CHAPTER FOUR

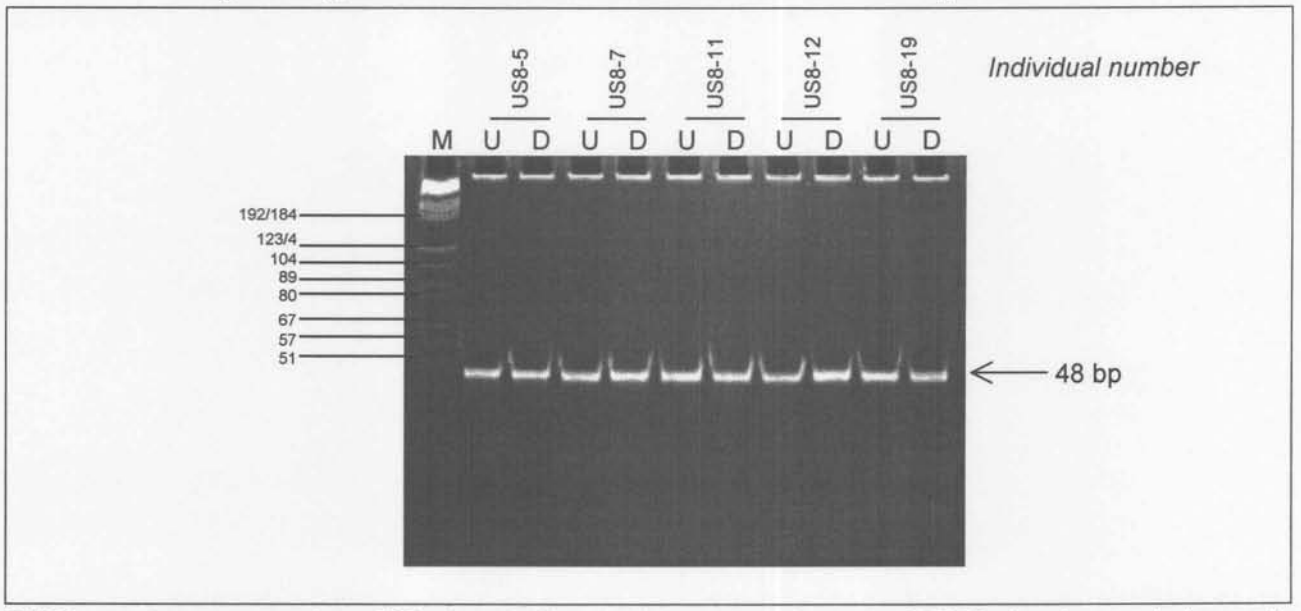
RESULTS AND DISCUSSION

Nine reported missense mutations in the skeletal muscle ryanodine receptor (RYR1) gene were investigated in this study. Results obtained for the seventeen families included in this study are presented and discussed in the respective paragraphs of this chapter. PCR was performed as described in chapter three paragraph 3.3. All templates were amplified via PCR at an optimised annealing temperature of 64°C unless otherwise stated.

4.1 Cys35Arg mutation in the skeletal muscle ryanodine receptor (RYR1) gene

The Cys35Arg mutation results in the gain of an *Aci I* restriction site. In homozygous normal individuals the 48 bp PCR amplified DNA fragment would not be cleaved by the *Aci I* restriction endonuclease (Figure 4.1). However, three fragments (48 bp, 25 bp and 23 bp respectively) will be observed after *Aci I* restriction if the mutation is present in a heterozygous affected individual. Only the two smaller fragments (25 bp and 23 bp) would be observed if the individual carries the mutation in the homozygous state.

Figure 4.1: Photographic representation of results generated for detection of the Cys35Arg mutation within exon two of the RYR1 gene



RFLP fragments were separated on a 20% polyacrylamide gel, which was electrophoresed at 250 V for three hours and subsequently stained with EtBr for 30 min. "M" indicates the molecular weight marker (pBR322/*Hae III*) used to size the fragments. "U" indicates the undigested PCR product and "D" the PCR product digested with 10 units of *Aci I*.

