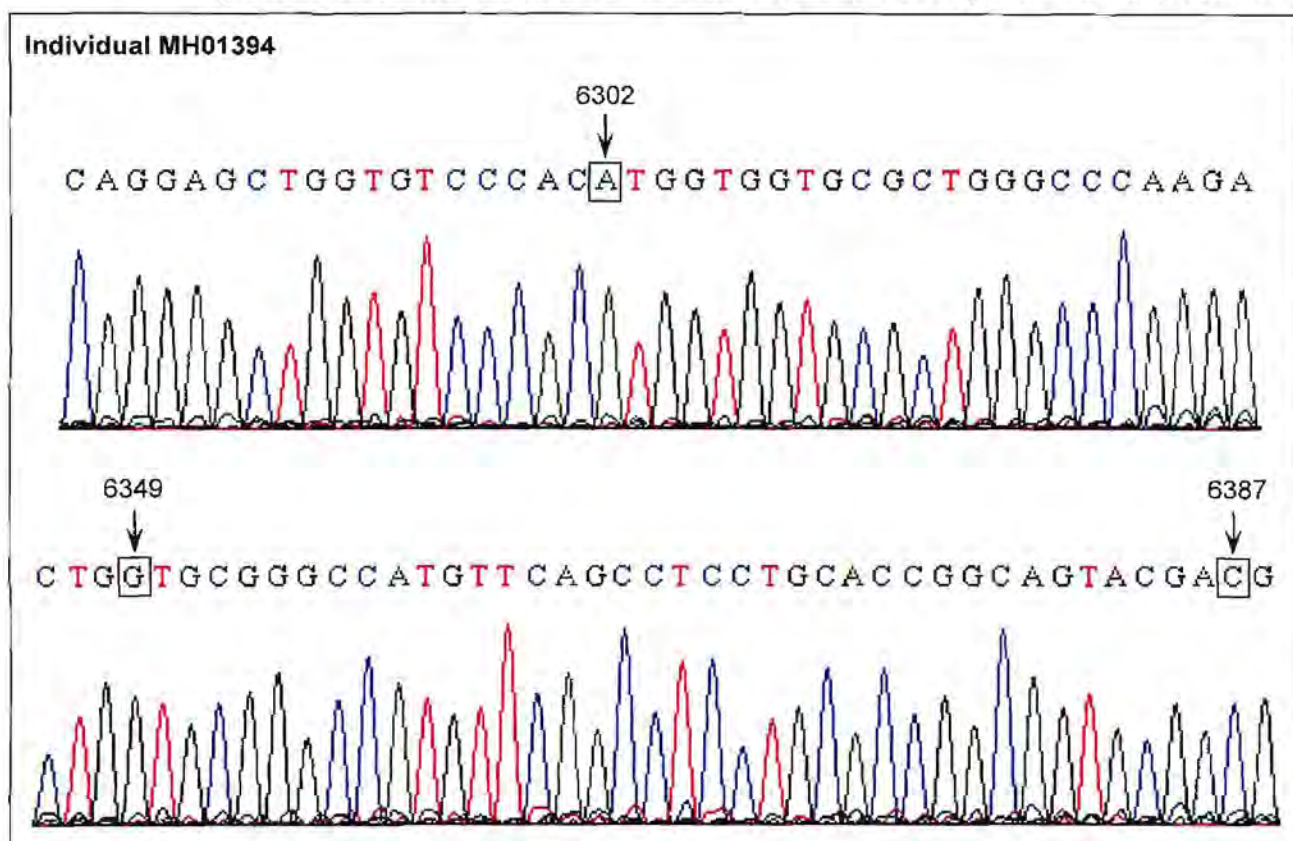
Figure 4.47: Photographic representation of amplified PCR products encompassing exon 39



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 fragments appeared distorted.

PCR products obtained from all 15 samples were purified and sequenced, in order to investigate for the presence of the eight reported mutations as well as to identify possible novel mutations that may be present in this region of the RYR1 gene. Sequencing was conducted using the forward primer (RYRex39bF), according to the standard protocol. A representative electropherogram obtained for individual MH01394, depicting the nucleotide positions of previously reported alterations Met2101Leu, Val2117Leu and Asp2129Glu observed in exon 39, is indicated in Figure 4.48.

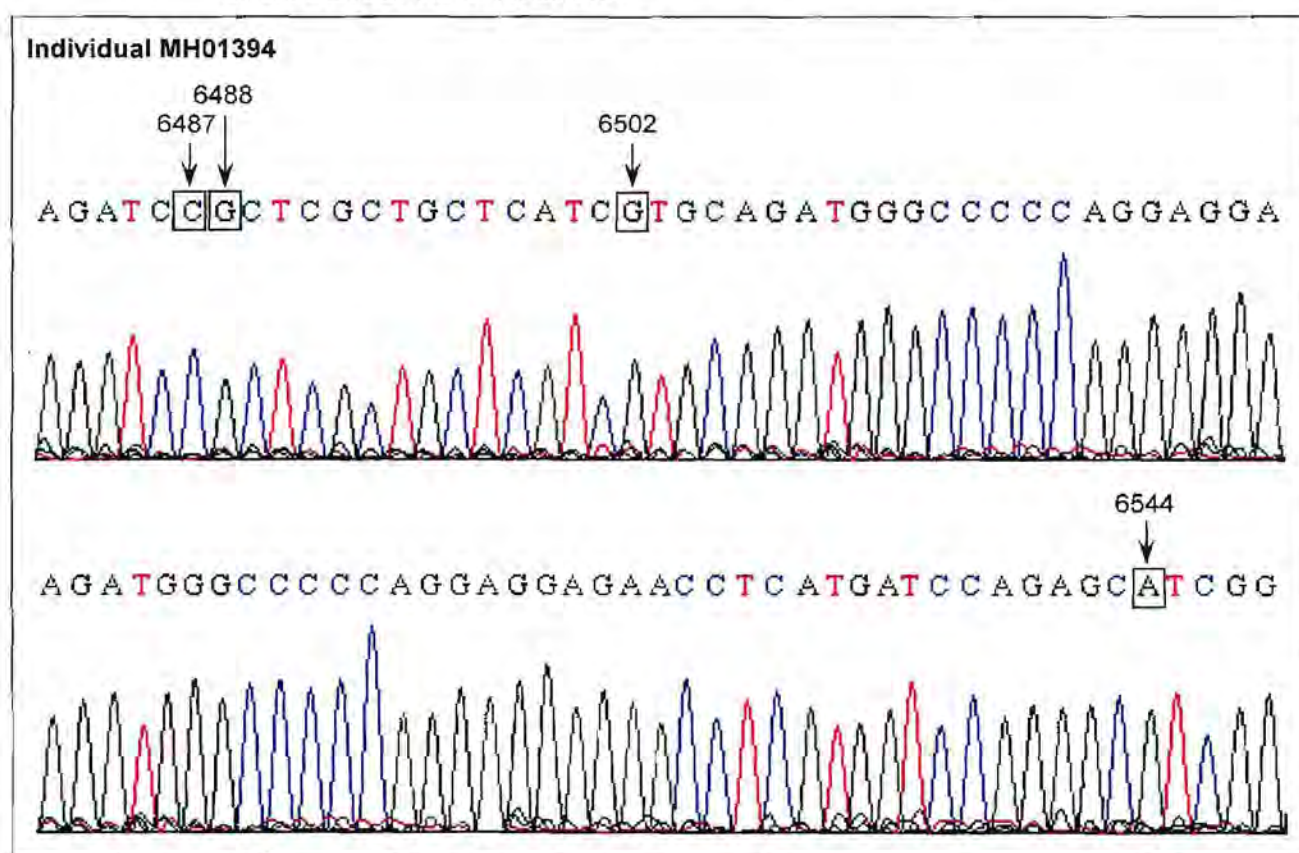
Figure 4.48: Representative electropherogram of exon 39 indicating the nucleotide positions of the Met2101Leu, Val2117Leu and Asp2129Glu alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Met2101Leu at nucleotide 6302; Val2117Leu at nucleotide 6349 and Asp2129Glu at nucleotide 6387. Background peaks were observed for sequence results obtained for the amplified region of exon 39 in all 15 samples, as discussed in Section 4.5.

None of the 15 MHS probands analysed in Phase 3 harboured any reported or novel alterations in exon 39 of the RYR1 gene. Several alterations have been reported to occur in this region of the RYR1 gene. Five of the alterations, i.e. Val2117Leu, Asp2129Glu, Met2101Lys, Arg2163Pro and Ile2182Phe, are likely to be family-specific, as they have to date only been reported in single families (Fortunato *et al.*, 2000; Rueffert *et al.*, 2001; Rueffert *et al.*, 2002; Tammaro *et al.*, 2003). A representative electropherogram obtained for individual MH01394, illustrating the nucleotide positions of alterations Arg2163Cys, Arg2163Pro, Arg2163His, Val2168Met and Ile2182Phe previously reported in exon 39, is depicted in Figure 4.49.

Figure 4.49: Representative electropherogram of exon 39 indicating the nucleotide positions of the Arg2163Cys, Arg2163Pro, Arg2163His, Val2168Met and Ile2182Phe alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Arg2163Cys and Arg2163Pro at nucleotide 6487; Arg2163His at nucleotide 6488; Val2168Met at nucleotide 6502 and Ile2182Phe at nucleotide 6544.

Three alterations, namely Arg2163Cys, Arg2163His and Val2168Met, have been reported to occur in several families. Functional analysis of all three of these alterations via recombinant *in vitro* expression on a defined genetic background or via assays of RYR1 function in *ex vivo* tissues (Tong *et al.*, 1997), as discussed in Section 5.6 (page 413), has indicated that they result in a modification in function of the mutated protein (Tong *et al.*, 1997; Yang *et al.*, 2003), as listed in Appendix A (page 443). Therefore, alterations Arg2163Cys, Arg2163His and Val2168Met have been classified as causative, as they all comply with the criteria set out by the EMHG (Halsall and Robinson, 2004). Manning *et al.* (1998a) identified the Arg2163Cys alteration in two unrelated families from Europe. A subsequent study conducted by Robinson *et al.* (2003b) reported the Arg2163Cys alteration in one MH family from Belgium, one MH family from Germany and one MH family from the UK. In addition, Brandt *et al.* (1999) observed the alteration in one German family. Manning *et al.* (1998a) suggested that the mutation was either very common in the European population or arose through a founder effect.

The Arg2163His alteration has been observed in families originating from North America (Sambuughin *et al.*, 2005), Italy (Galli *et al.*, 2002; Tammaro *et al.*, 2003) and Europe (Manning *et al.*, 1998a; Robinson *et al.*, 2002). The Val2168Met alteration has been reported in eight MH families from Switzerland and in families of Italian, European, French, German and Taiwanese descent (Manning *et al.*, 1998b; Brandt *et al.*, 1999; Tammaro *et al.*, 2003; Monnier *et al.*, 2005; Yeh *et al.*, 2005). These three alterations, as well as a fourth alteration, Thr2206Met, collectively account for 11% of MH cases (Manning *et al.*, 1998a). Therefore, it could be concluded that these are not family-specific alterations. As the alterations Arg2163Cys, Arg2163His and Val2168Met have been reported in several families from several populations, it is likely that they play a role in the development of MH in the South African population. This observation is supported by previous findings (Neumann, 2002). The Val2168Met alteration has previously been reported in a single MH proband from South Africa (Neumann, 2002), that was not included in the study presented here. However, the Arg2163Cys, Arg2163His and Val2168Met alterations are not responsible for the MH phenotype in any of the 15 MH probands analysed in the study presented here, but may be present in other South African probands diagnosed with MH.

4.8.2 Exon 40 of the RYR1 gene

Exon 40 harbours six known alterations that have previously been identified in patients diagnosed with MH, as listed in Table 4.5. All six alterations reside in the central portion of the RYR1 gene, which represents the second mutation hotspot of the RYR1.

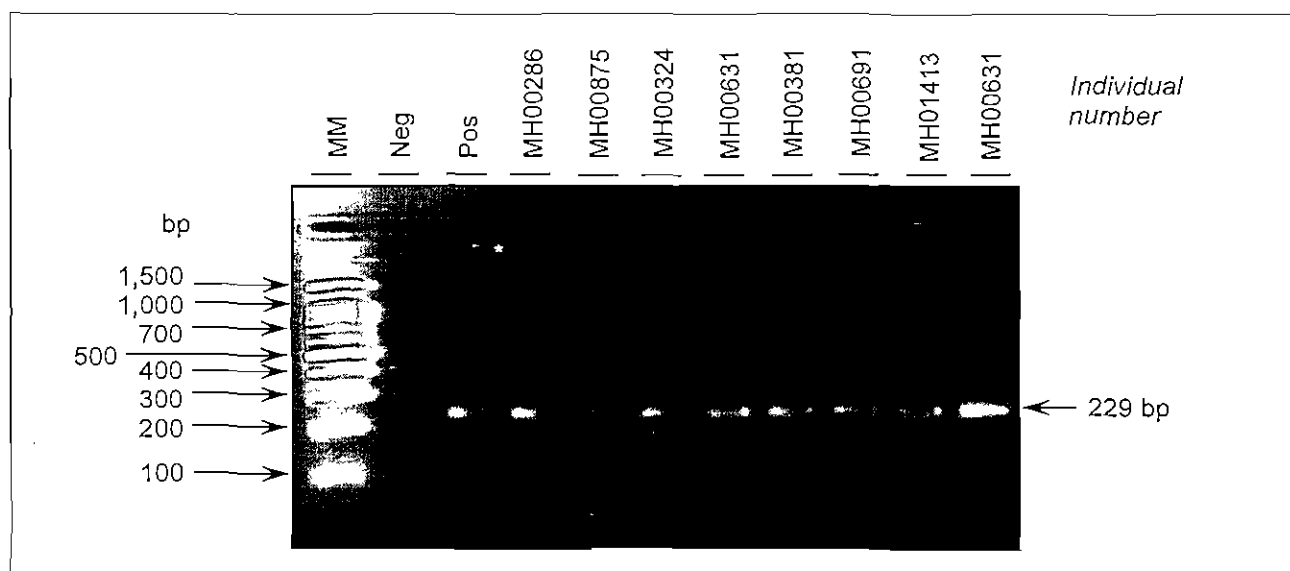
Table 4.5: Reported alterations in exon 40 of the RYR1 gene

Amino acid change	Nucleotide change	Reference	Amino acid change	Nucleotide change	Reference
Ala2200Val	C6599T	Halsall and Robinson, 2004	Val2210Phe	G6628T	Sambuughin <i>et al.</i> , 2005
Thr2206Met	C6617T	Manning <i>et al.</i> , 1998b	Val2212Asp	T6635A	Galli <i>et al.</i> , 2006
Thr2206Arg	C6617G	Brandt <i>et al.</i> , 1999	Val2214Ile	G6640A	Sambuughin <i>et al.</i> , 2001b

Exon 40 has previously been screened for reported alterations in South African MH probands. In Phase 1, 42 individuals were analysed for the Thr2206Met and Thr2206Arg alterations via sequencing or RFLP strategies (Havenga, 2000; Neumann, 2002). In Phase 2, an additional 13 individuals were sequenced in order to detect reported alterations as well as to identify novel mutations that may occur in the amplified region (Dalton, 2004). In the study presented here, five MH probands were analysed for

alterations that may occur in exon 40. PCR conditions were optimised, as discussed in Section 4.2 (page 159). Results obtained for PCR amplification are depicted in Figure 4.50.

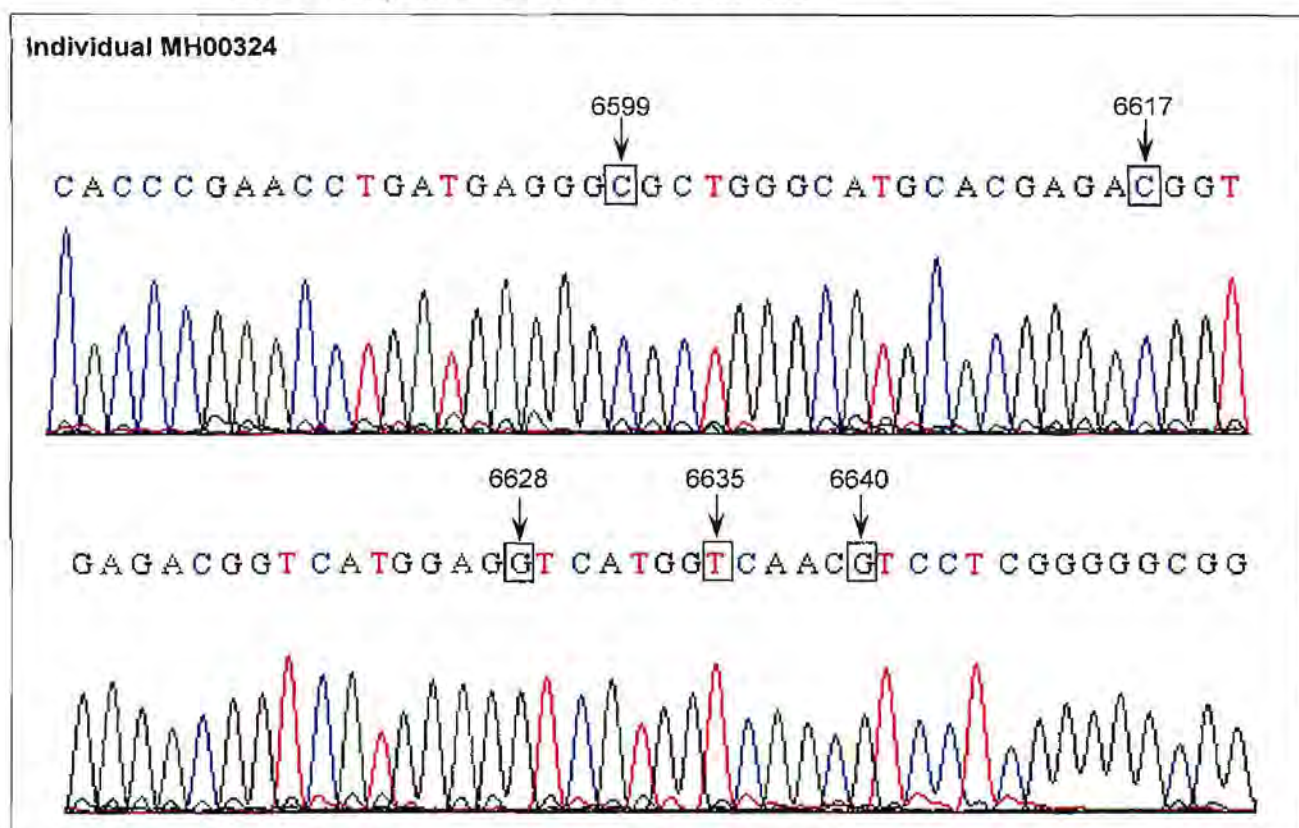
Figure 4.50: Photographic representation of amplified PCR products encompassing exon 40



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 (*), distortion of amplified fragments, distortion of the MM and variation in amplification efficiency were observed, as discussed in Sections 4.2 and 4.3.

Following PCR amplification the PCR product was purified and sequenced using the reverse primer (RYRE40R). The sequence is therefore depicted as the reverse complement. Figure 4.51 is a representative electropherogram obtained for individual MH00324, indicating the nucleotide positions of alterations previously reported to occur in exon 40.

Figure 4.51: Representative electropherogram of exon 40 indicating the nucleotide positions of the Ala2200Val, Thr2206Met, Thr2206Arg, Val2210Phe, Val2212Asp and Val2214Ile alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Ala2200Val at nucleotide 6599; Thr2206Met and Thr2206Arg at nucleotide 6617; Val2210Phe at nucleotide 6628; Val2212Asp at nucleotide 6635 and Val2214Ile at nucleotide 6640. Low level background peaks were observed, as discussed in Section 4.5.

The six known alterations were not observed in any of the MH individuals analysed in Phase 1, 2 or 3. In addition, novel alterations that may be associated with the MH phenotype were also not observed in the amplified region. Alterations Ala2200Val, Val2210Phe, Val2212Asp and Val2214Ile have thus far only been observed in single families. This may indicate that they are unique to those specific families (Sambuughin *et al.*, 2001a; Halsall and Robinson, 2004; Sambuughin *et al.*, 2005; Galli *et al.*, 2006).