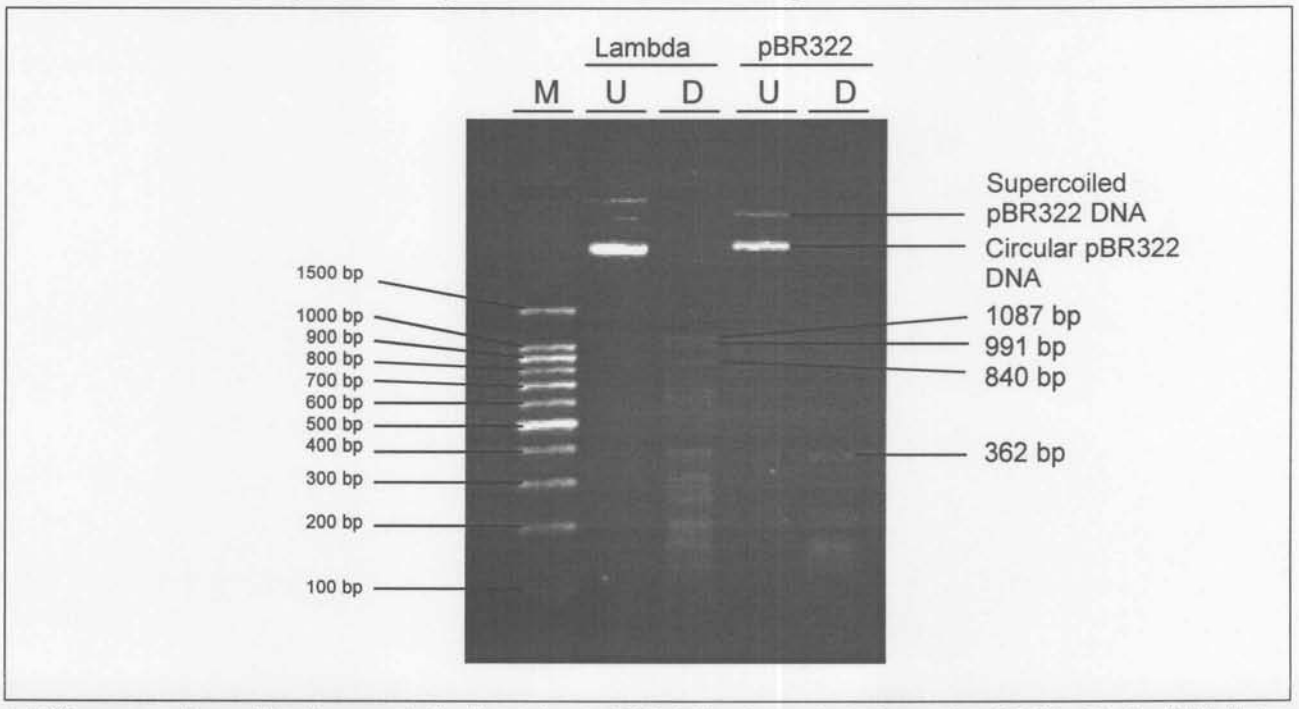
The Cys35Arg mutation was not observed in any of the seventeen families investigated in this study. Results obtained for individuals from family US-8 have been used to illustrate

the typical results generated for MHS families included in this study and are presented in Figure 4.1.

This figure illustrates that the 48 bp PCR product was not digested with the *Aci I* restriction enzyme. It was subsequently concluded that the individuals included in this study were negative for the mutation. Unfortunately no positive control for the Cys35Arg mutation was available. It is, therefore, possible that the 48 bp fragments were not digested due to the loss of, or decrease in, enzyme activity. For this reason the enzyme activity of the *Aci I* enzyme required verification.

Lambda DNA and pBR322 DNA were digested with the *Aci I* enzyme and electrophoresed on an agarose gel. These results are presented in Figure 4.2. Lambda DNA and pBR322 DNA has 516 and 67 recognition sites for *Aci I* respectively (Polisson and Morgan, 1990). The fragment sizes for digested lambda DNA ranged from 1087 bp to 4 bp and for digested pBR322 DNA from 362 bp to 6 bp. A smear was observed on the gel in the lane that contained the digested lambda DNA. This is due to the large number of *Aci I* recognition sites (516) present in the lambda DNA. In the lane containing the undigested pBR322 DNA two fragments were observed, representing supercoiled and circular DNA, as indicated in Figure 4.2. The DNA from both the lambda DNA and the pBR322 DNA were completely digested. The exact fragment sizes could not be determined after digestion.