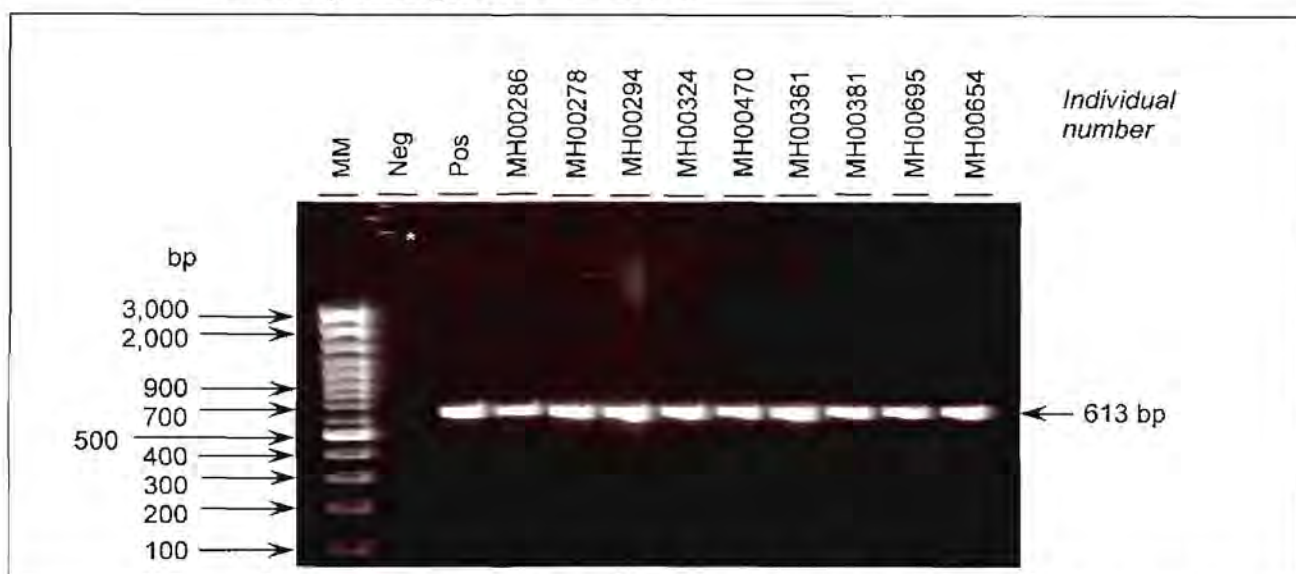
The Thr2206Met alteration has been reported in several populations, including North American (Sambuughin *et al.*, 2001b, Sei *et al.*, 2004; Sambuughin *et al.*, 2005), Italian (Galli *et al.*, 2002), European (Manning *et al.*, 1998b), French (Monnier *et al.*, 2005) and German (Brandt *et al.*, 1999). In addition, the Thr2206Arg alteration has been reported in the European population (Manning *et al.*, 1998a) and in probands from Taiwan (Yeh *et al.*, 2005). Both alterations have been reported as causative of MHS (Wehner *et al.*, 2002; Monnier *et al.*, 2005) and a summary of the functional characterisation studies conducted for these alterations is listed in Appendix A (page 443). As South African probands in the

cohort analysed in the study presented here are Caucasian and of Western European descent (Saunders, 1983), it would be expected that alterations previously identified in certain European populations would be observed in the South African population. The absence of the Thr2206Met and Thr2206Arg alterations may be due to the small sample size included in the study presented here. Collins and Schwartz (2002) indicated the number of chromosomes that should be analysed when searching for alterations in disease genes. The authors indicated that in order to detect alterations that may occur in 1% of the population with 95% power, a minimum of 340 chromosomes should be examined. For an alteration that occurs in 0.1% of the population, a minimum of 3,910 chromosomes should be screened in order to obtain 95% power. Therefore, alterations that may be associated with MH may not be detected if a small number of individuals are screened for the alteration. The study presented here includes the largest number of South African probands screened for novel and reported alterations in the coding region of the RYR1 gene, to date. Unfortunately, screening of the entire coding region of the RYR1 gene via a sequencing strategy is not cost-effective. Denaturing high performance liquid chromatography (DHPLC) is an advancement of technology that has been suggested for the diagnosis of MH as it has many advantages, including high sensitivity, speed and cost-effectiveness, but it requires large capital outlays, as discussed in Section 5.5.4 (page 411), and is to this extent thus prohibitive in the present environment.

4.8.3 Exons 41 and 42 of the RYR1 gene

A 613 bp PCR product including exons 41 and 42 from hotspot two was amplified in order to identify novel and reported alterations that may occur in this region of the RYR1 gene. Thus far, alterations that may result in the MH phenotype have not been reported for exon 41. However, two alterations have been reported to occur in exon 42. Galli *et al.* (2002) reported a Val2280Ile alteration in one family with MH and Zhou *et al.* (2005) reported an Asn2283His alteration in a family diagnosed with CCD. The PCR reaction was optimised as described in Section 4.2 (page 159). Results obtained for PCR amplification are depicted in Figure 4.52.

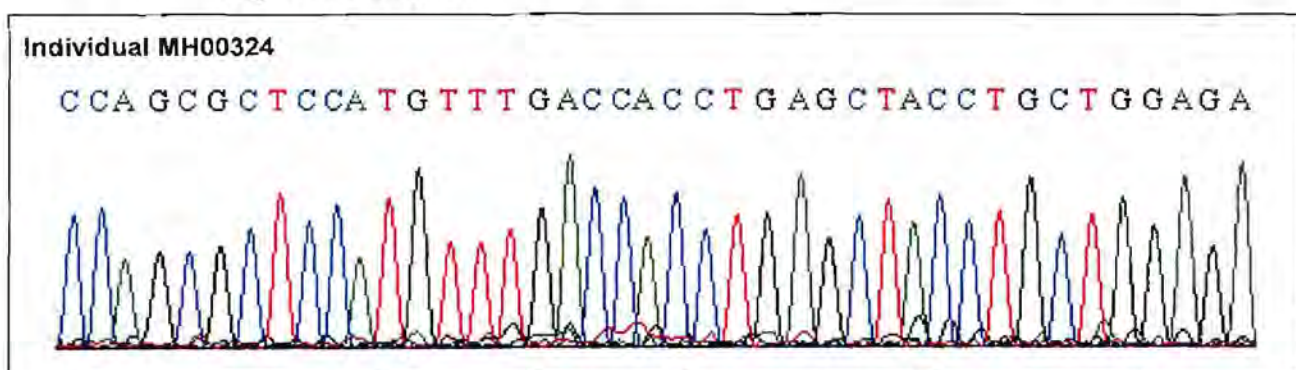
Figure 4.52: Photographic representation of amplified PCR products encompassing exons 41 and 42



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, fragment smear and fragment distortion were observed as discussed in Sections 4.2 and 4.3.

The PCR product was subsequently purified and all 15 MH probands were successfully sequenced using the reverse primer (RYRex41R). The sequence is therefore illustrated as the reverse complement. A representative electropherogram obtained for individual MH00324, illustrating a portion of the amplified region of exon 41, is indicated in Figure 4.53.

Figure 4.53: Representative electropherogram illustrating a portion of the amplified region of exon 41

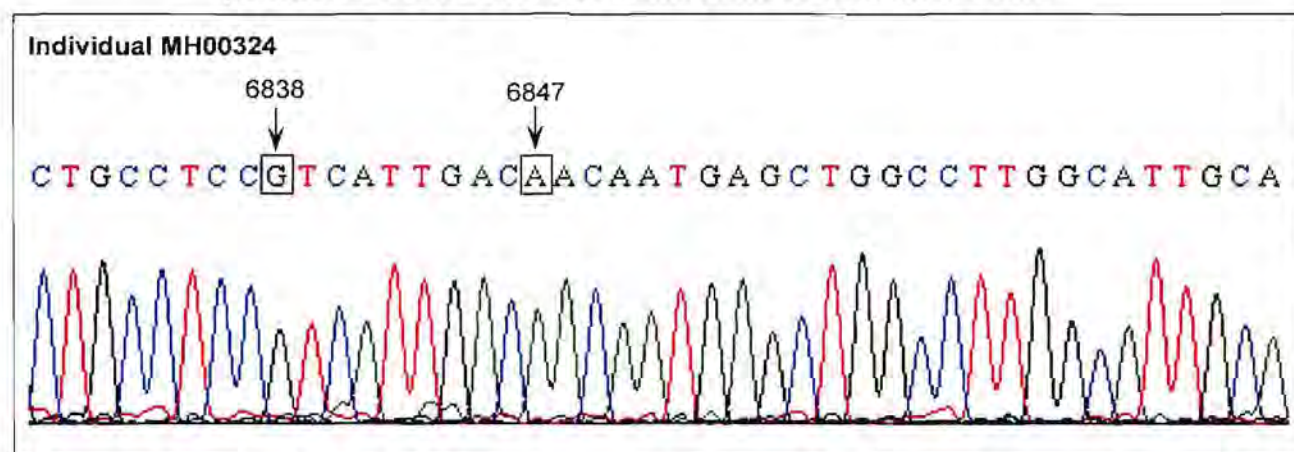


A = adenine; C = cytosine; G = guanine; T = thymine. As discussed in Section 4.5, low level background peaks were observed.

A representative electropherogram obtained for individual MH00324, illustrating the nucleotide positions of the Val2280Ile and Asn2283His alterations previously reported to occur in exon 41 of the RYR1 gene, is indicated in Figure 4.54. Thus far only one alteration that may be associated with the MH phenotype, namely Val2280Ile occurs in

this region of the RYR1 gene (Galli *et al.*, 2002). As this alteration has only been observed in a single family, it could indicate that it is specific to that family. In addition, the Asn2283His alteration (Zhou *et al.*, 2005) has thus far only been detected in a single family diagnosed with CCD. As none of the probands included in this study displayed the CCD phenotype, it would be expected that the alteration was not observed. In addition, the Asn2283His alteration has only been identified in a single family, thus it may represent a family-specific alteration.

Figure 4.54: Representative electropherogram of exon 42 indicating the nucleotide positions of the Val2280Ile and Asn2283His alterations

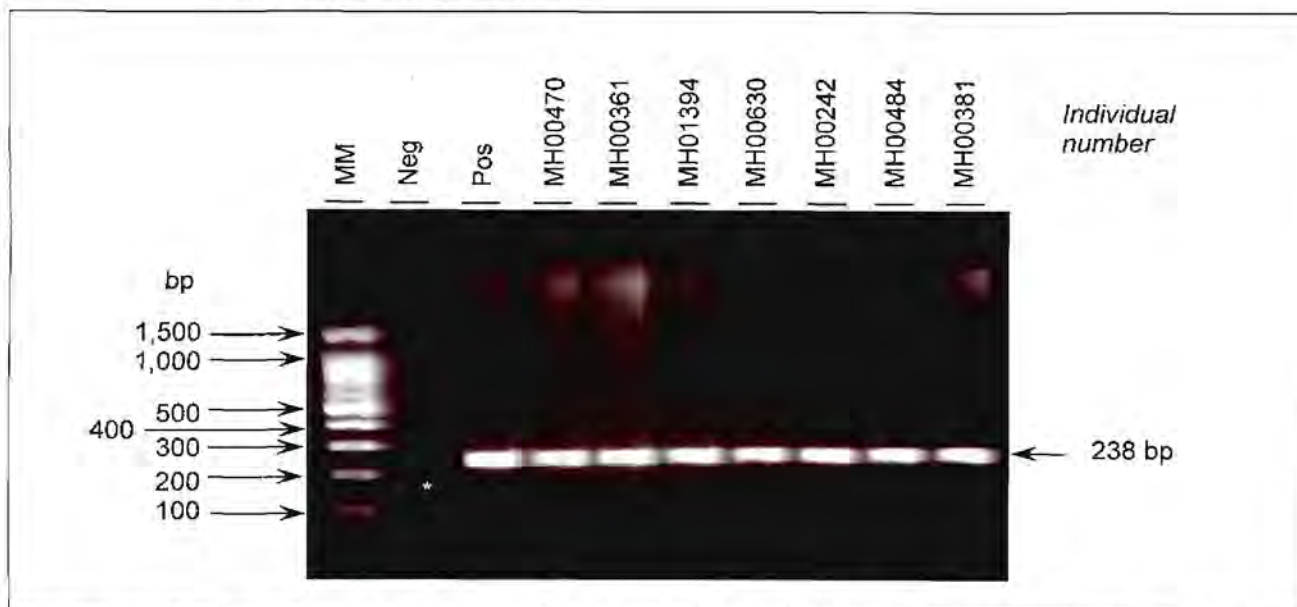


A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Val2280Ile at nucleotide 6838 and Asn2283His at nucleotide 6847.

4.8.4 Exon 43 of the RYR1 gene

Two alterations, Asn2342Ser and Arg2336Gln, have been reported to occur in exon 43 of the RYR1 gene. The alterations are located within the second mutational hotspot and occur due to an A7025G nucleotide substitution and a G7007A transition, respectively. The Asn2342Ser alteration has been identified in two UK MH families (Halsall and Robinson, 2004) and the Arg2336Gln alteration has been identified in two MH families from Italy (Galli *et al.*, 2006). Exon 43 was therefore analysed via sequencing in order to detect these alterations and other novel alterations that may occur in this region. A 238 bp region encompassing these mutations was amplified using optimised PCR conditions listed in Table 4.1 (page 161). The results are depicted in Figure 4.55. The PCR product was subsequently purified and sequenced according to the standard sequencing protocol.

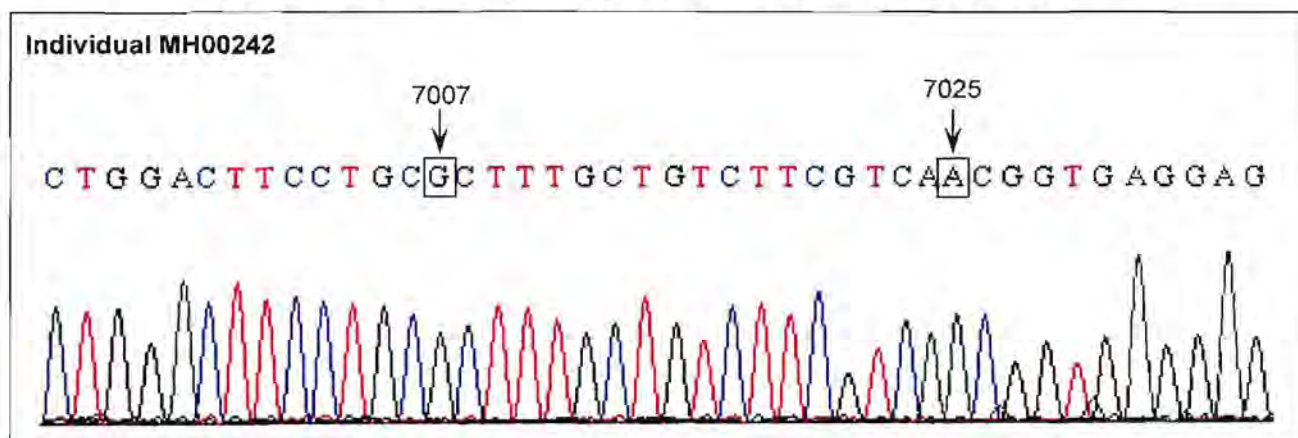
Figure 4.55: Photographic representation of amplified PCR products encompassing exon 43



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 following artefacts were observed on the gel namely, non-specific amplification, background smears, amplified fragment distortion, MM overloading and an artefact in the gel matrix, as indicated via the white asterisk (*).

Sequencing was conducted using the reverse primer (RYRex43R) and sequences are depicted as the reverse complement. A representative electropherogram obtained for individual MH00242, indicating the nucleotide positions of the reported Arg2336Gln and Asn2342Ser alterations in exon 43, is illustrated in Figure 4.56.

Figure 4.56: Representative electropherogram of exon 43 indicating the nucleotide positions of the Arg2336Gln and Asn2342Ser alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Arg2336Gln at nucleotide 7007 and Asn2342Ser at nucleotide 7025.

The reported Asn2342Ser alteration was not detected in any of the 15 South African probands analysed and may represent a family-specific alteration. However, the

Arg2336Gln alteration was observed in a single South African MH proband, as discussed in Section 4.8.4.1 (page 222).

4.8.4.1 The Arg2336His alteration in the RYR1 gene

An Arg2336His alteration, which occurs due to a G7007A nucleotide transition, was detected in a single South African MH proband, i.e. MH 108-1 (MH00470). The remaining MH probands analysed in the study presented here did not harbour this alteration. The variant affects a well conserved amino acid and occurs in a highly conserved region of the RyR1 protein, indicating that the alteration resides in a functionally significant region of the RyR1 protein. Table 4.6 depicts the partial amino acid sequences of exon 43 of the RyR 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). The position of the alteration detected in this study (Arg2336His) is indicated in red and the position of the previously detected alteration (Asn2342Ser) is indicated in blue.

Table 4.6: Conserved amino acids obtained from different RyR isoforms and species surrounding the novel and reported mutations in exon 43 of the RyR1 protein

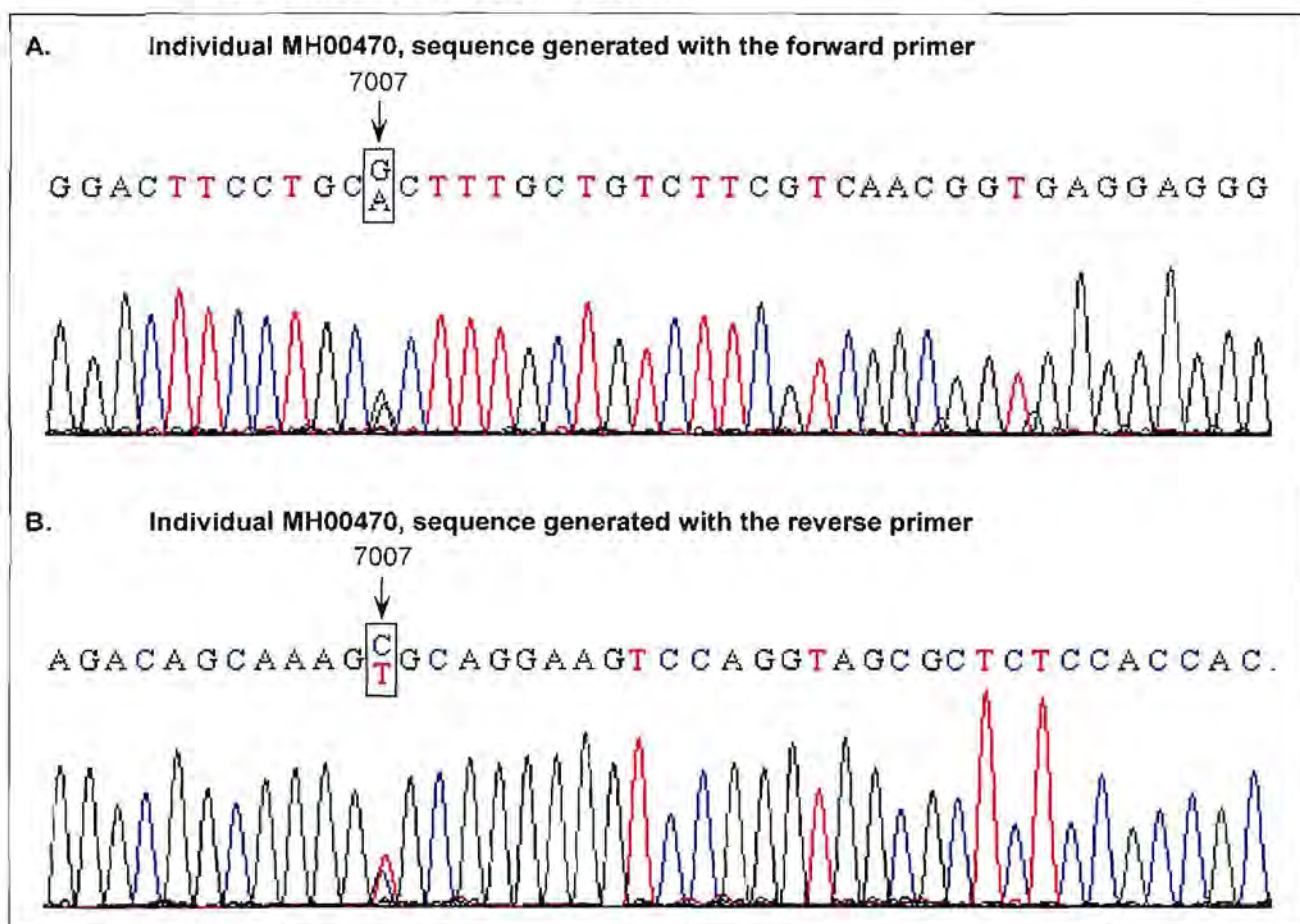
Isoform	Species	Alignment of RyR protein sequences
RyR1	Human	GCGLQSC PMLVAKGYPD IGWNPCGGER YLD ~~FL~~ RF AVF VNGESVEENA NVVVRL ~~L~~ IRK
RyR2	Human	GCGLQSC QMLVSKGYPD IGWNPVEGER YLD ~~FL~~ RF AVF CNGESVEENA NVVVRL ~~L~~ IRR
RyR3	Human	GCGLQSC PMLLAKGYPD VGWNPPIEGEY YLS ~~FL~~ RF AVF VNSSESVEENA SVVVKLLIRR
<i>ryr1</i>	Pig	GCGLQSC PMLLAKGYPD IGWNPCGGER YLD ~~FL~~ RF AVF VNGESVEENA NVVVRL ~~L~~ IRK
<i>ryr1</i>	Rabbit	GCGLQSC PMLLAKGYPD IGWNPCGGER YLD ~~FL~~ RF AVF VNGESVEENA NVVVRL ~~L~~ IRK

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 Arg amino acid at nucleotide 7007 is indicated in red and the site of the conserved reported Asn amino acid at nucleotide 7025 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.

As presented in Figure 4.57, at nucleotide position 7007, two peaks of similar intensity representing two different bases, namely G and A, were detected. The two different nucleotides correlated to two distinct codons, which code for different amino acids, Arg and His, respectively. As the Arg2336His alteration was detected in individual MH00470, four other family members, i.e. MH108-2 (MH00471), MH108-3 (MH00472), MH108-4 (MH00473) and MH108-5 (MH00474), were subsequently screened. The wife of the proband (MH00471) was diagnosed as MHN via the IVCT and did not harbour the alteration. The three children of the proband (MH00472, MH00473 and MH00474) were all diagnosed as MHS via the IVCT and all three individuals harboured this alteration.

Therefore, the alteration segregates with the MHS phenotype in the pedigree investigated here.

Figure 4.57: Representative electropherograms indicating the Arg2336His alteration observed in exon 43



A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Arg2336His at nucleotide 7007.

An Arg2336Gln alteration, which is due to a G7007A nucleotide transition, has recently been reported in two MH probands from Italy (Galli *et al.*, 2006). The authors incorrectly identified the Arg2336His alteration as an Arg2336Gln alteration, as the unaltered nucleotide sequence CGC encodes the amino acid Arg and the altered nucleotide sequence CAC encodes the amino acid His. The amino acid Gln would only occur if the nucleotide sequence was altered to either CAA or CAG. Therefore, the Arg2336His alteration observed in the study presented here and the Arg2336Gln alteration reported by Galli *et al.* (2006) actually refer to the same alteration. Galli *et al.* (2006) indicated that the mutation occurred in a highly conserved region of the RyR1 and that the amino acids were conserved across the three reported RyR proteins and among different species of *ryr1*. The alteration was also not detected in more than 100 chromosomes obtained from the Italian population.

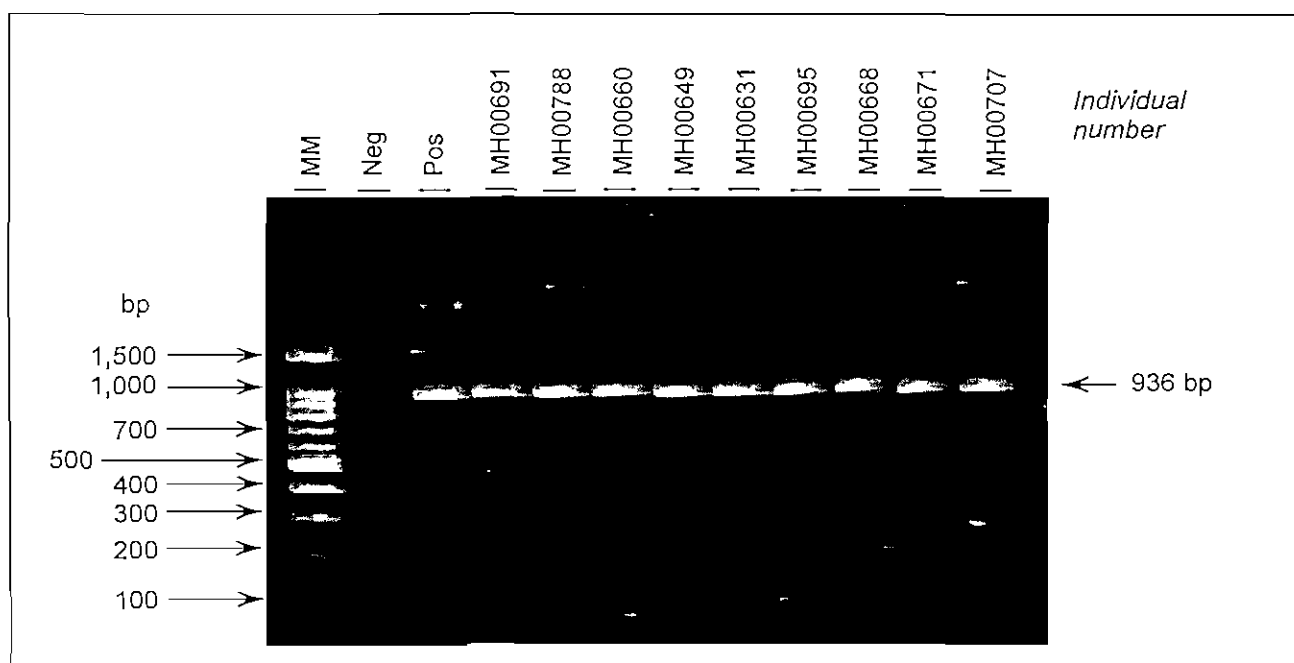
The alteration satisfies three out of four of the criteria set by the EMHG (Halsall and Robinson, 2004), as it occurs in a conserved region of the RyR1 protein, is absent from 100 controls (Galli *et al.*, 2006) and segregates with the MH phenotype in two unrelated families from Italy (Galli *et al.*, 2006) and in one family from South Africa (in the study presented here). However, the alteration will have to be functionally characterised via recombinant *in vitro* expression on a defined genetic background or via assays of RYR1 function in *ex vivo* tissues (Tong *et al.*, 1997) prior to its adoption in a predictive genetic test. Although the Arg2336His alteration was absent in 100 controls from Italy the alteration cannot be used in a predictive genetic test in South Africa unless it is absent in control individuals from South Africa. As discussed in Section 5.6 (page 413), because of the population-specific nature of alterations, ethnically and geographically matched controls should be used in the analysis (Collins and Schwartz, 2002) and the control population should be sufficiently large and adequately matched. In addition, the criteria set by the EMHG (Halsall and Robinson, 2004), indicate that the alteration may only be included in a predictive genetic test if it segregates with the MH phenotype in two unrelated pedigrees. However, as discussed in Section 5.6 (page 413), the size of the pedigree required to determine the genetic transmission of an alteration is not indicated. Burns *et al.* (1984) indicated that samples composed of three-generation pedigrees of intermediate size are required in order to test hypotheses about the mode of inheritance of an alteration.

The study presented here is the first survey in which the South African population was screened for alterations in exon 43, and represents the first description of this alteration in South Africa. If the alteration acquires causative status, as discussed in Section 5.6 (page 413), the detection of this transition in the South African population may have significant implications for the future development of a molecular diagnostic test for MH in South Africa. In addition, the alteration may contribute to the understanding of the aetiology of MH in the South African population.

4.8.5 Exons 44 and 45 of the RYR1 gene

A region of 936 bp was amplified, as discussed in Section 4.2 (page 159), and the results are depicted in Figure 4.58. The amplified product was subsequently sequenced in order to detect reported alterations, as well as to allow for the identification of possible novel mutations that may occur in this region of the RYR1 gene.

Figure 4.58: Photographic representation of amplified PCR products encompassing exons 44 and 45



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. Artefacts such as an artefact in the gel matrix, as indicated by the white asterisk (*), fragment distortion, overloaded MM and variation in amplification efficiency, were observed as discussed in Sections 4.2 and 4.3.

Sequencing was conducted using the reverse primer (Glu2434R) according to the standard protocol and the sequences are therefore depicted as the reverse complement. Thus far, 11 alterations have been reported in exon 44 and eight known alterations have been reported in exon 45, as listed in Table 4.7.

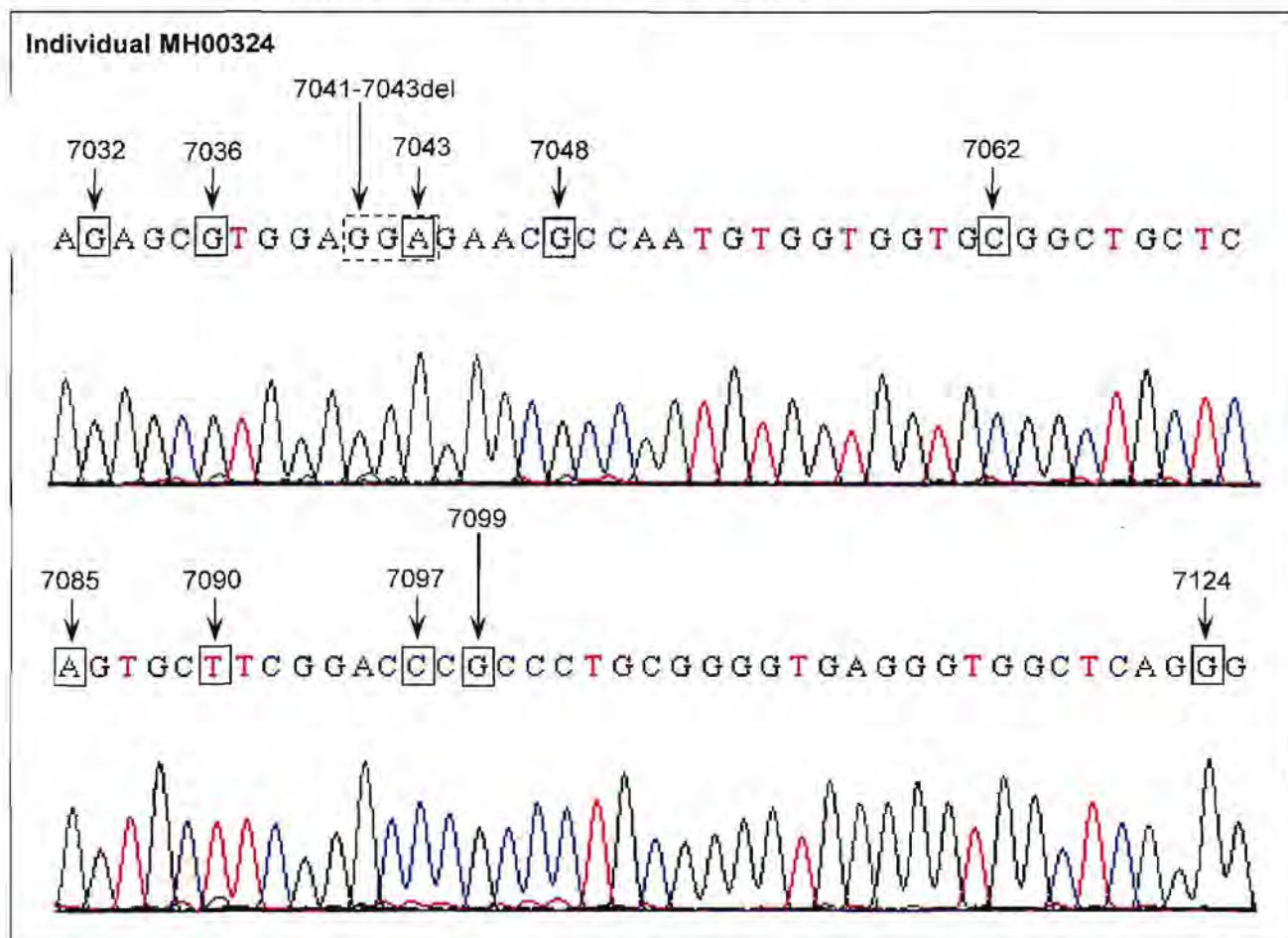
Table 4.7: Reported alterations in exons 44 and 45 of the RYR1 gene

Amino acid change	Nucleotide change	Exon	Reference
Glu2344Asp	G7032C	44	Monnier <i>et al.</i> , 2005
Val2346Met	G7036A	44	Halsall and Robinson, 2004
Glu2347del	7041delGGA	44	Sambuughin <i>et al.</i> , 2001c
Glu2348Gly	A7043G	44	Halsall and Robinson, 2004
Ala2350Thr	G7048A	44	Sambuughin <i>et al.</i> , 2001b
Arg2355Cys	C7062T	44	McWilliams <i>et al.</i> , 2002
Glu2362Gly	A7085G	44	Galli <i>et al.</i> , 2006
Phe2364Val	T7090G	44	Halsall and Robinson, 2004
Pro2366Arg	C7097G	44	Ibarra <i>et al.</i> , 2006
Ala2367Thr	G7099A	44	Sambuughin <i>et al.</i> , 2001a
Gly2375Ala	G7124C	44	Wehner <i>et al.</i> , 2004
Met2423Lys ¹	T7268A	45	Zhou <i>et al.</i> , 2005
Ala2428Thr	G7282A	45	Monnier <i>et al.</i> , 2005
Asp2431Asn	G7291A	45	Sambuughin <i>et al.</i> , 2001a
Gly2434Arg	G7300A	45	Keating <i>et al.</i> , 1994
Arg2435His	G7304A	45	Zhang <i>et al.</i> , 1993
Arg2435Leu ¹	G7304T	45	Barone <i>et al.</i> , 1999
Ala2436Val	C7310T	45	Galli <i>et al.</i> , 2006
Glu2439Asp	G7317C	45	Galli <i>et al.</i> , 2006

del = deletion, ¹ = indicates alterations observed in probands clinically diagnosed with CCD.

The presence of novel and reported alterations in exon 44 was previously investigated in South African MH probands in Phase 1 and Phase 2. In Phase 3, an additional four MH probands (MH00324, MH00361, MH01394 and MH01626) for whom sequence data were not available were analysed for alterations that may occur in this region of the RYR1. A representative electropherogram obtained for individual MH00324, depicting the nucleotide positions of alterations that have previously been reported in exon 44, is illustrated in Figure 4.59.

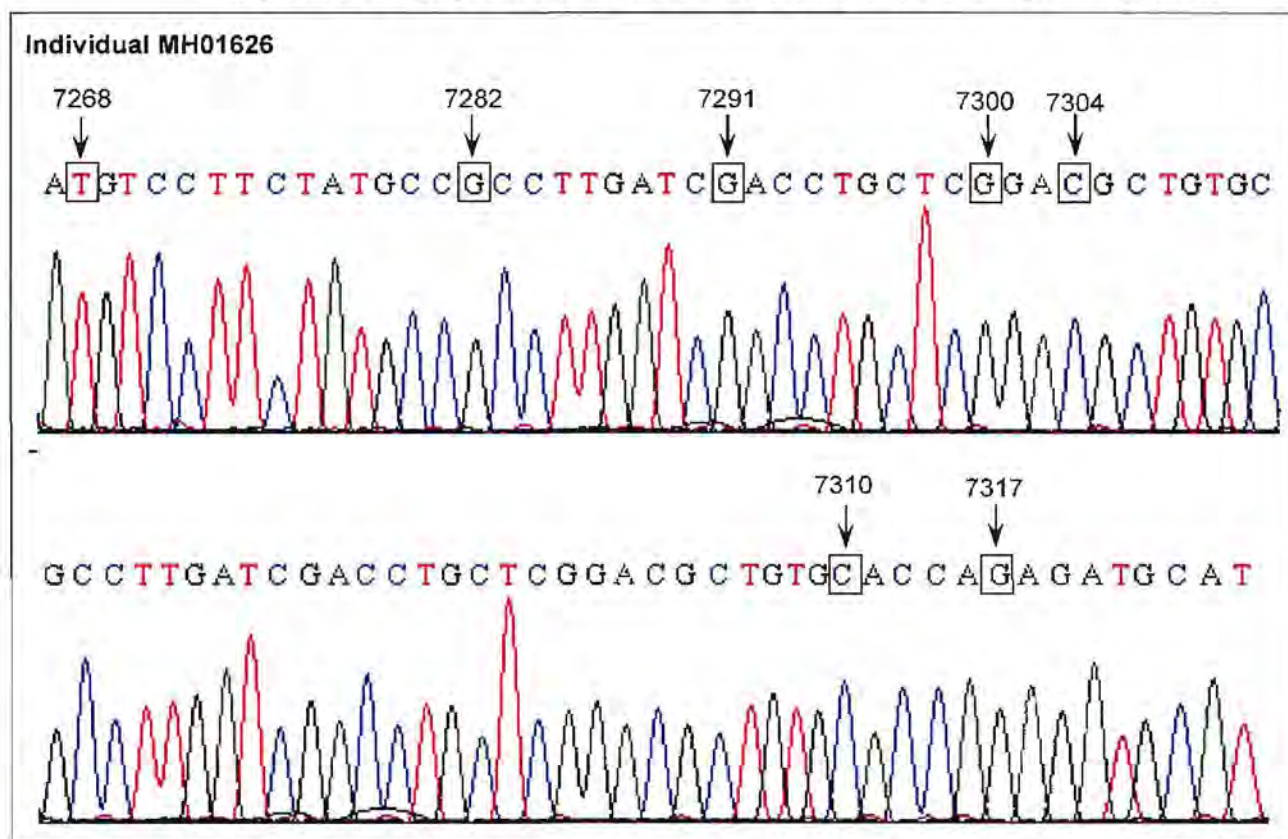
Figure 4.59: Representative electropherogram of exon 44 indicating the nucleotide positions of the Glu2344Asp, Val2346Met, Glu2347del, Glu2348Gly, Ala2350Thr, Arg2355Cys, Glu2362Gly, Phe2364Val, Ala2367Thr, Gly2375Ala and Pro2366Arg alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Glu2344Asp at nucleotide 7032; Val2346Met at nucleotide 7036; Glu2347del at nucleotides 7041-7043; Glu2348Gly at nucleotide 7043; Ala2350Thr at nucleotide 7048; Arg2355Cys at nucleotide 7062; Glu2362Gly at nucleotide 7085; Phe2364Val at nucleotide 7090; Pro2366Arg at nucleotide 7097; Ala2367Thr at nucleotide 7099 and Gly2375Ala at nucleotide 7124.

The Gly2434Arg and Arg2435His alterations have previously been analysed in Phase 1 and Phase 2. Both the Gly2434Arg and Arg2435His alterations were analysed via an RFLP strategy in 31 MH individuals in Phase 1 and were detected by the gain of a *Dde* I restriction site and by the loss of the *Hga* I site, respectively. In Phase 2, both alterations, in addition to the Ala2428Thr, Asp2431Asn and Arg2435Leu alterations, were screened for in South African MH probands via a sequencing strategy. Figure 4.60 is a representative electropherogram obtained for individual MH01626 depicting the nucleotide positions of previously reported alterations in the amplified region of exon 45.

Figure 4.60: Representative electropherogram of exon 45 indicating the nucleotide positions of the Ala2428Thr, Met2423Lys, Asp2431Asn, Gly2434Arg, Arg2435His, Arg2435Leu, Ala2436Val and Glu2439Asp alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Met2423Lys at nucleotide 7268; Ala2428Thr at nucleotide 7282; Asp2431Asn at nucleotide 7291; Gly2423Arg at nucleotide 7300; Arg2435His and Arg2435Leu at nucleotide 7304; Ala2436Val at nucleotide 7310 and Glu2439Asp at nucleotide 7317.

None of the reported alterations were detected in any of the MH individuals analysed in Phase 2. In addition, novel alterations were also absent from the sequenced region. However, in Phase 3, one individual was observed to harbour the Phe2364Val alteration, as discussed in Section 4.8.5.2 (page 231).

The alterations Glu2344Asp, Val2346Met, Glu2347del, Glu2348Gly, Glu2362Gly; Pro2366Arg, Ala2367Thr, Gly2375Ala, Ala2428Thr, Asp2431Asn, Ala2436Val and Glu2439Asp have each been reported in single MH families (Sambuughin *et al.*, 2001a; Sambuughin *et al.*, 2001b; Sambuughin *et al.*, 2001c; Halsall and Robinson, 2004; Wehner *et al.*, 2004; Monnier *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006). It has still not been determined whether these mutations represent family-specific alterations or if they occur frequently in the various populations in which they were described. It would be expected that alterations Met2423Lys and Arg2435Leu would not be observed in any of the 15 South African MH probands, as they have thus far only been identified in patients clinically diagnosed with CCD (Barone *et al.*, 1999; Zhou *et al.*, 2005).

The Ala2350Thr alteration is currently being used in the genetic diagnosis of MH susceptibility in the UK, and the causative status of this alteration has been determined (Wehner *et al.*, 2004), as listed in Appendix A (page 443). Halsall and Robinson (2004) reported the Ala2350Thr alteration in three MH families from the UK and determined that the frequency of this alteration in the British population was 0.69. The Ala2350Thr alteration was not observed in the cohort of South African MH probands analysed in the study presented here, but may be present in other South African probands diagnosed with MH. As discussed in Section 4.8.2 (page 215), in order to detect an alteration that has a frequency between 0.1 - 1, with 95% power, approximately 340 - 3,910 chromosomes would have to be analysed (Collins and Schwartz, 2002).

The Arg2355Cys alteration was first reported by McWilliams *et al.* (2002) in a large Brazilian MH family and was subsequently reported in six MH families from the UK (Halsall and Robinson, 2004). As the alteration has been observed in two different populations this may indicate that the families have a common ancestor. Phylogenetic analysis of both mitochondrial DNA and the Y-chromosome could be used in order to estimate the age of an alteration, to determine if the alteration represents an ancestral mutation present in several different populations (Rannala and Bertorelle, 2001). It would be difficult to determine the reason the alteration was observed in these two very different populations. However, the observation may be a coincidental occurrence, as nine of the reported alterations in the central region of the RYR1 involve Arg, and four of these result in an Arg-to-Cys alteration. This could indicate that the positively charged amino acids play an important regulatory role in this part of the RyR1 protein.

The Gly2434Arg alteration was first reported in four unrelated Caucasian families with MHS (Keating *et al.*, 1994). The mutation was subsequently identified in MH families from Italy, France, Germany, Switzerland and the UK (Robinson *et al.*, 2003b). The Arg2435His alteration was observed in a CCD pedigree from Canada (Zhang *et al.*, 1993) and was subsequently reported in MH families from France, Italy, Germany and the UK (Robinson *et al.*, 2003b). The authors indicated that the alteration was not detected in families from Belgium and Switzerland, as well as observing that the alterations were frequently detected in different populations of European origin. Both alterations have been described as causative (Richter *et al.*, 1997; Tong *et al.*, 1997), as listed in Appendix A (page 443) and were not observed in the South African MH probands analysed in Phase 1, Phase 2 or Phase 3. Due to the wide-spread detection of these alterations, it is evident that neither of the alterations is localised to a single population.