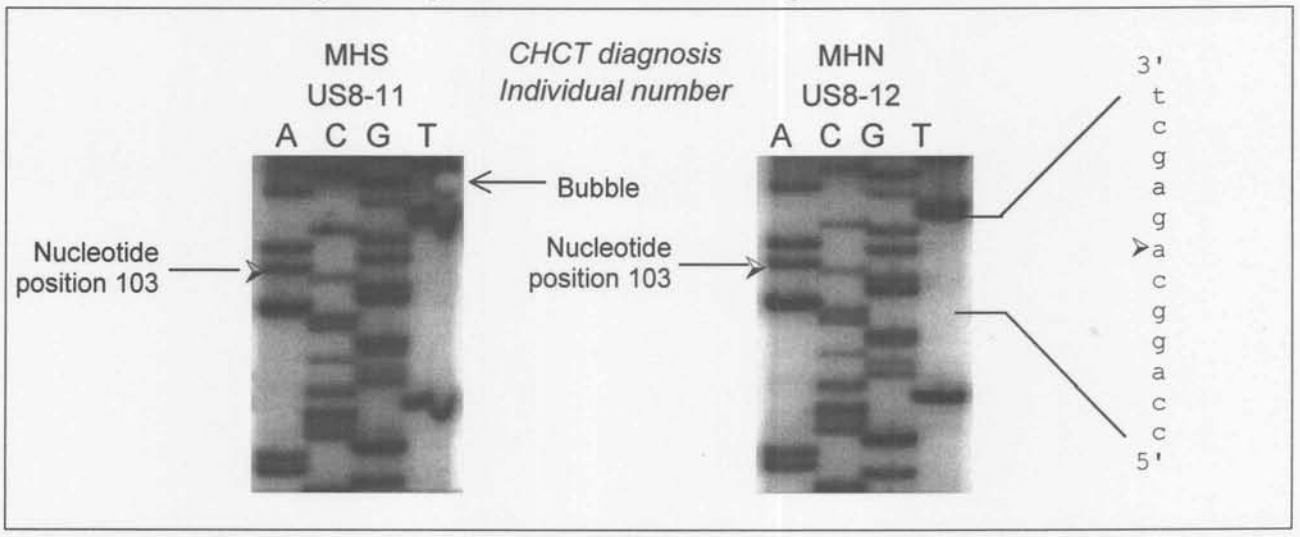
Figure 4.2: Photographic representation of *Aci I* digested lambda and pBR322 DNA



A 1.6% agarose gel was utilised to separate the digested vector DNA. This gel was electrophoresed at 100 V for 30 min. "M" indicates the molecular weight marker - 100 bp ladder (Promega), "U" the undigested vector DNA and "D" the digested vector DNA.

One MH positive (US8-11) and one MH negative (US8-12) individual from family US8 were selected for sequencing to confirm the obtained RFLP analysis results. The sequence generated for these two individuals is presented in Figure 4.3. An additional fragment at position 103 in the G lane of the sequence would indicate the presence of the Cys35Arg mutation in a heterozygous affected individual. A homozygous affected individual would display only the additional fragments in the G lane and no fragment in the A lane of the sequencing gel. No additional fragments were observed for either of the individuals of whom results are presented in Figure 4.3. This confirmed the results obtained via RFLP analysis for the individuals included in this study, as listed in Table 4.3 (page 128).

Figure 4.3: Autoradiographs of chain termination sequencing utilised for detection of the Cys35Arg mutation in the RYR1 gene



MHN = MH normal; MHS = MH susceptible; CHCT = Caffeine halothane contracture test; A = adenine; C = cytosine; G = guanine and T = thymidine. The reverse primer was selected as the sequencing primer and the samples were labelled with α^{32}P-dCTP. Gels were electrophoresed for 3 hours at 60 W. An arrowhead (➢) indicates nucleotide position 103. The bubble in the gel is responsible for the aberrant migration pattern in the T lane.

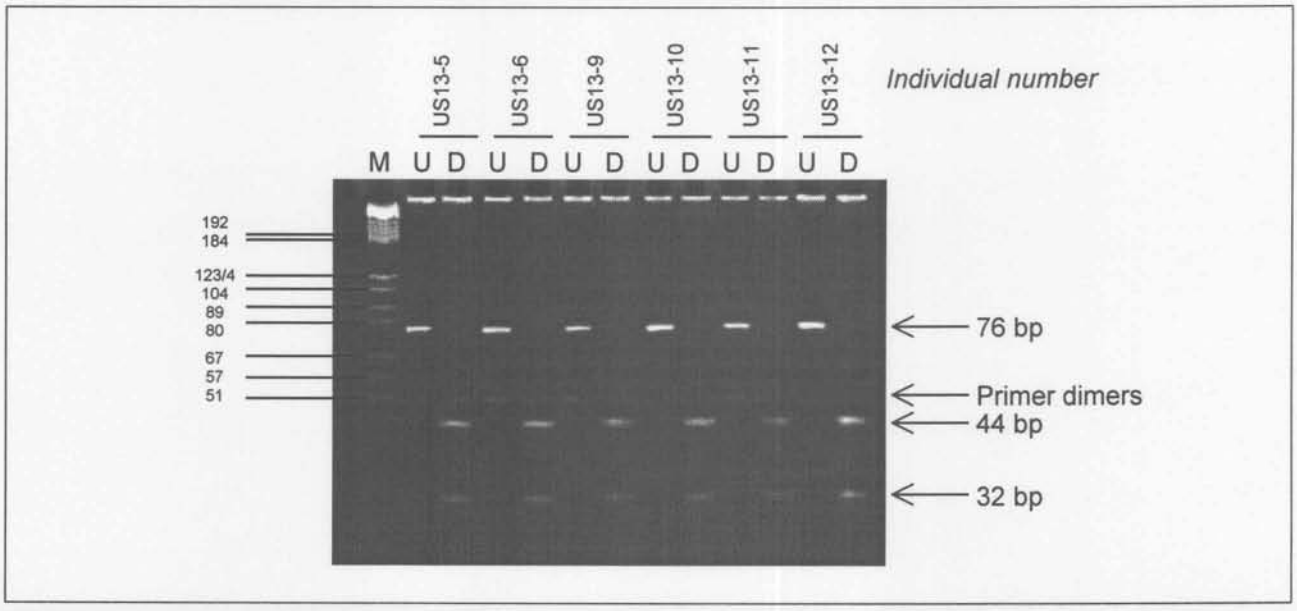
The Cys35Arg mutation is the most N-terminal mutation of the RYR1 protein detected to date. This mutation has only been reported in one MHS family which is of Sicilian origin (Lynch *et al.*, 1997). These authors did not observe the Cys35Arg mutation in 65 unrelated MHS patients investigated and suggested that this mutation might be unique to that particular Sicilian family. The absence of this mutation in the seventeen families investigated in this study, and the MHS individuals screened worldwide by other investigators, supports the theory that the mutation may be family specific. This implies that the Cys35Arg mutation may be a recent mutational event that occurred within the large family in which it was described by Lynch *et al.* (1997). If this mutation did indeed originate within this Sicilian family and no family members harbouring the mutation emigrated to other regions the mutation would not be expected to be present in other populations.