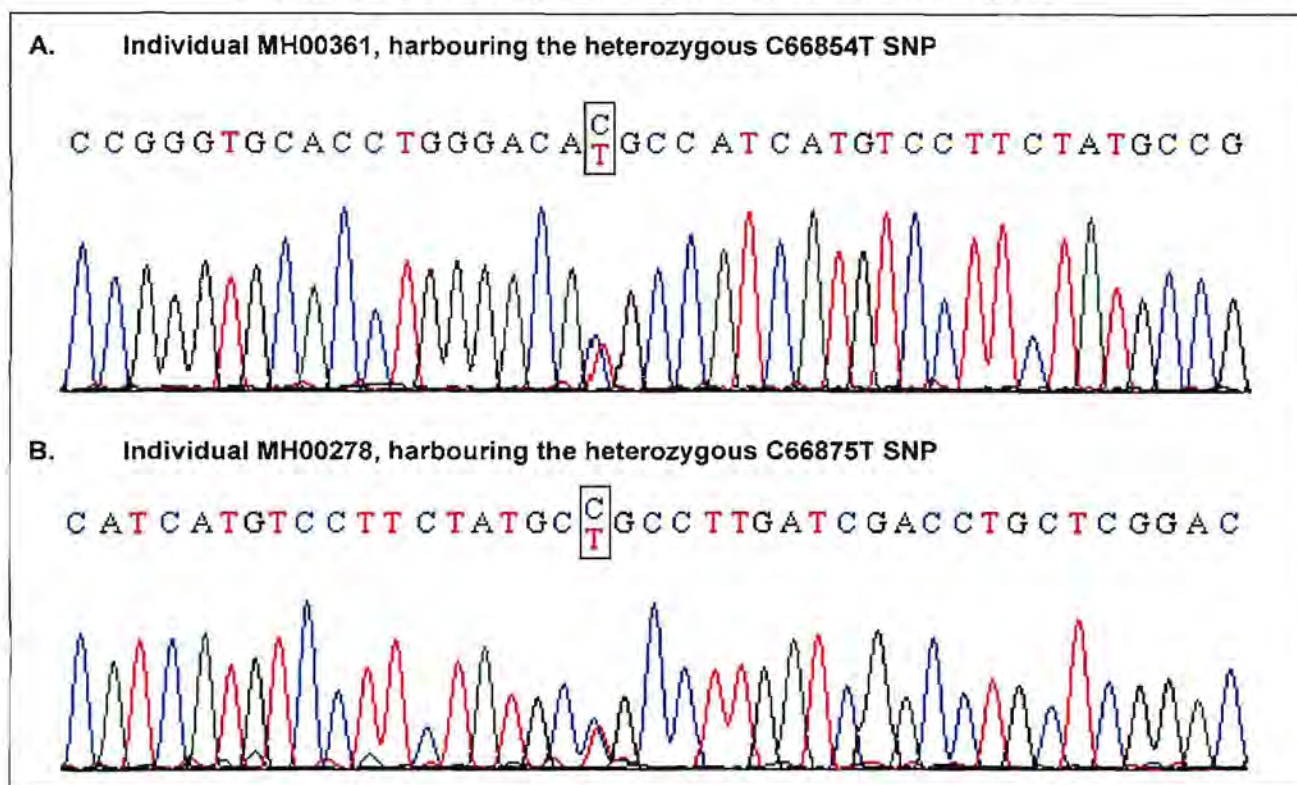
4.8.5.1 Synonymous substitutions in the amplified region of exons 44 and 45 of the RYR1 gene

Two SNPs were identified in the amplified regions of exons 44 and 45. The C66854T SNP was identified in exon 45, in two South African MH probands, as listed in Appendix B (page 447). In addition, a novel C66875T SNP was identified in a single MH proband. Synonymous SNPs are continually being reported in the coding region of the RYR1 gene. Recently, Galli *et al.* (2006) identified eight novel alterations in Italian MH probands. Therefore, although the C7281T alteration has thus far only been identified in a single individual, it may not represent a SNP that is unique to the South African population. In addition, as discussed in Section 4.7.10.1.2 (page 208), the alteration may assist in providing information about population substructure. However, if the SNP is observed to be rare, this may indicate that it resides in a functionally important region of the RyR1 protein and may play a role in disease. Cargill *et al.* (1999) indicated that selection acts against deleterious alterations during human evolution, therefore functionally important SNPs may be rarer. Synonymous SNPs, although they do not result in an amino acid change, could alter splicing, regulation and transcript stability. However the mechanism of action of these events has been difficult to determine (Cargill *et al.*, 1999). As discussed in Section 4.7.4.1 (page 185), synonymous substitutions observed in the RYR1 gene may not be directly associated with the MH phenotype but via epistasis may contribute to the development of the disorder.

4.8.5.1.1 SNP C66854T

In two South African MH probands, i.e. MH00361 and MH00242, two peaks representing two different nucleotides i.e. C and T, were identified. The C66854T SNP is a synonymous substitution, as it occurs at the third codon position and does not result in an alteration in the amino acid encoded by the gene. 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 rs12973632). However, the allele and genotype frequencies for the C66854T SNP have not been determined worldwide. The C66854T nucleotide transition retains the His amino acid. A representative electropherogram of the C66864T SNP is depicted in Figure 4.61A, The remaining individuals included in the study presented here did not exhibit the SNP.

Figure 4.61: Representative electropherograms indicating the C66854T and C66875T SNPs observed in exon 45 of the RYR1 gene



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

4.8.5.1.2 SNP C66875T

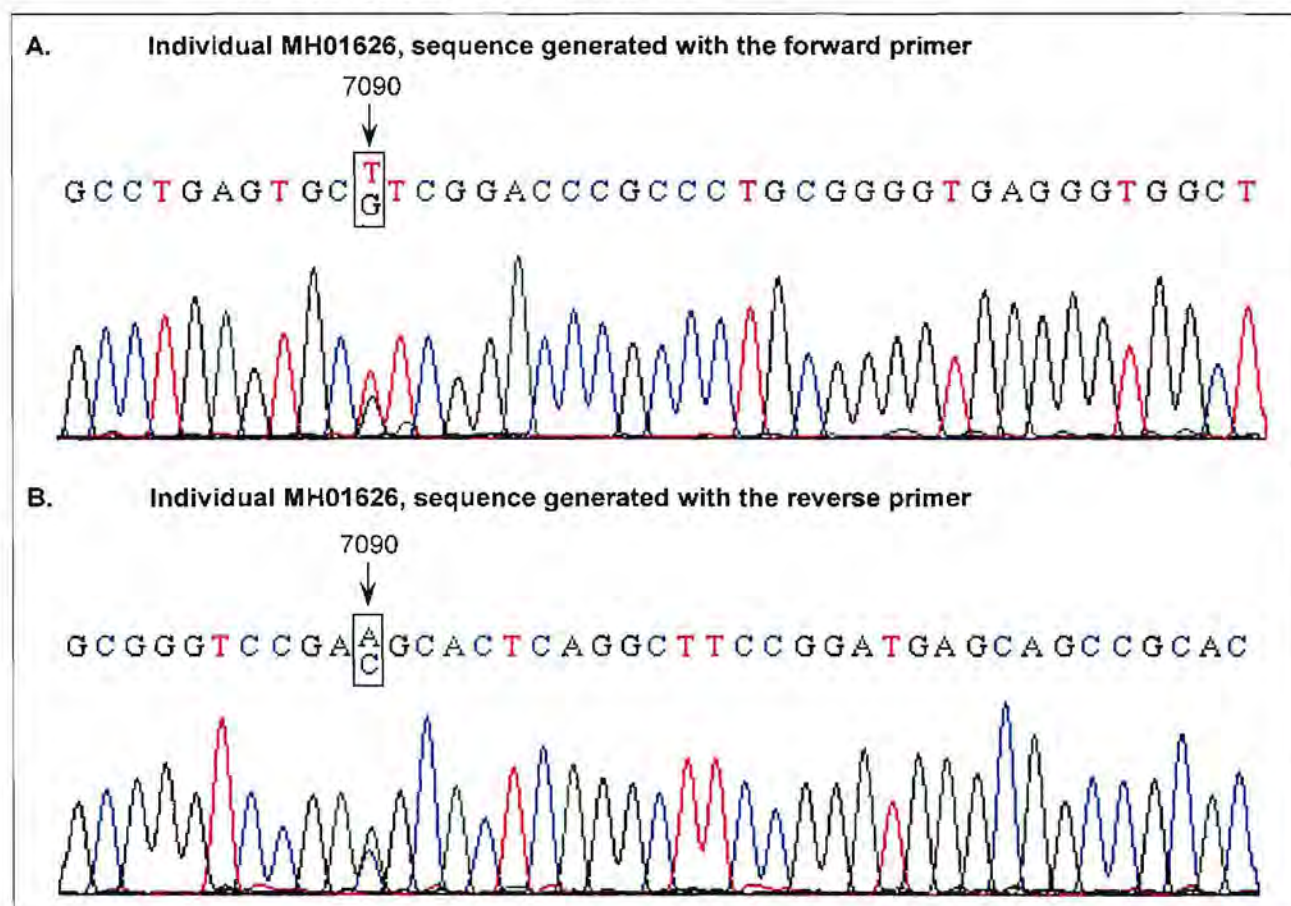
In addition, a novel C66875T SNP was detected in exon 45 in one South African MH proband (MH00278), as illustrated in Figure 4.61B. The alteration is synonymous and does not result in an alteration in the amino acid Ala. The individual analysed for the alteration was identified as being heterozygous, due to the presence of two different nucleotides, namely C and T. The C66875T nucleotide transition was not detected in any of the other probands analysed in the study presented here, and has never been reported in any other population.

4.8.5.2 The Phe2364Val alteration in the RYR1 gene

In Phase 3, one individual (MH01626) that had not previously been analysed in Phase 1 or Phase 2 yielded a positive result for the previously reported Phe2364Val alteration in exon 44. At nucleotide position 7090, two peaks were observed that indicate the presence of two bases, namely G and T. The different bases code for two different amino acids, namely Phe and Ala, respectively. The sequence depicted on the electropherogram of Figure 4.62A indicates the alteration observed using the forward primer and similarly the

sequence depicted on the electropherogram of Figure 4.62B indicates the alteration observed using the reverse primer. Sequencing of exon 44 in the forward and reverse directions confirmed the presence of the Phe2364Val alteration.

Figure 4.62: Representative electropherograms indicating the Phe2364Val alteration observed in exon 44



A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Phe2364Val at nucleotide 7090.

Table 4.8 depicts the partial amino acid sequence of exon 44 of the RyR1 protein. Multiple sequence alignments encompassing the position of the Phe2364Val 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 MHS. The region also exhibits homology to the InsP₃R and is highly conserved through evolution and among different RyR1 species.

Table 4.8: Conserved amino acids obtained from different RyR isoforms and species surrounding the reported mutations in exon 44 of the RyR1 protein

Isoform	Species	Alignment of RyR protein sequences
RyR1	Human	IGWNPCCGERY LDFLRFVAVFV NGESVEENAN VVRLIRKP ECFGPAALRGE GGSGLL
RyR2	Human	IGWNPVEGERY LDFLRFVAVFC NGESVEENAN VVRLIRRP ECFGPAALRGE GGNGLL
RyR3	Human	VGWNPPIGERY LSFLRFVAVFV NESVEENAS VVKLLIRRP ECFGPAALRGE GGNGLL
<i>ryr1</i>	Pig	IGWNPCCGERY LDFLRFVAVFV NGESVEENAN VVRLIRKP ECFGPAALRGE GGSGLL
<i>ryr1</i>	Rabbit	IGWNPCCGERY LDFLRFVAVFV NGESVEENAN VVRLIRKP ECFGPAALRGE GGSGLL

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 Phe amino acid at nucleotide 7090 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 as follows: Glu2344Asp = red; Val2346Met = green; Glu2348Gly = pink; Ala2350Thr = orange; Arg2355Trp = dark purple; Glu2362Gly = light blue; Pro2366Arg = light green and Ala2367Thr = light purple. The accession numbers are as follows: RyR1 human = P21817; RyR2 human = Q92736; RyR3 human = NP_001027; *ryr1* pig = P16960 and *ryr1* rabbit = P11716.

In the study presented here, the Phe2364Val alteration was not observed in any other family members of this pedigree. The presence of the alteration in the proband (MH001626) was verified via re-amplification and sequencing of the DNA obtained from the proband.

The Phe2364Val alteration is the second alteration identified in family MH125-1. The Glu3583Gln alteration was identified in exon 73 of the RYR1 gene in this family, as discussed in Section 4.10.36.1 (page 365). Neither the Phe2364Val nor the Glu3583Gln alterations observed in this family segregate with the MH phenotype. The daughter of the proband (MH125-2) has been diagnosed as MHS via the IVCT but does not harbour the Phe2364Val alteration. The MH status of her two sons (MH125-3 and MH125-4) is currently unknown. However, an MHS status is suspected in the one child (MH125-3), as he displayed symptoms of MH during an anaesthetic procedure. The other child (MH125-4) has been diagnosed with API. To date, no association between API and MH has been reported. The absence of both alterations in family members of this pedigree diagnosed as MHS may indicate the possibility that a currently unidentified genetic alteration is responsible for the MH phenotype in this family.

The Phe2364Val alteration was previously reported in two MH families from the UK, and the frequency of the alteration was determined to be 0.46% (Halsall and Robinson, 2004). However, to date functional studies in an experimental system have not been conducted for the Phe2364Val alteration. Therefore, the causative status of this alteration has not been determined. Caution should be taken when determining whether a newly discovered amino acid change is disease-causing or a polymorphism, as this will have drastic

consequences for an affected carrier whose diagnosis is confirmed via identification of the mutation. This is the first report of the Phe2364Val alteration within the South African population. This finding may have significant implications for the molecular diagnosis of MH in the South African population if the alteration is determined to be causative of the MH phenotype.

4.8.6 Exon 46 of the RYR1 gene

A 242 bp product encompassing exon 46 was amplified via PCR. This region of the RYR1 gene harbours seven alterations that have all been observed in patients diagnosed with MH, as listed in Table 4.9. Exon 46 is located in the central portion of the RYR1 gene, in hotspot two. In Phase 1, the Arg2458Cys and Arg2458His alterations were analysed via sequencing in 34 South African MH probands. However, the Arg2452Trp alteration was not analysed in Phase 1 as it was only reported in 2004 (Halsall and Robinson, 2004). As sequencing information was available for 24 MH probands from Phase 1, the sequence was subsequently re-analysed for the Arg2452Trp alteration in Phase 2. In addition, seven MH probands that were not investigated previously for these alterations were analysed in Phase 2. In Phase 3, two individuals that had not been analysed for alterations in this region of the RYR1 were subsequently analysed.

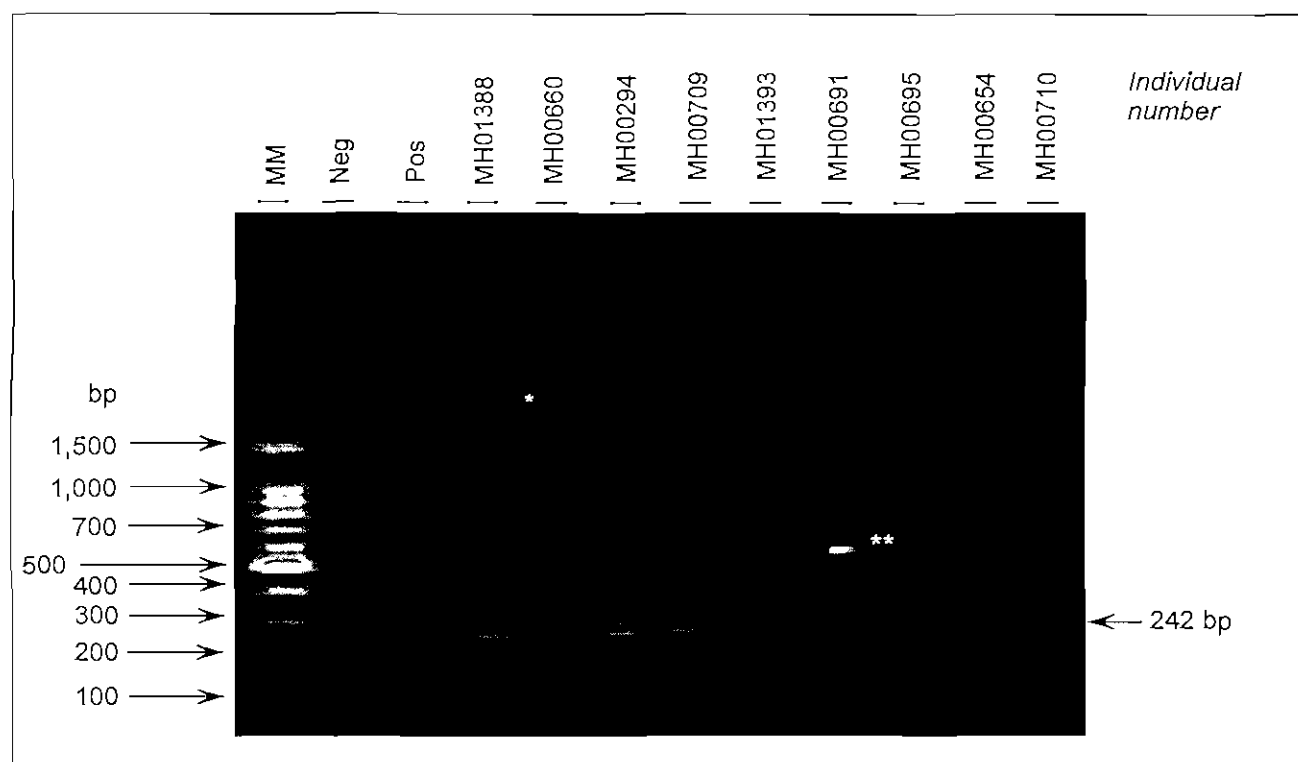
Table 4.9: Reported alterations in exon 46 of the RYR1 gene

Amino acid change	Nucleotide change	Reference	Amino acid change	Nucleotide change	Reference
Arg2452Trp	C7354T	Chamley <i>et al.</i> , 2000	Arg2454His	G7361A	Barone <i>et al.</i> , 1999
Arg2452Gln	G7355A	Ibarra <i>et al.</i> , 2006	Arg2458Cys	C7372T	Manning <i>et al.</i> , 1998b
Ile2435Thr	T7358C	Rueffert <i>et al.</i> , 2004	Arg2458His	G7373A	Manning <i>et al.</i> , 1998b
Arg2454Cys	C7360T	Brandt <i>et al.</i> , 1999	---	---	---

--- = indicates no information given.

Successful amplification was achieved for both of the samples investigated using amplification conditions listed in Table 4.1 (page 161). Figure 4.63 is a photographic representation of the amplicon encompassing exon 46 that was obtained in the Phase 2 study.

Figure 4.63: Photographic representation of amplified PCR products encompassing exon 46



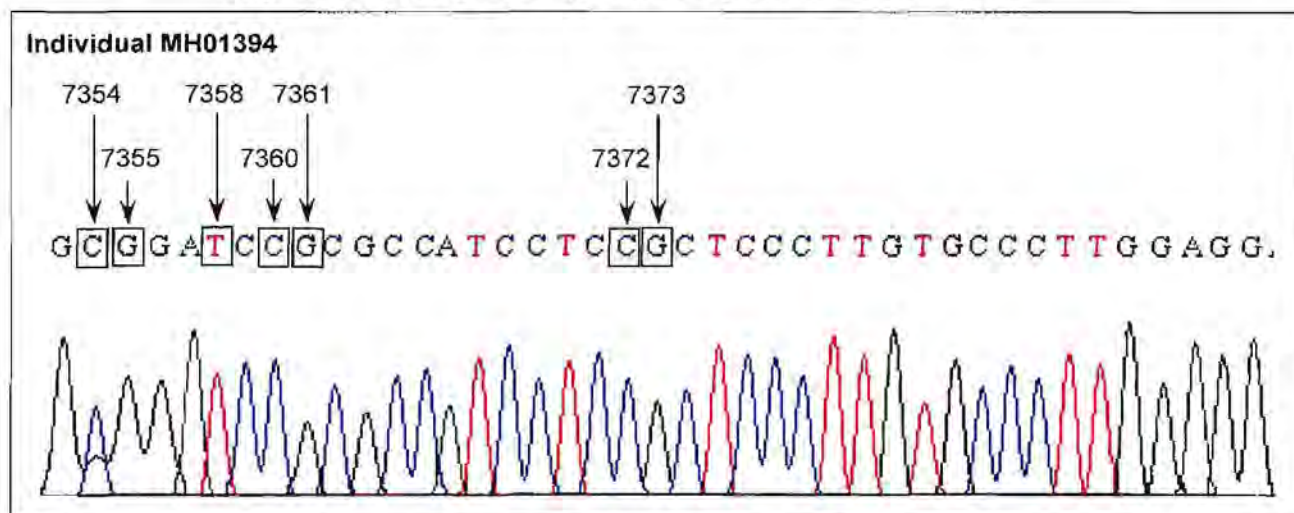
Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular weight marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. The following artefacts were detected on the gel: an artefact in the gel matrix, as indicated by the white asterisk (*), fragment distortion, overloaded MM and variation in amplification efficiency, as discussed in Sections 4.2 and 4.3. An unidentified artefact as indicated by two white asterisks (**) was detected in the gel in the lane of the sample obtained from individual MH00691.

The amplified region was subsequently sequenced using the forward primer (RYRE46F), in order to investigate for the presence of seven published causative mutations in this region, as well as to identify possible novel mutations. A representative electropherogram obtained for individual MH01394, illustrating the amplified region of exon 46, indicating the nucleotide positions of previously reported alterations, is depicted in Figure 4.64.

The Arg2452Trp alteration was first reported in a six-month-old male from New Zealand, who developed symptoms associated with MH during surgery to repair his cleft palate (Chamley *et al.*, 2000). The alteration was also identified in the mother and sibling of the proband. The mutation was subsequently reported in one UK MH family (Halsall and Robinson, 2004). The Ile2453Thr alteration has thus far only been identified in a single family that was diagnosed with MH (Rueffert *et al.*, 2004). The Arg2452Gln alterations have been identified in a single MH proband from Japan (Ibarra *et al.*, 2006). The Arg2452Trp, Ile2453Thr and Arg2452Gln alterations were not detected in any of the South African MH probands analysed in Phase 2 or 3. However, as the mutations have thus far only been detected in one or two families, it is likely that they are specific to those families.

Although the frequency of each alteration varies, all mutations should initially be screened for in the different populations to determine the frequency of each alteration.

Figure 4.64: Representative electropherogram of exon 46 indicating the nucleotide positions of the Arg2452Trp, Arg2452Gln, Ile2435Thr, Arg2454Cys, Arg2454His, Arg2458Cys and Arg2458His alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Arg2452Trp at nucleotide 7354; Arg2452Gln at nucleotide 7355; Ile2435Thr at nucleotide 7358; Arg2454Cys at nucleotide 7360; Arg2454His at nucleotide 7361; Arg2458Cys at nucleotide 7372 and Arg2458His at nucleotide 7373.

The Arg2454Cys, Arg2454His, Arg2458Cys and Arg2458His alterations were not detected in any of the South African MH individuals analysed in Phase 1, Phase 2 or Phase 3. The alteration Arg2454Cys was first reported in a single MH pedigree (Brandt *et al.*, 1999) and an Arg2454His alteration has been observed in one family with MH (Barone *et al.*, 1999). It has subsequently been identified in MH probands from North America (Sambuughin *et al.*, 2001b; Sei *et al.*, 2004), Italy (Tammaro *et al.*, 2003) and France (Monnier *et al.*, 2005). The Arg2458Cys mutation was first reported by Manning *et al.* (1998b), and was detected in a single MH Swiss and Italian pedigree. The authors observed that this mutation segregated in both MHS and MHE individuals. The mutation was subsequently reported in two MH families from the UK (Halsall and Robinson, 2004), in three unrelated MH families from Italy, two unrelated families from Germany and five unrelated families from Switzerland (Robinson *et al.*, 2003b). The alteration is currently being used for genetic diagnosis of MH susceptibility. The Arg2458His alteration was first reported in a single large French MH pedigree (Manning *et al.*, 1998b) and was subsequently reported in six unrelated MH families from the UK (Halsall and Robinson, 2004), two unrelated MH families from France and one MH family from Germany (Robinson *et al.*, 2003b). Although the alteration occurs at a lower frequency than the Arg2458Cys alteration, it is also used in the genetic diagnosis of MH susceptibility. The Arg2454Cys, Arg2454His, Arg2458Cys

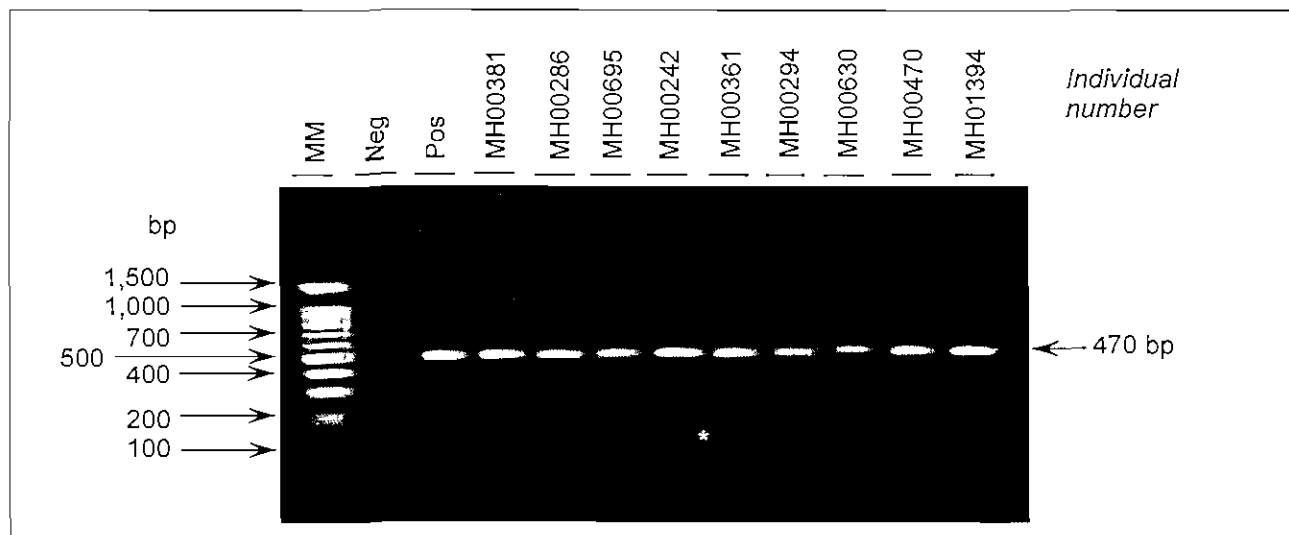
and Arg2458His alterations have been detected in several MH families from several different populations, thus they are unlikely to be specific to the families in which they have been identified. In addition, all four alterations have been functionally analysed and result in an alteration in Ca^{2+} release following pharmacological action (Tong *et al.*, 1997; Yang *et al.*, 2003; Monnier *et al.*, 2005), as listed in Appendix A (page 443). The absence of these alterations in the cohort of South African MH probands investigated in Phases 1, 2 and 3 indicates that they are not responsible for the phenotype in these individuals. It is, however, possible that in the future these alterations may be detected in South African MHS individuals that have thus far not been screened for them.

4.9 HOTSPOT THREE OF THE RYR1 GENE

Amplification of all exons in hotspot three in the study presented here led to the identification of several SNPs and two alterations, namely Thr4826Ile in exon 100 and Gly4935Ser in exon 102. Hotspot three harbours several functional domains, as discussed in Section 2.11.3.3.3 (page 58). This region of the RyR1 protein contains three Ca^{2+} binding sites within residues that are encoded by exons 91 and 92 (Takeshima *et al.*, 1989), while both an ATP binding site and a CaM binding site have also been identified within residues that are encoded by exon 91. In addition, residues encoded by exons 92, 93 and 94 are involved in CICR (Chen *et al.*, 1992). As this region of the RyR1 has several functional domains, the entire coding region of hotspot three was analysed in order to identify any reported or novel alteration that may result in the MH phenotype observed in 15 South African MH probands. The study presented here is the first analysis of all exons that reside in hotspot three of the RYR1 gene in South African MH probands.

4.9.1 Exon 90 of the RYR1 gene

PCR was conducted, as discussed in Section 4.2 (page 159), in order to amplify a 470 bp region of exon 90 of the RYR1 gene. Results obtained for PCR amplification are depicted in Figure 4.65. Thus far, two alterations have been observed to occur in this exon, Asn4119Tyr (Sambuughin *et al.*, 2005) and Arg4136Ser (Galli *et al.*, 2002). This exon resides in the third mutational hotspot and was amplified and subsequently sequenced in order to identify reported and novel alterations that may occur in this region of the RYR1 gene, in samples obtained from 15 South African MHS probands. The role that this region of the RYR1 gene plays in the functioning of the RyR1 is described in Chapter 2.11.3.3.3 (page 58).

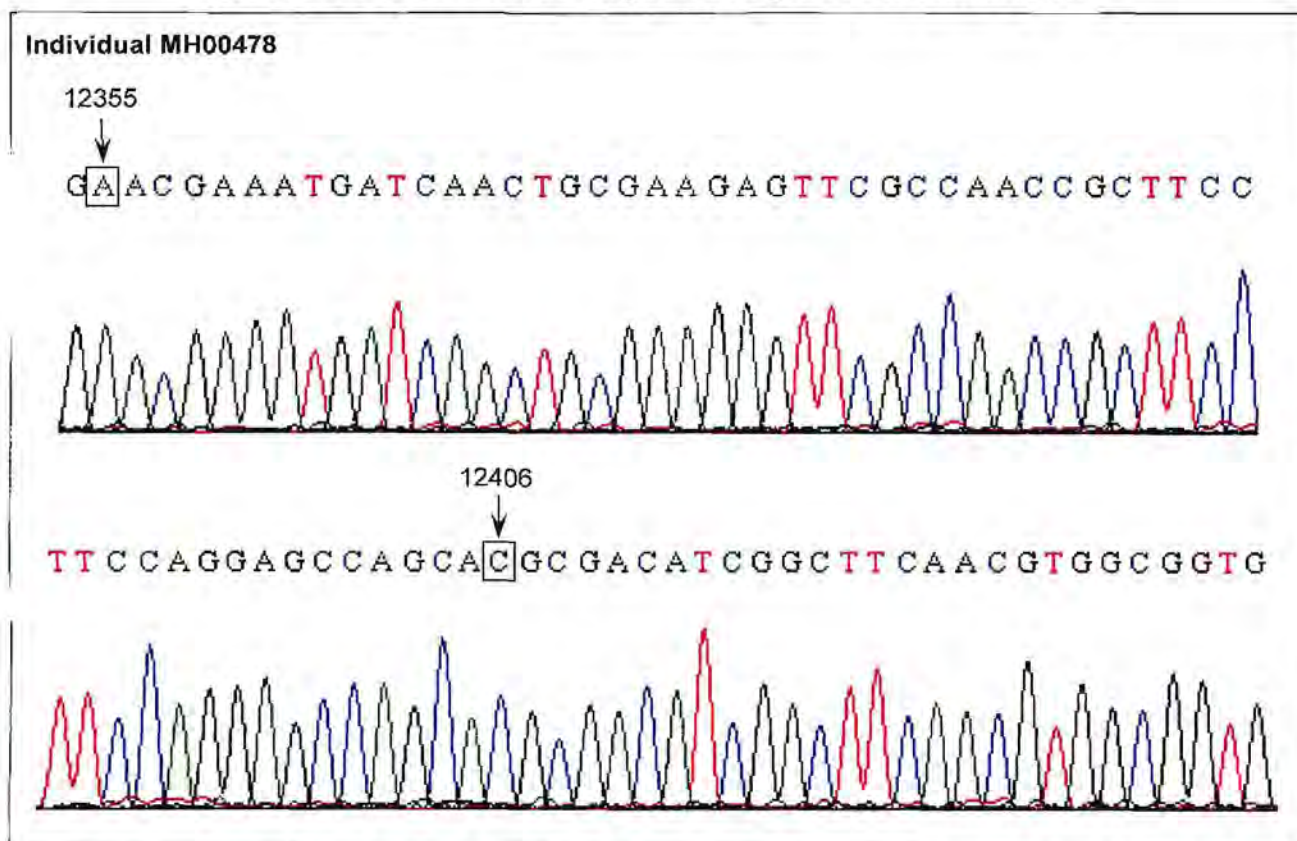
Figure 4.65: Photographic representation of amplified PCR products encompassing exon 90

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 (*) and variation in the amplification efficiency were observed as discussed in Sections 4.2 and 4.3.

Following PCR amplification, MH probands were successfully sequenced using the reverse primer (RYRex90R). The sequence is therefore represented as the reverse complement. A representative electropherogram obtained for individual MH00478, indicating the nucleotide positions of the reported Asn4119Tyr and Arg4126Ser alterations, is depicted in Figure 4.66. A total of 15 samples were successfully amplified and sequenced, in which neither the Asn4119Tyr alteration nor the Arg4136Ser alteration was detected.

The Asn4119Tyr alteration has thus far only been detected in one North American pedigree (Sambuughin *et al.*, 2005), while the Arg4136Ser alteration has only been identified in a single Italian pedigree (Galli *et al.*, 2006). These two investigations (Sambuughin *et al.*, 2005; Galli *et al.*, 2006) are the only reports of the Asn4119Tyr and Arg4136Ser alterations, to date. The lack of identification in other populations or within the South African population indicates that these two mutations may be family-specific. However, as discussed in Section 4.8.2 (page 215), analysis in a larger number of individuals may be required in order to detect alterations that occur in less than 1% of the population. Therefore, further screening of these alterations in additional individuals would determine if they are family-specific or if they occur more frequently.

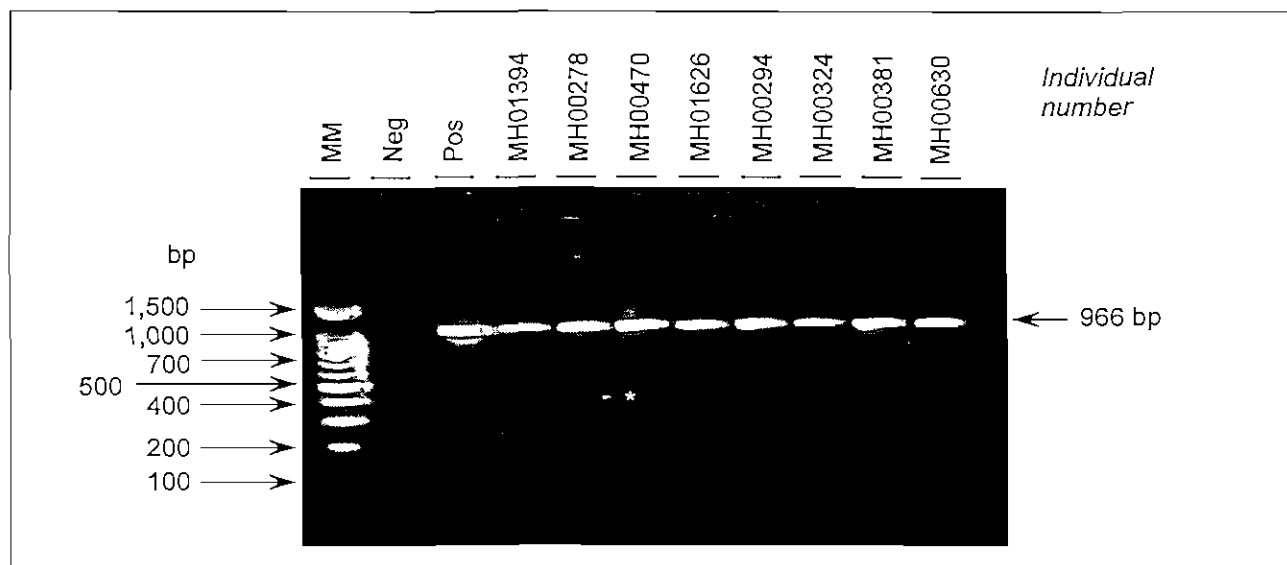
Figure 4.66: Representative electropherogram of exon 90 indicating the nucleotide positions of the Asn4119Tyr and Arg4136Ser alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Asn4119Tyr at nucleotide 12355 and Arg4126Ser at nucleotide 12406.

4.9.2 Exon 91 of the RYR1 gene

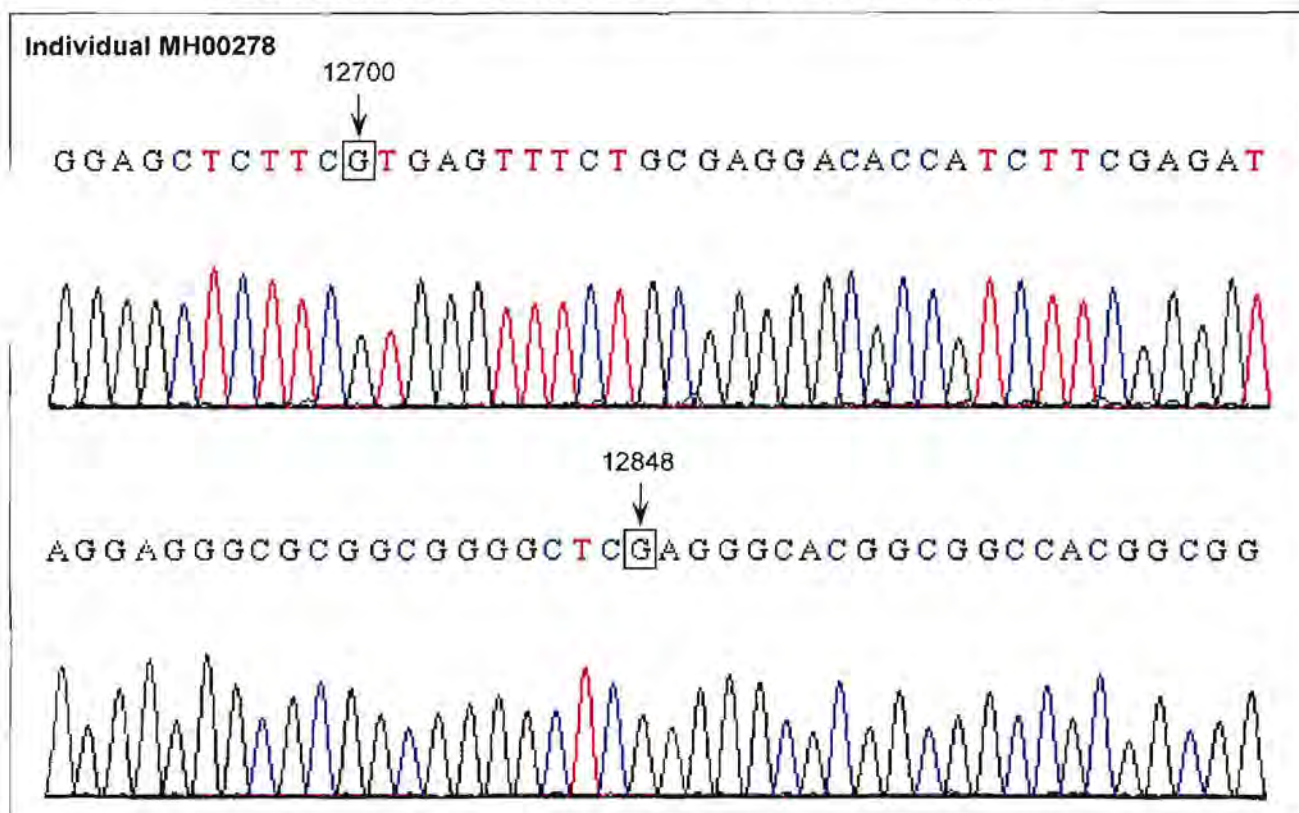
Exon 91 harbours two reported alterations, Val4234Leu and Glu4283Val. The first alteration (Val4234Leu) was observed in a single MH family from Italy (Galli *et al.*, 2006) and the second alteration (Glu4283Val) was reported in an MH family from Japan (Ibarra *et al.*, 2006). PCR amplification was performed to amplify the entire region of exon 91 and was achieved using FastStart® Taq DNA polymerase as discussed in Section 4.2.2 (page 159). All 15 samples from the South African probands were successfully amplified using the altered PCR protocol. The amplified product was electrophoresed on a 2% (w/v) mini agarose gel and the results are represented in Figure 4.67.

Figure 4.67: Photographic representation of amplified PCR products encompassing exon 91

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. Artefacts including fragment distortion, an artefact in the gel matrix (as indicated by the white asterisk), non-specific amplification, fragment smear and MM overloading were observed, as discussed in Sections 4.2 and 4.3.

Due to the large size of the amplified region, all samples were sequenced using both the forward (RYRex91F) and reverse (RYRex91R) primers. A representative electropherogram obtained for individual MH00278, illustrating the nucleotide positions of the previously reported alterations detected in exon 91, is presented in Figure 4.68. None of the 15 South African individuals analysed for reported or novel alterations in exon 91 harboured any mutations that may result in the MH phenotype. Thus far, only two alterations associated with MHS have been identified in two different families, namely Val4234Leu (Galli *et al.*, 2002) and Glu4234Leu (Ibarra *et al.*, 2006). Therefore, both alterations could represent private mutations that are only present in the two above-mentioned families.

Figure 4.68: Representative electropherogram of exon 91 indicating the nucleotide positions of the Val4234Leu and Glu4283Val alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Val4234Leu at nucleotide 12700 and Glu4283Val at nucleotide 12848.

4.9.2.1 Synonymous substitution in the amplified region of exon 91 of the RYR1 gene

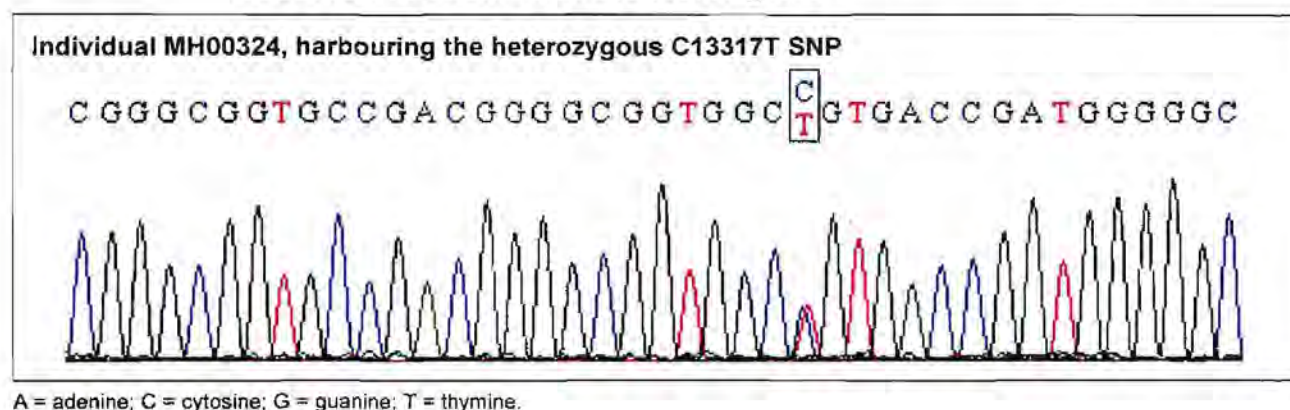
In exon 91, a synonymous C13317T SNP was observed in one MHS proband (MH00324) from South Africa. Exon 91 has a very high GC content (75%), and contains several important functional domains, including Ca^{2+} binding sites (Takeshima *et al.*, 1989), a CaM binding site (Zorzato *et al.*, 1990) and an ATP binding site (Takeshima *et al.*, 1989). The observed SNP occurs between a Ca^{2+} binding site and an ATP binding site. Due to the high GC content in this region, it would be expected that there would be a higher synonymous substitution rate, as synonymous substitution rates are positively correlated with nucleotide composition (Bielawski *et al.*, 2000). Thus far, only two SNPs have been identified in this region. This could indicate that due to the many functional domains observed in this region of the RYR1 gene, alterations are not readily tolerated as they could have a negative impact on protein function. The presence of a synonymous alteration could have many functional effects by altering splicing, regulation or transcript stability (Stephan and Glueck, 2002). It is unlikely that the SNP plays a primary role in the development of MH, however, it may play a secondary role in the development of this

disorder via epistasis. *In vitro* functional studies of the channel mutant would have to be conducted in order to determine the exact role that this SNP plays in the development of the disorder.