The Cys35Arg mutation meets most of the requirements of a causative missense mutation as discussed in paragraph 2.6.1.2. It could, however, not be demonstrated to be causative with transfection studies (Tong *et al.*, 1997), whilst other mutations within the RYR1 gene resulted in an increase in sensitivity to caffeine and halothane. It is therefore possible that the Cys35Arg mutation may be a rare polymorphism even though it was not observed in the 200 normal chromosomes originally analysed by Lynch *et al.* (1997). These authors stated that the normal chromosomes were obtained from healthy individuals and it was assumed that they were representative of the general population.

4.2 Arg163Cys mutation in the skeletal muscle ryanodine receptor (RYR1) gene

Genomic DNA was amplified via PCR at an optimised annealing temperature of 68°C. A 76 bp PCR amplified product was subsequently digested with the *Bst UI* restriction enzyme. The Arg163Cys mutation involves a C to T nucleotide substitution, resulting in the loss of a *Bst UI* restriction site. Therefore, upon digestion with *Bst UI* two fragments of 44 bp and 32 bp respectively were observed in homozygous normal individuals (Figure 4.4). Detection of an additional fragment of 76 bp would indicate heterozygous affected individuals. In homozygous MH affected individuals only the 76 bp fragment would be observed as the mutation results in the loss of the restriction endonuclease site in both copies of the RYR1 gene.

Figure 4.4: Photographic representation of results generated for detection of the Arg163Cys mutation within exon six of the RYR1 gene



RFLP fragments were separated on a 20% polyacrylamide gel, which was electrophoresed at 250 V for three hours and subsequently stained with EtBr for 30 min. "M" indicates the molecular weight marker (pBR322/*Hae III*) used to size the fragments. "U" indicates the undigested PCR product and "D" the PCR product digested with 10 units of *Bst UI*.

The RFLP results obtained for individuals investigated in this study are listed in Table 4.3 (page 128). Only the 44 bp and 32 bp fragments were observed. It was therefore concluded that the Arg163Cys mutation was not present in any of these individuals. The results obtained for family US13 are presented in Figure 4.4.

An additional fragment was observed in the undigested samples from individuals US13-6 and US13-11. These fragments are due to secondary structures formed by the primers, such as primer dimers - including self-dimers and cross dimers. The primer dimers migrate through the gel at a size equivalent to ca. 50 bp as indicated in Figure 4.4. All the secondary structures that could be formed by the primers are depicted in Figure 4.5 as well as the calculated free energy (ΔG) for each primer dimer.

Figure 4.5: Secondary structures formed by the R163CF1 and R163CR1 primers

Self-dimers

R163CF1 forward primer dimers

(a)
```
   5' tccaagcagaggtctgaaggagaa 3'
      |||                 :::
3' aagaggaagtctggagacgaacct
```
ΔG = -5.64 kcal.mol^{-1}
2nd Most stable dimer

(b)
```
  5' tccaagcagaggtctgaaggagaa 3'
       :   ||||  ::::   :
3' aagaggaagtctggagacgaacct 5'
```
ΔG = -5.12 kcal.mol^{-1}

(c)
```
5' tccaagcagaggtctgaaggagaa 3'
             |||     :::
       3' aagaggaagtctggagacgaacct 5'
```
3' Dimer
ΔG = -4.17 kcal.mol^{-1}

R163CR1 reverse primer dimers

(d)
```
                  5' agcgctcggaggagacactgacaa 3'
                     ||||||
3' aacagtcacagaggaggctcgcga
```
ΔG = -13.08 kcal.mol^{-1}
Most stable dimer