4.9.2.1.1 SNP C13317T

The SNP occurs due to a C13317T nucleotide transition, and retains the amino acid Ala. The SNP has thus far not been reported in GenBank®. However, Galli *et al.* (2006) identified the novel SNP in MH probands from Italy. The C13317T nucleotide transition was reported by the authors to have a minor allele frequency of 0.3, as estimated from analysis of 100 chromosomes. The sequence illustrated on the electropherogram of Figure 4.69, generated with the reverse primer, depicts the heterozygous SNP. The remaining individuals included in the study presented here did not exhibit the SNP.

Figure 4.69: Representative electropherogram indicating the C13317T SNP observed in exon 91 of the RYR1 gene

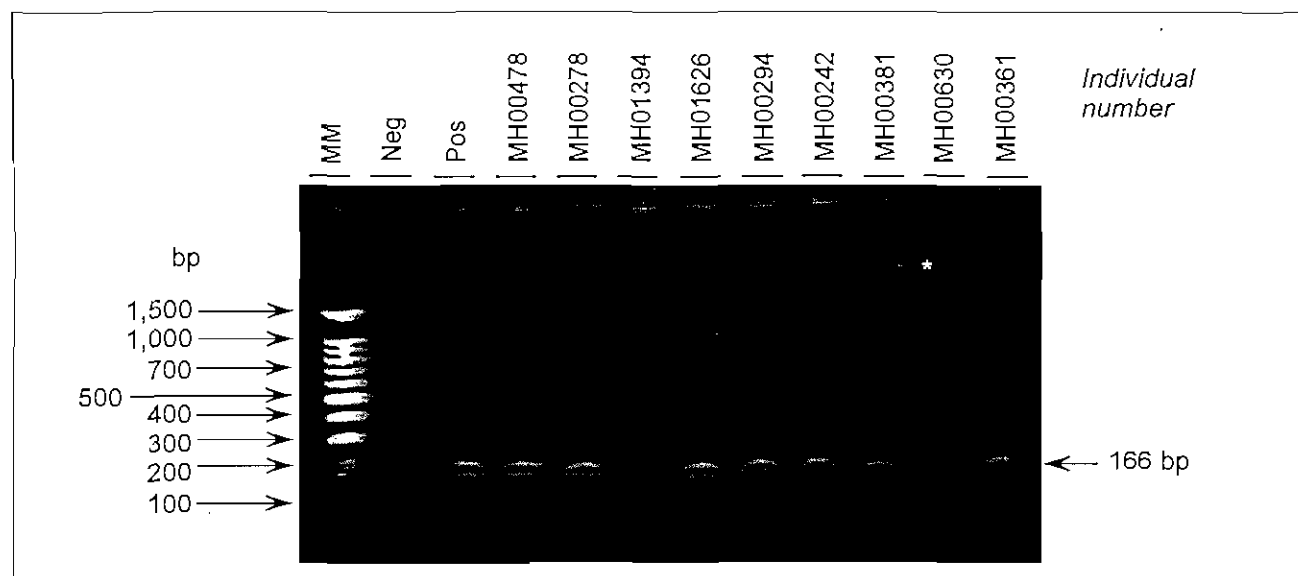


4.9.3 Exon 92 of the RYR1 gene

PCR was conducted in order to amplify a 166 bp region of exon 92 of the RYR1 gene. Thus far, alterations associated with MHS have not been reported worldwide in exon 92, even though it resides in the third hotspot of the RYR1 gene. Therefore, this exon was sequenced in order to identify novel alterations that may occur in this region, in 15 South African MHS probands. This domain of the gene plays an important role in channel conductance and has both high- and low-affinity Ca^{2+} binding sites (Chen and MacLennan, 1998). The role that this region plays in the functioning of the RyR1 is described in Chapter 2.11.3.3.3 (page 58).

The PCR conditions were optimised, as discussed 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 to amplify the fragments, as discussed in Section 3.5 (page 92). The amplified product was subsequently electrophoresed on a 2% (w/v) mini agarose gel. Results obtained for PCR amplification are depicted in Figure 4.70. Sequencing was conducted following PCR amplification using the standard protocol and the reverse primer (RYRex92R). The sequence is thus represented as the reverse complement.

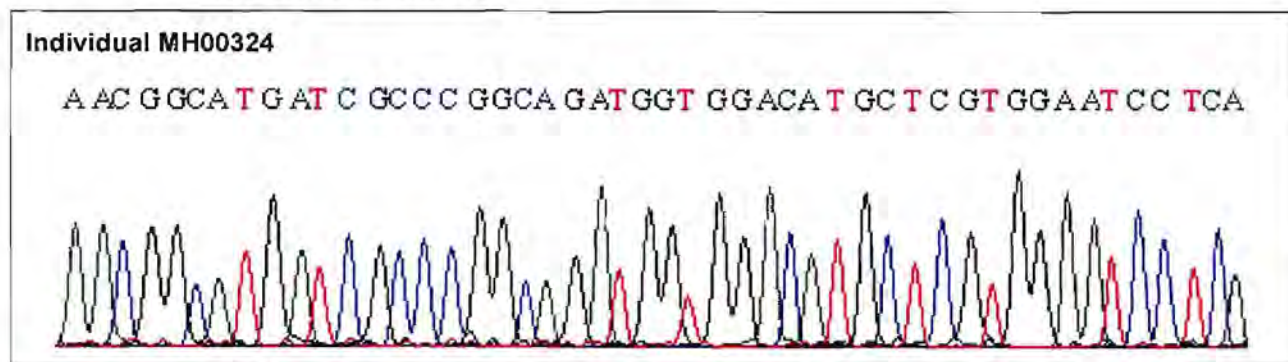
Figure 4.70: Photographic representation of amplified PCR products encompassing exon 92



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 (*), fragment distortion and overloaded MM were detected, as discussed in Sections 4.2 and 4.3. Variation in amplification efficiency was observed, as discussed in Section 4.3.

A representative electropherogram obtained for individual MH00324 is illustrated in Figure 4.71. Although exon 92 resides in hotspot three of the RYR1 gene, alterations that may be associated with the MH phenotype were not observed in any of the 15 South African probands studied. Thus far, alterations in this exon that are associated with MHS have also not been described for any other population analysed worldwide. This region of the RyR1 protein contains residues that are involved in CICR and within this region, a Ca^{2+} binding site exists that is required for channel activation (Chen *et al.*, 1992). In view of the identification of several functional domains within this region of the RYR1 gene, an alteration may be deleterious and result in a threshold being reached which is not tolerated. This could explain the absence of alterations in all populations analysed thus far.

Figure 4.71: Representative electropherogram illustrating a portion of the amplified region of exon 92

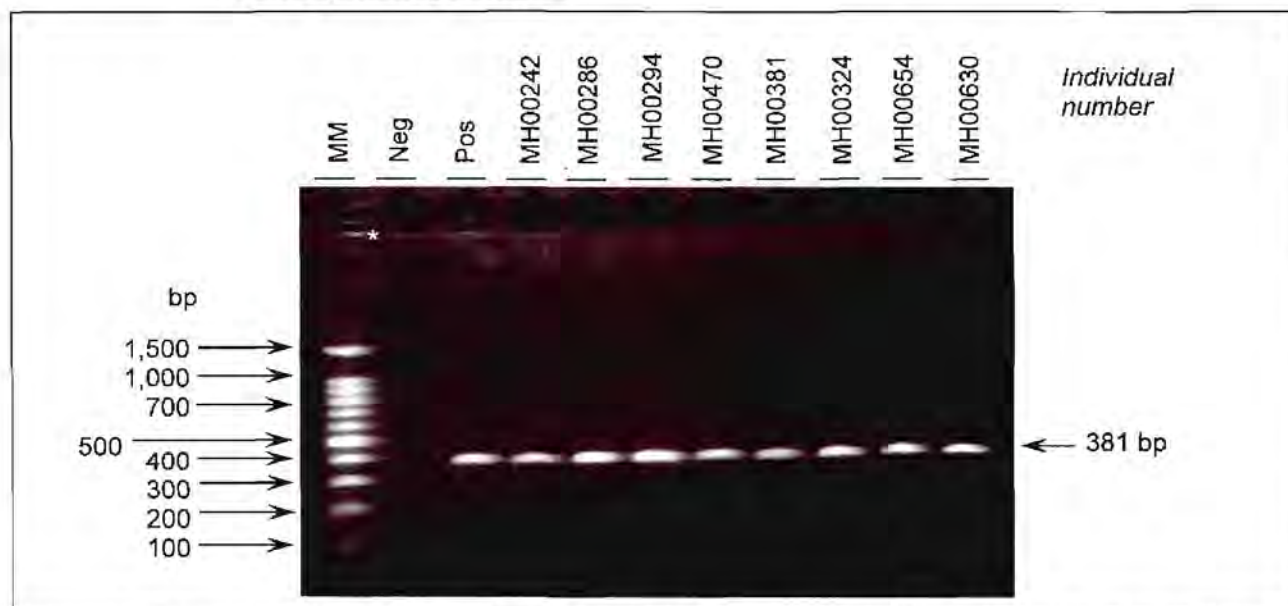


A = adenine; C = cytosine; G = guanine; T = thymine.

4.9.4 Exon 93 of the RYR1 gene

PCR was conducted in order to amplify a 381 bp region, which includes exon 93 of the RYR1 gene, as discussed in Section 4.2 (page 159). Results obtained for PCR amplification are depicted in Figure 4.72. Although exon 93 resides in the third mutational hotspot, alterations associated with MHS, as well as polymorphisms or SNPs, have never been reported to occur in this region of the RYR1 gene. Therefore, this exon was sequenced in order to identify novel alterations that may occur in this region of the RYR1 gene in 15 South African MHS probands.

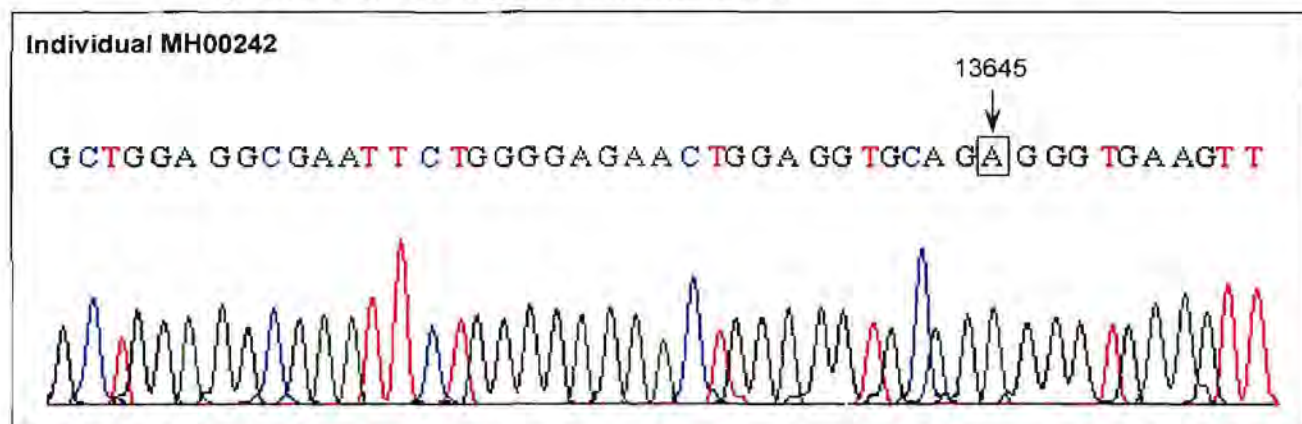
Figure 4.72: Photographic representation of amplified PCR products encompassing exon 93



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 via the white asterisk (*), fragment distortion and MM overloading were observed, as discussed in Sections 4.2 and 4.3. In addition, the fragments appear slanted, as discussed in Section 4.3.

Sequencing of the region encompassing exon 93 was conducted using the reverse primer (RYRex93R). Therefore, sequence results illustrate the reverse complement. A representative electropherogram obtained for individual MH00242, indicating the nucleotide position of the previously reported Arg4549Gln alteration, is illustrated in Figure 4.73.

Figure 4.73: Representative electropherogram of exon 93 indicating the nucleotide position of the Arg4549Gln alteration



A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Arg4549Gln at nucleotide 13645.

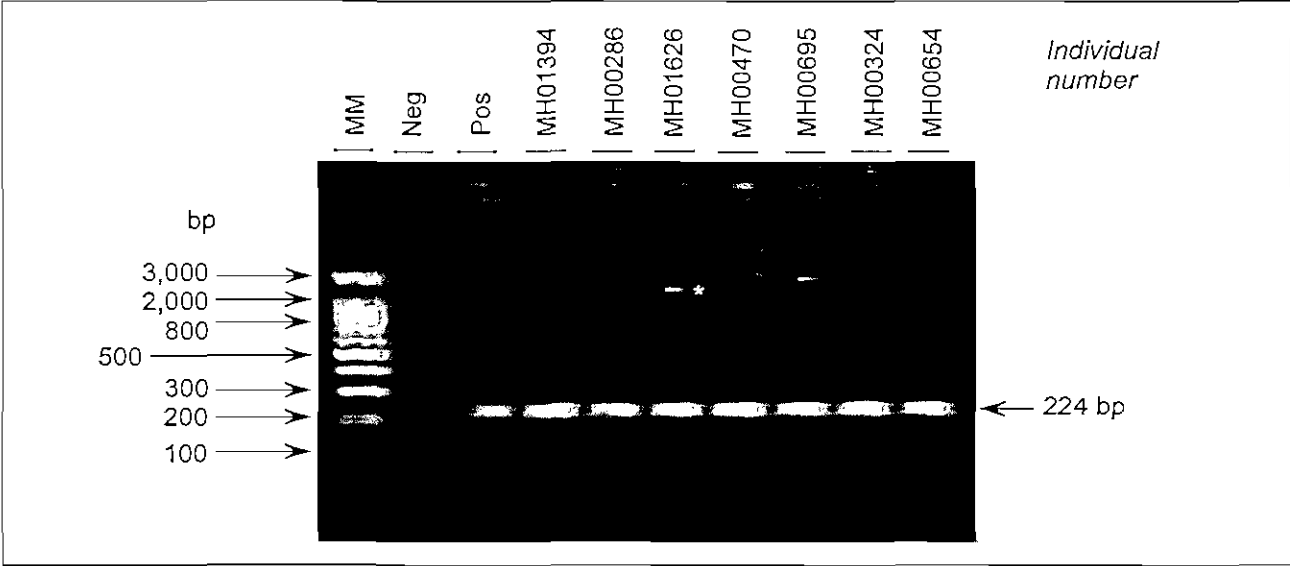
None of the 15 MH probands from South Africa harboured any reported or novel alterations associated with MH in this region of the RYR1 gene. Thus far only one alteration has been observed in exon 93 worldwide. The Arg4549Glu alteration was identified in a single proband diagnosed with CCD (Kossugue *et al.*, 2005). None of the 15 families included in the study presented here has any history of CCD. Therefore, the absence of this alteration would be expected, as it has thus far only been reported in a single proband clinically diagnosed with CCD and not MH. In addition, the absence of the Arg4549Glu alteration could also be due to the family-specific nature of alterations observed in the RYR1 gene. In contrast to CCD alterations observed in hotspots one and two, alterations in the C-terminal domain of the RYR1 gene display resting Ca^{2+} levels and SR Ca^{2+} content that are unaffected (Avila and Dirksen, 2001). The presence of alterations in this region of the protein may result in various degrees of functional uncoupling of sarcolemmal depolarisation from the efficient release of SR Ca^{2+} (Avila *et al.*, 2003). Therefore, alterations in this region of RYR1 may lead only to E-C uncoupling, which is a unique characteristic of CCD mutations. This observation is supported by the identification of the C-terminal transmembrane region of the RYR1 gene as a principal mutation hotspot for CCD alterations (Davis *et al.*, 2003).

4.9.5 Exon 94 of the RYR1 gene

To date, alterations in this region of the RYR1 have never been reported to be associated with MH. A single alteration, i.e. Leu4568Pro has been reported in one family diagnosed with CCD (Wu *et al.*, 2006). A 224 bp product encompassing exon 94 was amplified via PCR, as described in Section 4.2 (page 159). Figure 4.74 is a photographic representation of the amplicon encompassing exon 94.

The amplified product was subsequently 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 standard protocol. The reverse primer (RYRex94R) was used in the sequencing reaction and sequences are depicted as the reverse complement. A representative electropherogram obtained for individual MH00478, depicting the nucleotide position of the previously reported Leu4568Pro alteration, is illustrated in Figure 4.75.

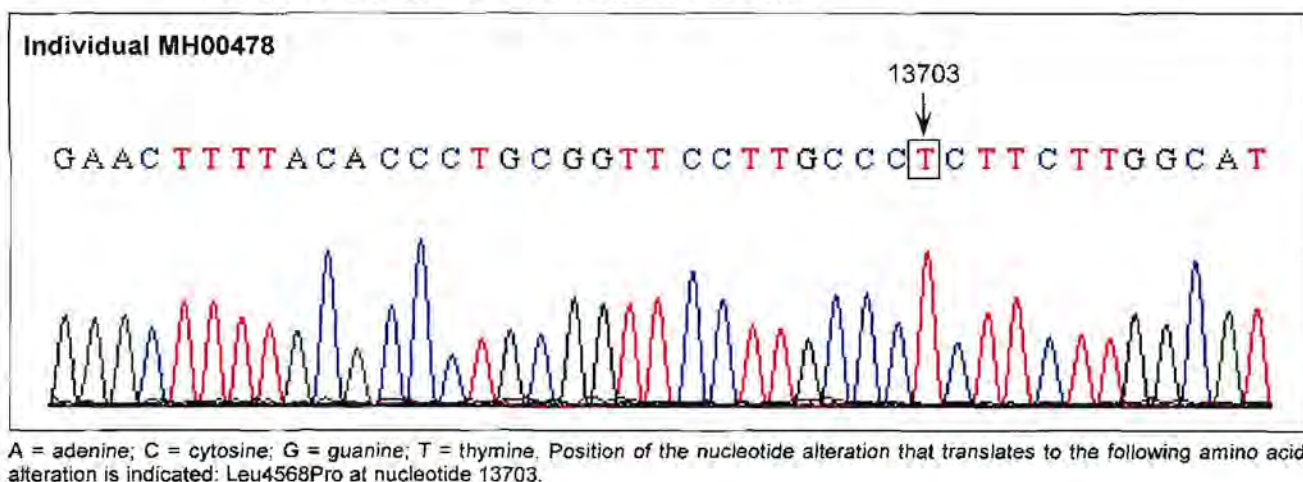
Figure 4.74: Photographic representation of amplified PCR products encompassing exon 94



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, fragment distortion and MM overloading were observed, as discussed in Sections 4.2 and 4.3.

Exon 94 was not observed to harbour any novel alterations associated with MH in any of the 15 MH probands analysed. Thus far, only one alteration has been observed in exon 94, namely Leu4568Pro, which was identified in a single proband diagnosed with CCD (Kossugue *et al.*, 2005).

Figure 4.75: Representative electropherogram of exon 94 indicating the nucleotide position of the Leu4568Pro alteration



As discussed in Section 4.9.4 (page 244), it was expected that probands analysed in the study presented here would not harbour the alteration, as it has thus far only been identified in a proband diagnosed with CCD. Alterations observed in this region of the RyR1 protein could lead to various degrees of E-C uncoupling, which is a unique characteristic of CCD mutations. In addition, the Leu4568Pro alteration may be family-specific.