Cross dimers

R163CF1 and R163CR1 primer dimers

(e)
```
5' tccaagcagaggtctgaaggagaa 3'
        |||  :       :::
  3' aacagtcacagaggaggctcgcga 5'
```
ΔG = -4.55 kcal.mol^{-1}
3rd Most stable dimer

(f)
```
5' tccaagcagaggtctgaaggagaa 3'
      :   ||||
   3' aacagtcacagaggaggctcgcga 5'
```
ΔG = -4.51 kcal.mol^{-1}

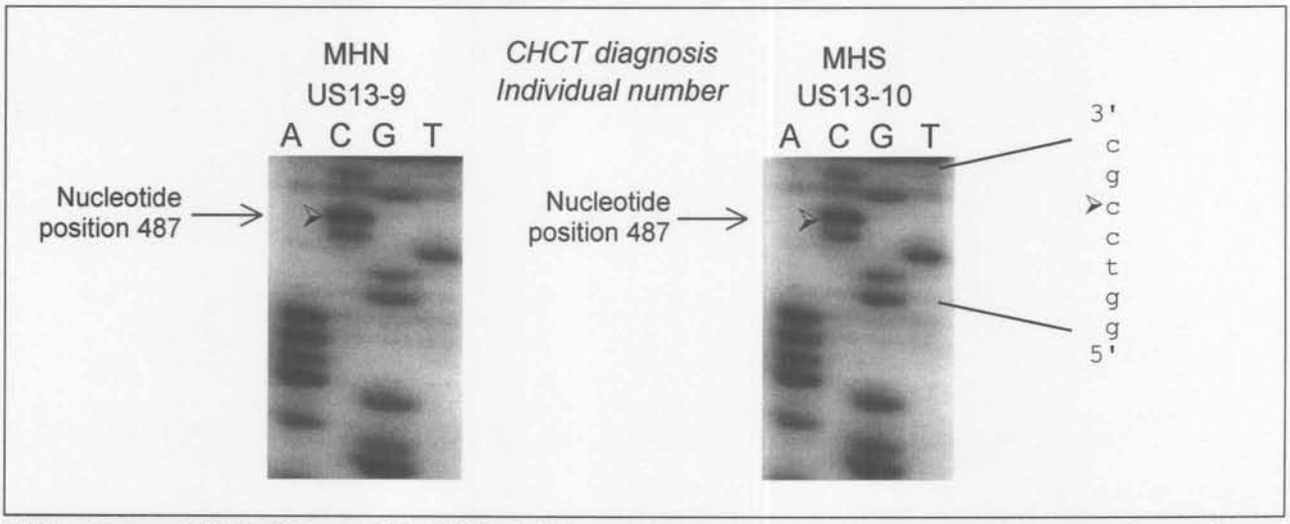
(g)
```
5' ttcaagcagaggtctgaaggagaa 3'
          |||  :
     3' aacagtcacagaggaggctcgcga 5'
```
ΔG = -3.91 kcal.mol^{-1}

Primer sequences are listed in Table 3.3. ΔG indicates the free energy.

The chemical stability of a primer dimer is directly related to the free energy - a thermodynamic property of the primer dimer (Bohinski, 1987). A negative ΔG value (-ΔG) occurs when chemical reactions release free energy. These reactions are exergonic, favoured thermodynamically and occur spontaneously (Bohinski, 1987; Armstrong, 1989). Therefore the lower the ΔG of the primer dimer, the more stable it will be. Primer dimers "a", "d" and "e" in Figure 4.5, indicate the most stable primer dimers, as determined by their ΔG values. A single 3' dimer, "c" in Figure 4.5, was identified. Formation of the 3' dimer is problematic as this primer dimer could allow extension at the 3' end dimer leading to the formation of a 34 bp product. This class of PCR artefacts may obscure analysis of the results obtained. However, the additional fragments are only observed in undigested samples and therefore do not interfere with the identification of the true PCR amplified fragments. If these additional fragments were, however, visible in the digested samples they would be larger than the two fragments (44 bp and 32 bp) generated after digestion with the *Bst UI* enzyme. Care should, however, be taken when analysing RFLP results generated with samples containing stable primer dimer structures.

Individuals US13-9 and US13-10 were selected for sequencing to verify the RFLP analysis results listed in Table 4.3 (page 128). Representative autoradiographs of the generated sequence are depicted in Figure 4.6. No additional fragments at position 487 in the T lanes of the sequence were observed. The sequencing data for individuals US13-9 and US13-10 confirmed that they were both homozygous normal, as determined via RFLP analysis.

Figure 4.6: Autoradiographs of chain termination sequencing utilised for detection of the Arg163Cys mutation in the RYR1 gene



MHN = MH normal; MHS = MH susceptible; CHCT = Caffeine halothane contracture test; A = adenine; C = cytosine; G = guanine and T = thymidine. The forward primer was selected as the sequencing primer and the samples were labelled with α^{32}P-dCTP. Gels were electrophoresed for 2 hours at 60 W. An arrowhead indicates nucleotide position 487.