4.9.5.1 Synonymous substitution in the amplified region of exon 94 of the RYR1 gene

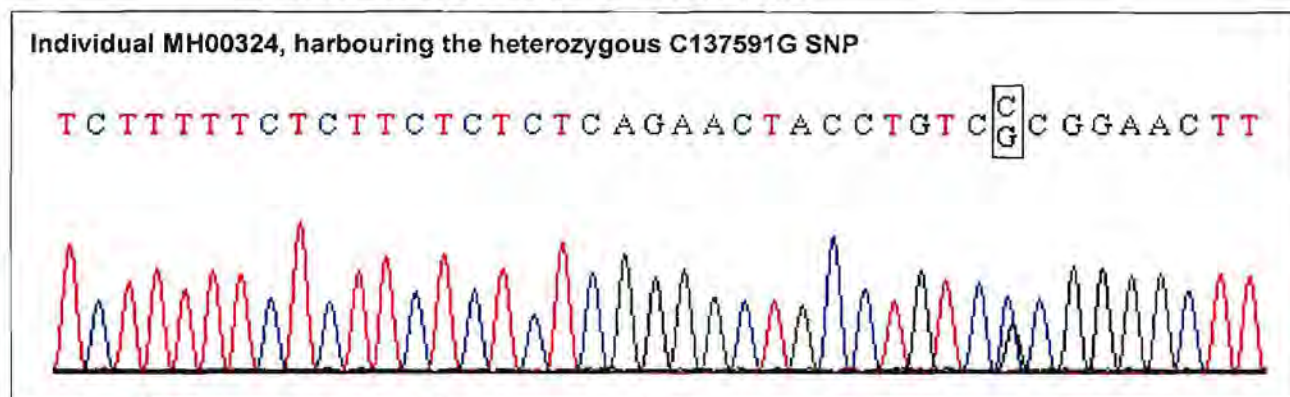
A single C137591G SNP was identified in one MH proband (MH00324) from South Africa in the coding region of exon 94. The remaining individuals included in the study presented here did not exhibit the SNP. The SNP may not play a primary role in the development of MHS in proband MH00324. However, it may, in combination with other alterations, contribute to the MH phenotype via epistasis. As discussed in Section 4.7.4.1 (page 185), synonymous alterations may have functional consequences. However, further investigation into the role of SNPs in the RYR1 gene would have to be conducted.

4.9.5.1.1 SNP C137591G

The SNP occurs due to a C137591G nucleotide transition, which retains the Ser amino acid. The synonymous substitution has been identified as a SNP of the RYR1 gene in GenBank® (International Human Genome Sequencing Consortium, 2004; with accession number rs35959206). The genotype and allele frequencies for the C137591G transition have been determined in the North American population (International Human Genome

Sequencing Consortium, 2004; with accession number rs35959206). In this population, the frequency of the C/C genotype and the C/G genotype has been identified as 0.97 and 0.03, respectively. Figure 4.76 depicts the sequence generated for the heterozygous C137591G SNP observed in individual MH00324.

Figure 4.76: Representative electropherogram indicating the C137591G SNP observed in exon 94 of the RYR1 gene



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

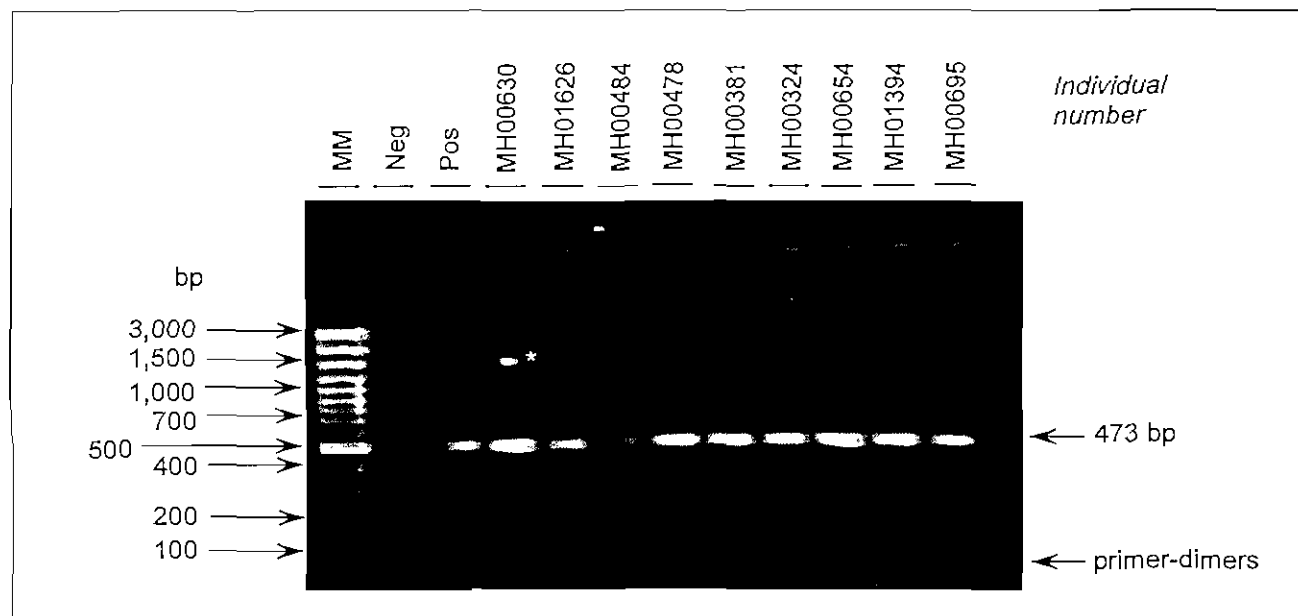
4.9.6 Exon 95 of the RYR1 gene

Exon 95 of the RYR1 gene has thus far been determined to harbour two alterations, i.e. Gly4638Met (Halsall and Robinson, 2004) and Arg4645Gln (Ibarra *et al.*, 2006) that may be associated with the MH phenotype. The Gly4638Met alteration was reported in two UK families diagnosed as MHS, and the Gly4638Asp alteration was identified in a single MH family from Japan. The region harbours nine alterations associated with CCD, and a deletion of two amino acids was observed in one CCD family, as discussed in Section 3.7.66 (page 148). The region resides in the C-terminal position of the RyR1 protein in the third mutation hotspot and represents a functionally significant domain.

In Phase 2, 43 South African MH probands were analysed for the presence of the Gly4638Met alteration as well as novel alterations that may occur in the amplified region. In Phase 3, a new reverse primer (RYRex95bR) was designed, as listed in Table 3.2 (page 86), in order to screen the entire exon in all 15 South African MH probands. Prior to sequencing, the PCR conditions were optimised, as discussed in Section 4.2 (page 159). Thereafter, the PCR products were electrophoresed on a 2% (w/v) agarose gel, as depicted in Figure 4.77. Sequencing was conducted according to the standard protocol, using the forward primer (*RYRex95F). Figure 4.78 depicts the nucleotide positions of the

previously reported alterations observed in exon 95 of the RYR1 gene for individual MH00242.

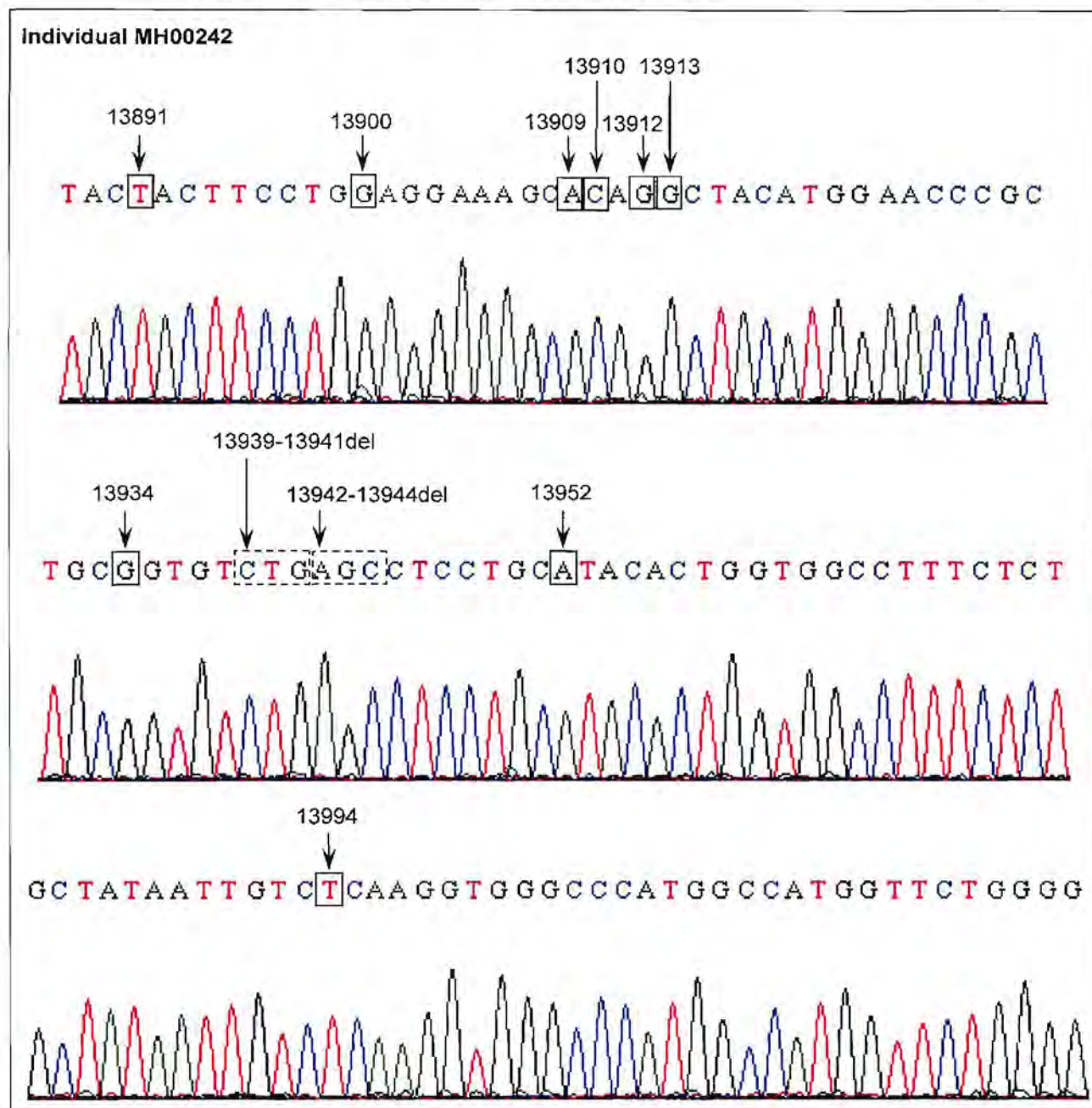
Figure 4.77: Photographic representation of amplified PCR products encompassing exon 95



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 (*), non-specific amplification, fragment distortion, MM overloading, variation in amplification efficiency and primer-dimers, as described in Sections 4.2 and 4.3.

None of the 15 MH probands analysed in the study presented here harboured any novel or reported alterations in exon 15. The Gly4638Met and Arg4645Gln alterations (Halsall and Robinson, 2004; Ibarra *et al.*, 2006) have to date only been identified in one or two families diagnosed with MH. Therefore, they may represent family-specific alterations. Thus far, more than 100 RYR1 mutations have been reported worldwide, however, a significant proportion have been described as specific to individual families (Halsall and Robinson, 2004). Several CCD alterations have been described to occur in this region of the RYR1 gene. The alterations described in this region may lead to functional E-C uncoupling, as discussed in Section 4.9 (page 237). Although certain alterations have been observed to be associated with MH and CCD, none of the above described mutations have been detected in individuals diagnosed with MH. Therefore, it is unlikely that the alterations associated with CCD in exon 95 would be observed in any of the analysed MH probands presented here.

Figure 4.78: Representative electropherogram of exon 95 indicating the nucleotide positions of the Tyr4631Asn, Glu4634Lys, Thr4637Ala, Thr4637Ile, Gly4638Ser, Gly4638Met, Arg4645Gln, Leu4647del, Ser4648del, His4651Pro and Leu4665Pro alterations



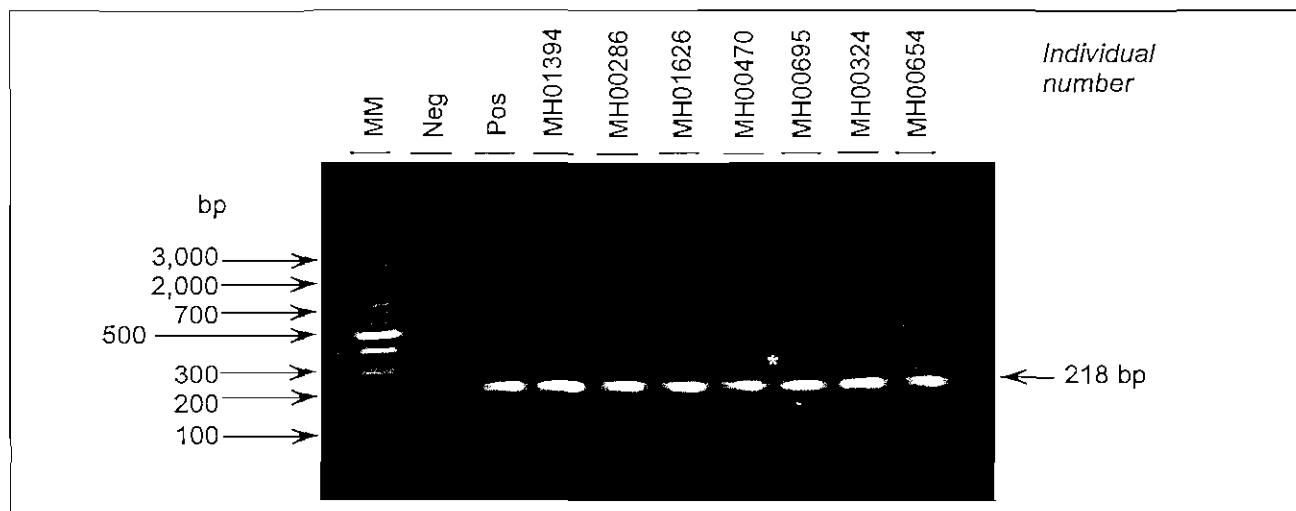
A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Tyr4631Asn at nucleotide 13891; Glu4634Lys at nucleotide 13900; Thr4637Ala at nucleotide 13909; Thr4637Ile at nucleotide 13910; Gly4638Ser at nucleotide 13912; Gly4638Met at nucleotide 13913; Arg4645Gln at nucleotide 13934; Leu4647del at nucleotides 13939-13941; Ser4648del at nucleotides 13942-13944; His4651Pro at nucleotide 13952 and Leu4665Pro at nucleotide 13994.

4.9.7 Exon 96 of the RYR1 gene

In order to detect reported or novel alterations, the entire exon 96 was analysed. Amplification of the 218 bp product via PCR was optimised, as discussed in Section 4.2

(page 159). Using these conditions, all 15 MH probands were effectively amplified and Figure 4.79 depicts the amplified products using the protocol stated above.

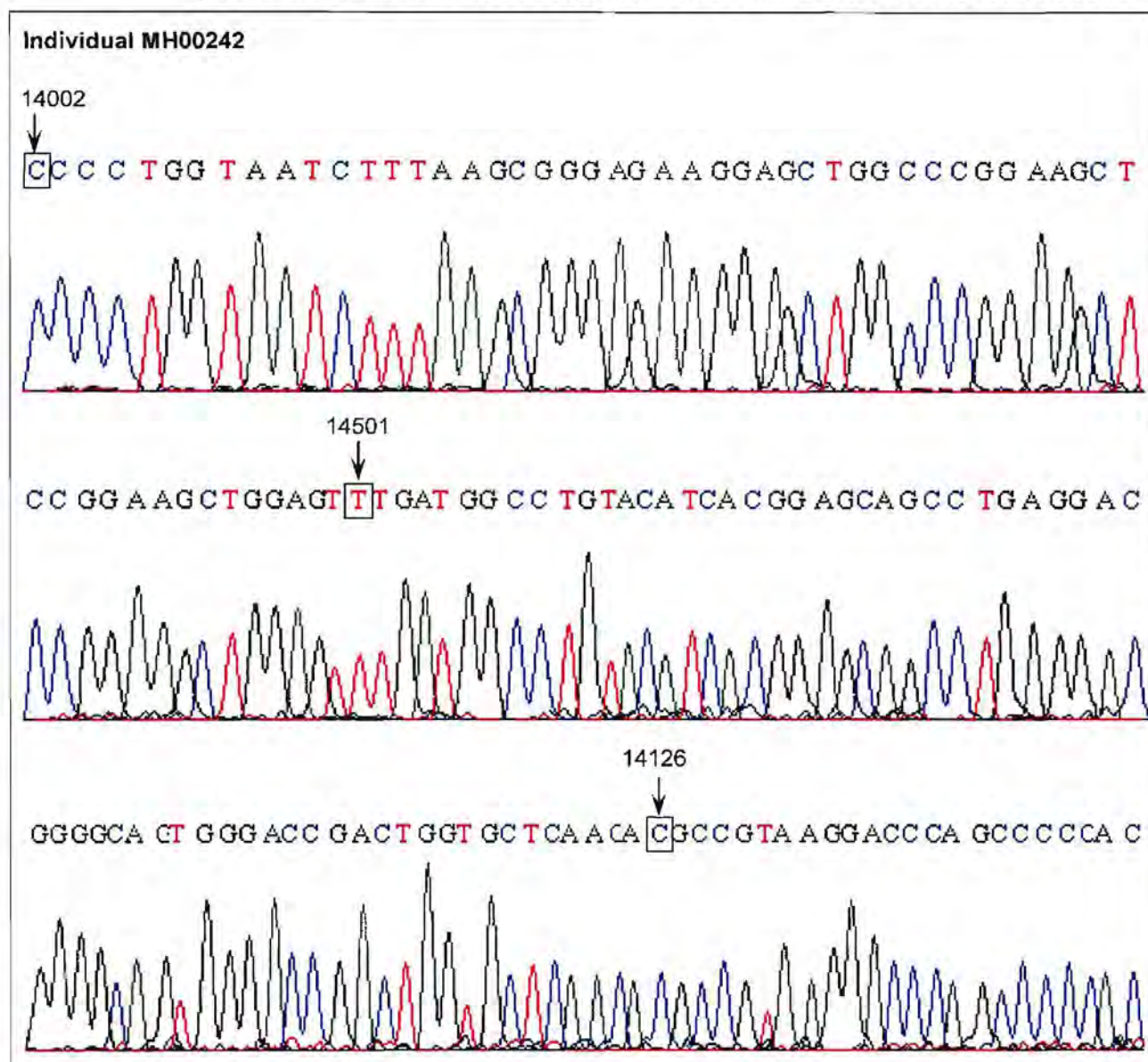
Figure 4.79: Photographic representation of amplified PCR products encompassing exon 96



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 MM overloading, as discussed in Sections 4.2 and 4.3 were observed. In addition, an artefact in the gel matrix, as indicated by the white asterisk (*), non-specific amplification and variation in amplification efficiency were observed, as described Sections 4.2 and 4.3.

The PCR product was subsequently sequenced, in order to investigate for the presence of reported mutations as well as to identify possible novel mutations that may be present in this region of the RYR1 gene. Sequencing was conducted using the forward primer (RYRex96F). A representative electropherogram obtained for individual MH00242, illustrating the nucleotide positions of previously reported alterations, is depicted in Figure 4.80. Exon 96 harbours two alterations that are associated with the MH phenotype. The Pro4668Ser alteration (Oyamada *et al.*, 2002) and the Phe4684Ser alteration (Monnier *et al.*, 2005) were respectively identified in single MH families. However, the Thr4709Met alteration was identified in a single CCD family (Zhou *et al.*, 2005), therefore all three alterations may be family-specific. Sequencing of all 15 MHS probands from South Africa indicated that none of the individuals analysed harboured any novel or reported alterations. Therefore, the observed alterations may be private to those specific families.

Figure 4.80: Representative electropherogram of exon 96 indicating the nucleotide positions of the Pro4888Ser, Phe4684Ser and Thr4709Met alterations



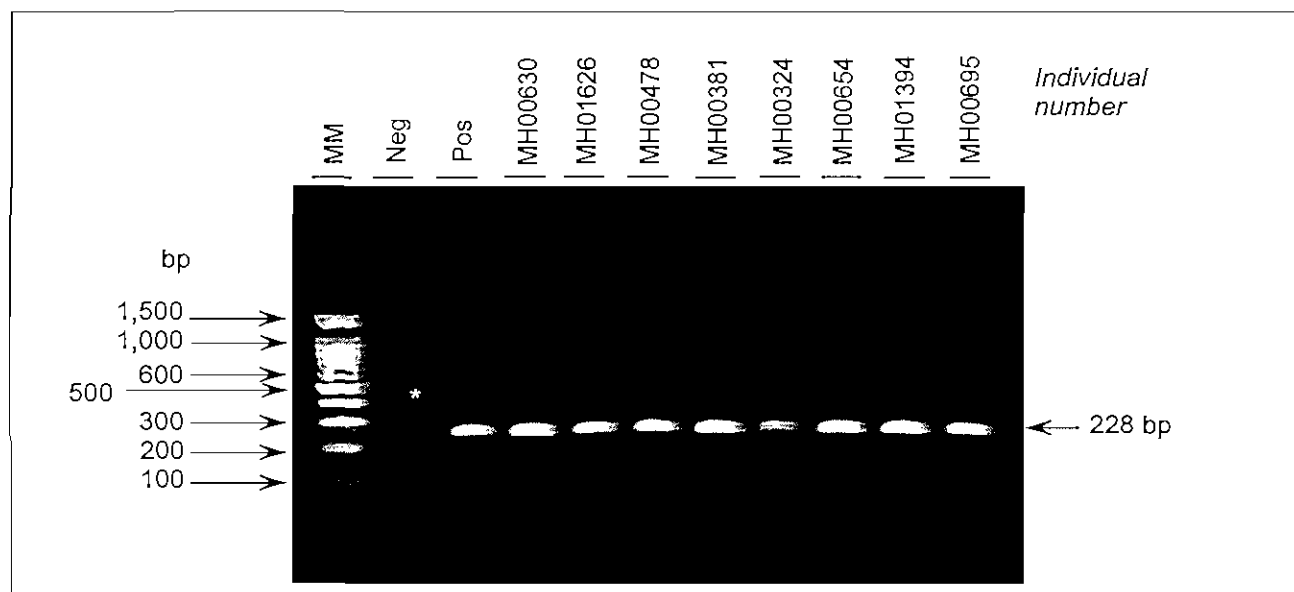
A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Pro4668Ser at nucleotide 14002; Phe4684Ser at nucleotide 14501 and Thr4709Met at nucleotide 14126. Sequences exhibited background peaks, which may have occurred due to reasons outlined in Section 4.5.