Censier *et al.* (1998) presented evidence that MHN derived primary muscle cell cultures transfected with the Arg163Cys mutation caused a two-fold increase in the sensitivity to halothane. This provided evidence that the Arg163Cys mutation is a causative mutation for MH. Fagerlund *et al.* (1994) reported the presence of this mutation in a Danish family with no history of CCD suggesting that the Arg163Cys mutation caused MHS in this family. Manning *et al.* (1998a) estimated that this mutation occurs in 2% of MHS families and is therefore one of the most frequent MHS mutations described to date.

The Arg163Cys mutation has also been observed in MHS families with a history of CCD (Quane *et al.*, 1993; Quane *et al.*, 1994a; Barone *et al.*, 1999). The possibility of an epistatic relationship between the different MH mutations and its contribution to the MH phenotype cannot be ignored. Consequently the individuals in this study were investigated for the presence of the Arg163Cys mutation even though only one family, MH105, displayed linkage to the chromosome 19q13.1 region where the RYR1 gene is located.

At the present it remains unclear whether the Arg163Cys mutation causes both MH and CCD. Quane *et al.* (1993) reported that this mutation is responsible for both MH and CCD. Although the mechanism by which the Arg163Cys mutation causes MH is unclear, the possibility that the mutation may also cause CCD, or contribute to the CCD phenotype, cannot be ruled out.

This mutation in the RYR1 gene results in hypersensitivity of the Ca^{2+}-release channel to triggering agents. The increased sensitivity of the RYR1 channel causes the release of excess Ca^{2+} into the cytosol from the SR due to the extended open-time of the RYR1 channel. The Arg163Cys mutation may therefore be responsible for the pathological changes observed in the SR and T-tubules of CCD patients. The RYR1 receptor is involved in the EC-coupling required for muscle contraction as described in paragraph 2.5. A mutation in the region involved in the interaction could result in diminished EC-coupling and therefore diminished muscle contraction, resulting in muscle weakness and poor reflexes associated with CCD. (Quane *et al.*, 1993)

However, segregation analysis performed by Quane *et al.*, (1993) suggests that the Arg163Cys mutation is causative of MH. It therefore seems plausible that the Arg163Cys mutation causes MH and predisposes individuals to CCD. However, other genetic factors such as genes coding for proteins involved in EC-coupling or the homeostasis of

intracellular Ca^{2+} levels could interact with the Arg163Cys mutation resulting in the expression of CCD and MH. (Quane *et al.*, 1993).

The identification of this mutation in subsequent MHS or CCD families might shed light on the mechanism by which the Arg163Cys mutation is responsible for MH or CCD. It may also indicate whether an epistatic relationship between this mutation and other mutations elsewhere contribute to the MH phenotype or the CCD phenotype.

4.3 Gly248Arg mutation in the skeletal muscle ryanodine receptor (RYR1) gene

The reported method of detection for the Gly248Arg substitution was direct sequencing. However, this was not the method of choice in this study (Gillard *et al.*, 1992). Direct sequencing was not cost effective even though it is the most sensitive and specific technique for the detection of mutations. Certain authors (Gillard *et al.*, 1992; Quane *et al.*, 1997; Barone *et al.*, 1999) utilised SSCP to detect the Gly248Arg mutation. This method, however, was not utilised for detection of the mutation in this study. As discussed below, SSCP is not specific for the Gly248Arg mutation nor is it sensitive enough.

Substitutions may alter the folding of a single stranded DNA molecule, even when a single base is involved, resulting in the formation of different three-dimensional conformers. The electrophoretic mobility of these different conformers influences the ability of single base substitutions that are detectable via SSCP. The sensitivity of SSCP to detect substitutions is also dependent on the length of the fragment, with longer fragments resulting in lower sensitivity. More than 90% of single base substitutions are detectable in fragments of 200 bp, and more than 80% in 400 bp fragments (Hayashi, 1992; Hayashi and Yandell, 1993; Hayashi, 1994; Hayashi, 1996). Since the amplified product for this particular mutation is 228 bp in length it should therefore be possible to detect the presence of a single base substitution in the PCR fragment with a sensitivity of between 80% and 90%. However, as SSCP is not specific for any mutation it is not possible to determine via SSCP analysis alone whether the aberrant conformation pattern that might be observed would be as a result of the known mutation, possible novel mutations or polymorphisms. SSCP was therefore not the preferred method of detection of the Gly248Arg mutation.