4.9.8 Exon 97 of the RYR1 gene

Currently, alterations resulting in MH have not been reported in exon 97 of the RYR1 gene, even though the exon resides in hotspot three. However, a single alteration that results in CCD susceptibility has been reported. The Lys4724Gln alteration occurs due to an A14170C nucleotide transition and has thus far been reported in one CCD family (Zhou *et al.*, 2005). As discussed in Section 3.5 (page 92), the two-step fast PCR protocol was used to amplify the fragment encompassing exon 97. The PCR conditions were optimised,

as discussed in Section 4.2 (page 159). Figure 4.81 is a photographic representation of the amplicon encompassing exon 97.

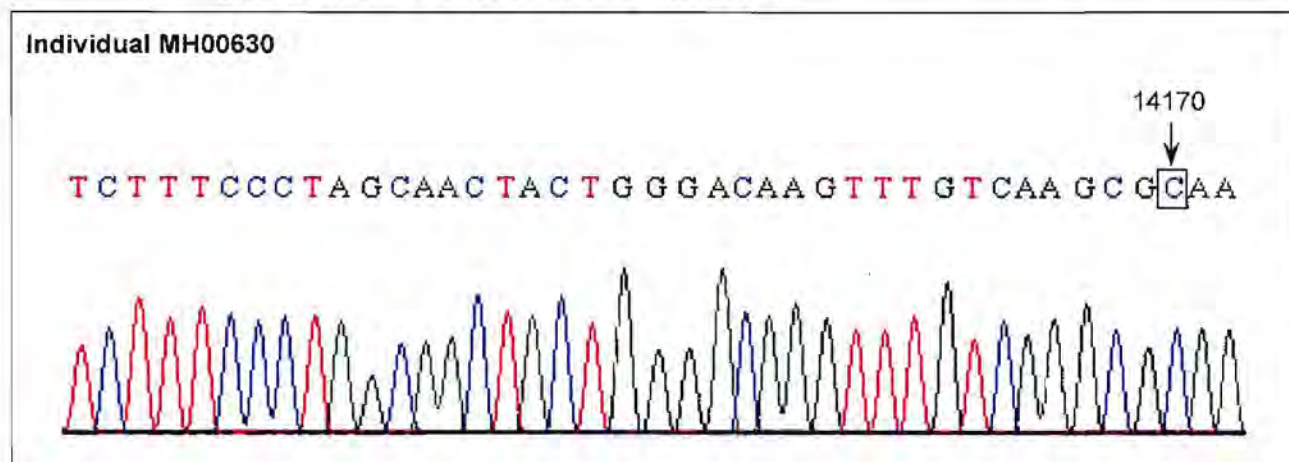
Figure 4.81: Photographic representation of amplified PCR products encompassing exon 97



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, fragment distortion and MM overloading were noted, as discussed in Sections 4.2 and 4.3.

A representative result generated via automated sequencing indicating the reported Lys4724Gln alteration is presented in Figure 4.82. None of the 15 MH probands that were sequenced harboured any novel alterations. Sequencing was conducted using the forward primer (RYRex97F). Thus far, alterations associated with MH have not been reported to occur in this region of the RYR1 gene. As discussed in Section 4.9.4 (page 244), since none of the 15 MH probands analysed in this study was diagnosed with CCD, it would be expected that the alteration would not be observed in any of the probands studied. In addition, alterations observed in exon 97 may result only in E-C uncoupling, which would result in the characteristic CCD phenotype.

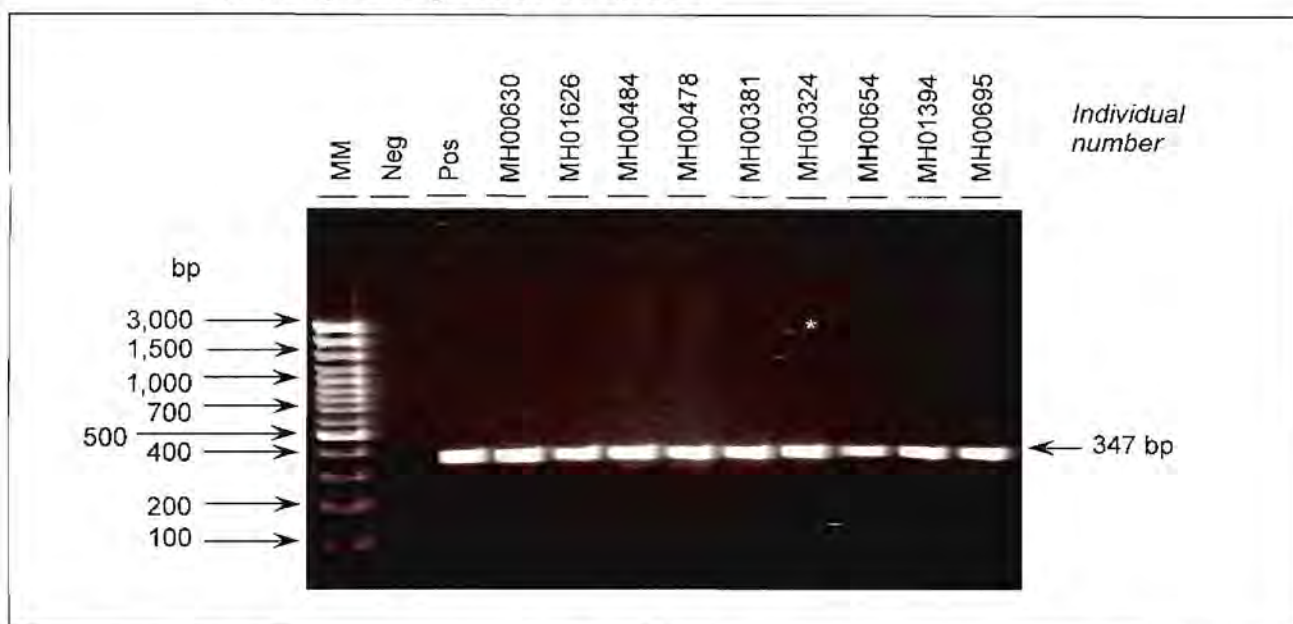
Figure 4.82: Representative electropherogram of exon 97 indicating the nucleotide position of the Lys4724Gln alteration



A = adenine; C = cytosine; G = guanine; T = thymine. Position of the nucleotide alteration that translates to the following amino acid alteration is indicated: Lys4724Gln at nucleotide 14170.

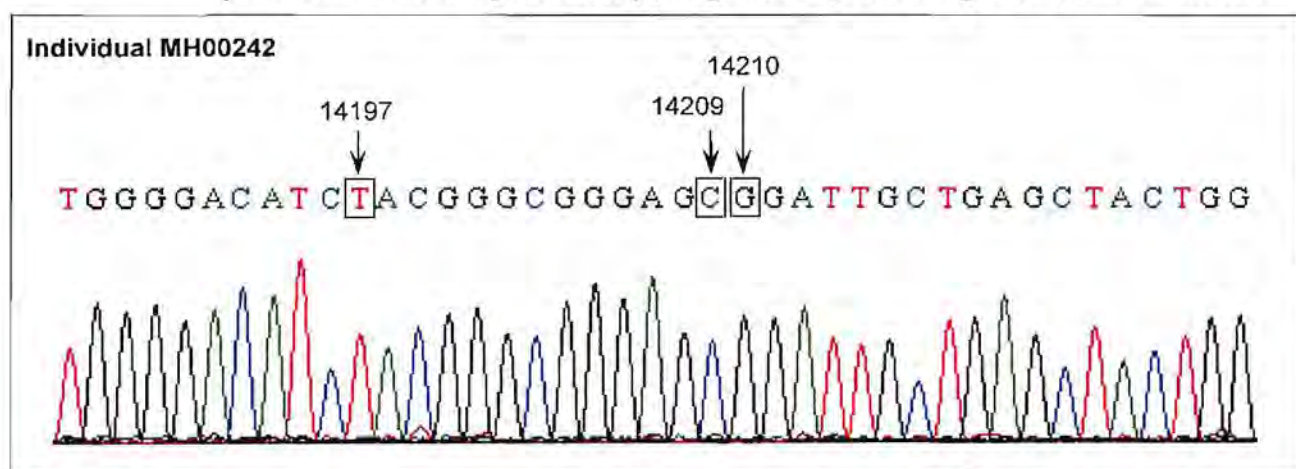
4.9.9 Exons 98 and 99 of the RYR1 gene

A 347 bp product encompassing both exons 98 and 99 was amplified via PCR using the protocol described in Section 4.2 (page 159). Figure 4.83 is a photographic representation of the amplicon encompassing exons 98 and 99. Exon 98 of the RYR1 gene harbours three alterations that may be associated with the MH phenotype, i.e. Arg2737Gln (Monnier *et al.*, 2005), Tyr4733Asp (Sambuughin *et al.*, 2005) and Arg4737Trp (Galli *et al.*, 2002). The alterations have been identified in one family from France, one family from North America and two families from Italy, respectively.

Figure 4.83: Photographic representation of amplified PCR products encompassing exons 98 and 99

Fragments were electrophoresed on a 2% agarose gel at $10 \text{ V} \cdot \text{cm}^{-1}$ 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. Artefacts such as an artefact in the gel matrix, as indicated by the white asterisk (*), fragment distortion, overloaded MM, non-specific amplification and fragment smears, as discussed in Sections 4.2 and 4.3 were encountered.

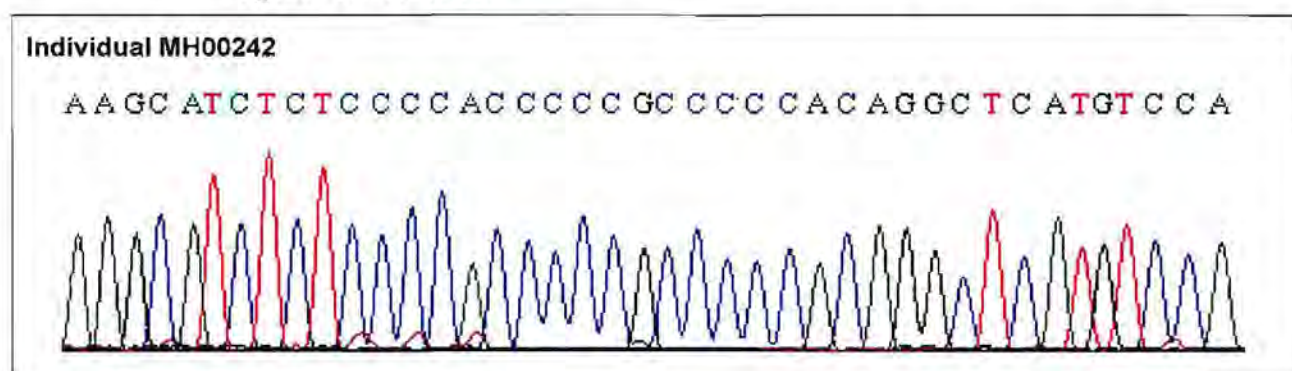
The amplified region was subsequently sequenced using the standard protocol. The reverse primer (RYRex98R) was used in the sequencing reaction, therefore sequences are depicted as the reverse complement. A representative electropherogram obtained for individual MH00242, indicating the nucleotide positions of previously reported alterations detected in exon 98, is indicated in Figure 4.84.

Figure 4.84: Representative electropherogram of exon 98 indicating the nucleotide positions of the Tyr4733Asp, Arg4737Trp and Arg4737Gln alterations

A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Tyr4733Asp at nucleotide 14197; Arg4737Trp at nucleotide 14209 and Arg4737Gln at nucleotide 14210.

A representative electropherogram obtained for individual MH00242, indicating a portion of the amplified region of exon 99, is indicated in Figure 4.85. None of the 15 individuals analysed in the study presented here harboured any reported or novel alterations associated with the MH phenotype. It is likely that the alterations that have been described for exon 98 are private to the specific family in which they were described. MHS alterations associated with exon 99 have never been reported in any population studied thus far. This finding could indicate that this region of the RYR1 gene does not play a role in the development of MH. However, as discussed in Section 4.8.2 (page 215), a minimum of 3,910 chromosomes should be screened in order to obtain 95% power. Therefore, alterations that may be associated with MH may not be detected if a small number of individuals are screened for the alteration.

Figure 4.85: Representative electropherogram illustrating a portion of the amplified region of exon 99



A = adenine: C = cytosine: G = guanine: T = thymine

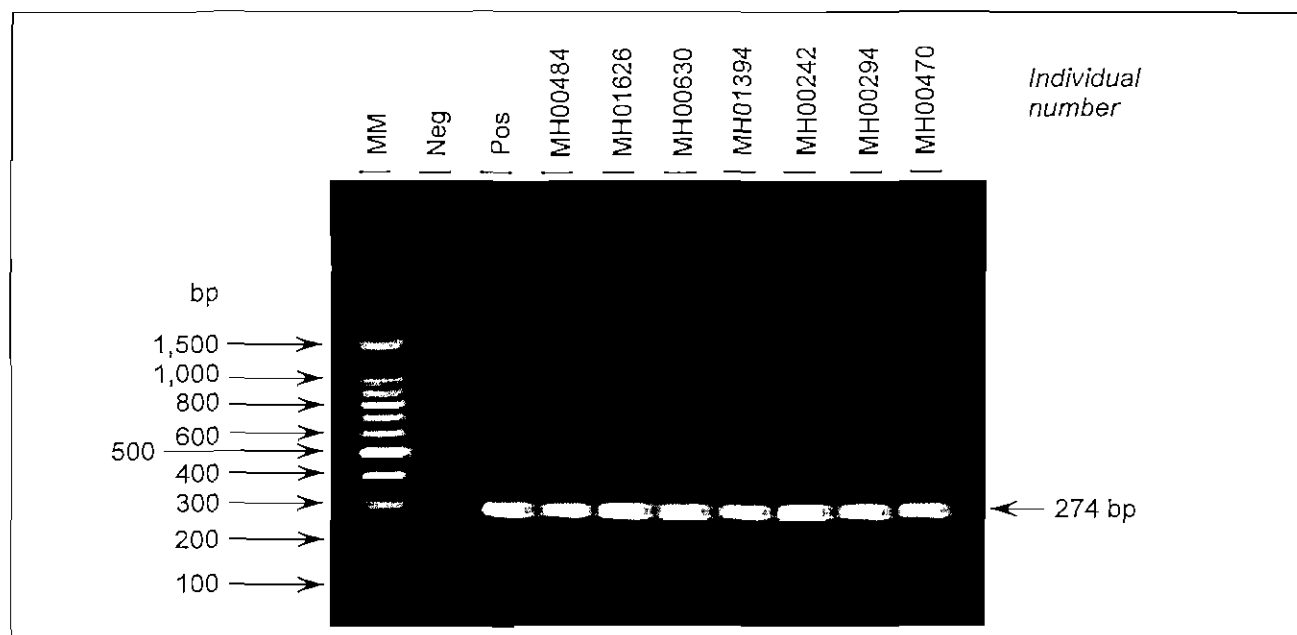
4.9.10 Exon 100 of the RYR1 gene

In order to detect novel alterations associated with MH in exon 100 in hotspot three, a region of 274 bp was analysed. This region harbours four alterations that may be associated with MH and four alterations that may be associated with CCD, as discussed in Section 3.7.70 (page 151). Alterations Leu4814Phe, Ile4817Phe and Leu4824Pro have been observed in UK pedigrees with MH (Halsall and Robinson, 2004). The alteration Thr4826Ile was identified in a large Maori pedigree and was described by Brown *et al.* (2000). PCR conditions were optimised as discussed in Section 4.2 (page 159) and the results are illustrated in Figure 4.86.

In Phase 2 of the MH research programme at the Centre for Genome Research, 43 samples obtained via PCR were successfully sequenced according to the standard protocol. In Phase 3 of the research programme, an additional two individuals were

analysed for novel and reported alterations that may occur in this region. As a mutation is observed in close proximity to the reverse primer, the forward primer (RYRex95F) was used for sequencing.

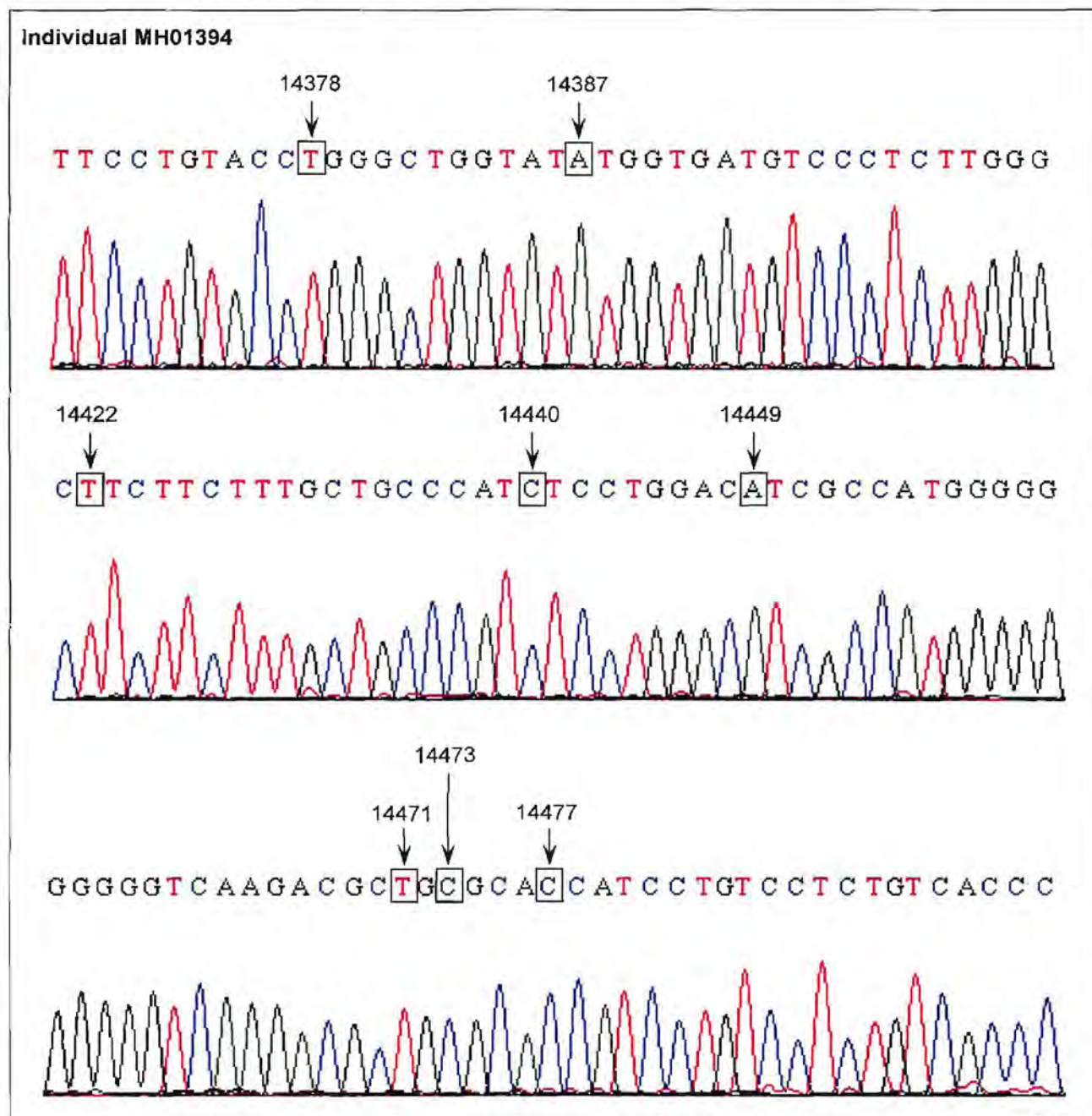
Figure 4.86: Photographic representation of amplified PCR products encompassing exon 100



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. Non-specific amplification, fragment distortion and MM overloading were observed, as discussed in Sections 4.2 and 4.3.

A representative electropherogram obtained for individual MH01394, illustrating the nucleotide positions of the previously reported alterations that were observed within the amplified region for exon 100, is presented in Figure 4.87. None of the MHS individuals that were sequenced during Phase 2 or Phase 3 harboured the Leu4814Phe, Ile4817Phe or Leu4824Pro alterations. All three alterations have been observed in single UK pedigrees (Halsall and Robinson, 2004) and may therefore indicate mutations specific to those families. In addition, alterations that have been reported in individuals diagnosed with CCD were not identified in the study presented here. As none of the 15 MH probands analysed presented with any history of the CCD phenotype, the absence of these alterations would be expected.

Figure 4.87: Representative electropherogram of exon 100 indicating the nucleotide positions of the Leu4793Pro, Tyr4796Cys, Phe4808Asn, Leu4814Phe, Ile4817Phe, Leu4824Pro, Arg4825Cys and Thr4826Ile alterations



A = adenine; C = cytosine; G = guanine; T = thymine. Positions of the nucleotide alterations that translate to the following amino acid alterations are indicated: Leu4793Pro at nucleotide 14378; Tyr4796Cys at nucleotide 14387; Phe4808Asn at nucleotide 14422; Leu4814Phe at nucleotide 14440; Ile4817Phe at nucleotide 14449; Leu4824Pro at nucleotide 14471; Arg4825Cys at nucleotide 14473 and Thr4826Ile at nucleotide 14477.