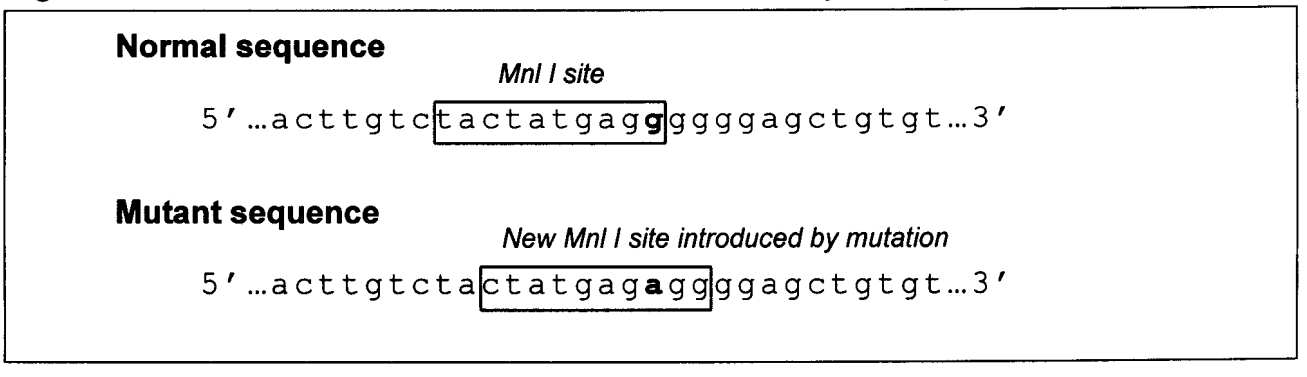
Zhang *et al.* (1993) developed a diagnostic assay to detect the mutation via the use of allele specific PCR amplification. Primers are designed with the forward primer differing by one base (the normal or the mutated base) at the 3' end. A common reverse primer is

used. If the forward primer has an A at the 3' end, representing the mutation, a 178 bp product is generated in affected individuals. Although the allele specific PCR amplification assay is highly specific it is not sensitive enough. The primers designed for this assay only amplifies one allele, either the mutant or the normal allele, depending on which allele the primer was designed to anneal to. Heterozygous and homozygous individuals can therefore not be distinguished unless a primer is designed for the opposite allele, and the assay repeated. Furthermore, the PCR conditions utilised to perform the reaction have to ensure high specificity and therefore high annealing temperatures are essential.

The absence of a fragment could imply the absence or presence of the mutation depending on which 3' base was incorporated in the primer. However, the absence of a PCR fragment could also be due to problems encountered during the PCR reaction. Optimisation and the reproducibility of these assays are imperative. It is therefore essential that controls be included during PCR to ensure that the results obtained are an accurate reflection of mutation status. This assay is useful in high throughput scenarios for diagnostic analyses when the presence or absence of the mutation is the only interest. Determination of the exact genotype and distinction between heterozygous and homozygous affected individuals is, however, essential in genetic diagnosis as it has implications for genetic counselling. For this reason, allele specific PCR amplification of only one allele (either normal or mutant) would be inappropriate as a detection method in a molecular diagnostic setting.

Sei *et al.* (1998) developed a PCR based RFLP method of detection for the Gly248Arg mutation via PCR-modified restriction site (PMRS) analysis. In principle this method involves the modification of a primer to introduce or eliminate a restriction site upon amplification (Haliassos *et al.*, 1989). The G to A substitution responsible for the Gly248Arg mutation eliminates a *MnlI* restriction site (N_6GAGG). However, the mutation also introduces a new *MnlI* site close to the original site as illustrated in Figure 4.7.

Figure 4.7: Restriction sites abolished and created by the Gly248Arg mutation



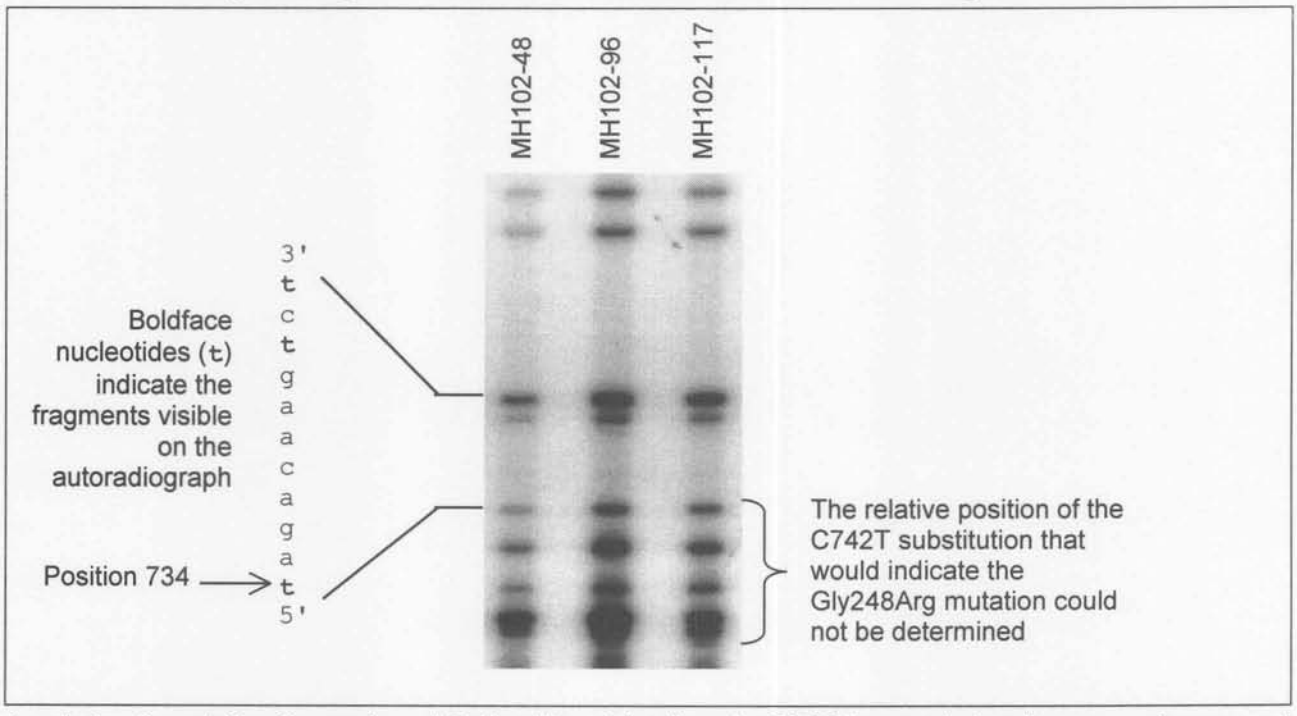
Abolishment of the restriction site could therefore not be utilised to detect this mutation. Designing a modified reverse primer that does not generate the new *Mnl I* site formed by the mutant but still abolish the *Mnl I* site in the normal sequence would, however, allow for the detection of the mutation via RFLP. This method of detection for the Gly248Arg mutation would be favoured, as it is highly sensitive and specific for this mutation. This modified RFLP assay for detection of the Gly248Arg mutation was first described by Sei *et al.* in 1998. At that time several individuals selected for this study were already screened via single lane sequencing (SLS). It was therefore decided that the remainder of the samples should be screened in a similar manner. Furthermore, SLS screening had the potential advantage of also detecting other possible substitutions involving an adenine nucleotide.

A single lane sequencing approach seemed the most appropriate mutation detection method at the time as such an approach was sensitive, specific and cost effective. The Gly248Arg mutation results in the substitution of a G to an A (Gillard *et al.*, 1992) and could therefore be detected via the BESS T analysis. This analysis is based on the principle that most point mutations involve changes in thymidine (Hawkins and Hoffman, 1997; Vaughan and McCarthy, 1998). Therefore if the T nucleotides were replaced with uracil (U) nucleotides the point mutations involving changes in the T nucleotides could be readily identified as the uracil nucleotides are removed from DNA by the DNA repair enzyme Uracil N-glycosylase. This results in the creation of an abasic site, located at the site of uracil incorporation. Defined fragments are generated by digestion of PCR product with the Endonuclease IV enzyme resulting in the cleavage of the phosphodiester bond at the abasic site. One of the primers is radioactivity end labelled with γ^{32}P-dATP and separated on a standard sequencing type gel. A DNA fragment ladder, identical to the T lane of a sequencing reaction, is generated. The presence or absence of a fragment, or the change in intensity from the normal fragments, would indicate the presence and location of a mutation. When fragments from a normal control are compared to other sample fragments, a difference in intensity would indicate the presence of a mutation in heterozygous individuals.

Since the aim of the study was the detection of specific reported mutations only the appropriate primer, in the case of the Gly248Arg mutation the reverse primer, was end labelled to detect a possible substitution of a cytosine with a thymidine at position 742 in the reverse sequence. The results obtained utilising this technique could not be analysed, as the mutation site is located too close to the end labelled primer, consequently obscuring

the region of interest. In Figure 4.8 the results obtained via BESS T analysis are displayed. No accurate interpretation could be made from the autoradiographs, as the relative position of C742 could not be determined on this autoradiograph. It seemed as though several additional fragments were present in the region close to the reverse primer. Selected samples were again sequenced with the reverse primer to determine whether there were indeed additional fragments in this region. However, many compressions were again observed in the 40 bp nucleotide sequence close to the reverse primer. It was therefore not possible to analyse the sequence close to the reverse primer. However, when the forward primer was utilised as the sequencing primer no additional fragments were observed in the A lane of the sequence. This indicated that the BESS T scan could not be used to detect the presence of the Gly248Arg mutation unless another reverse primer was designed that was further away from the codon (ggg) that codes for the Gly248 amino acid. The fragments could be analysed up to the T fragment at position 734. Investigation of the opposite strand with the forward primer would not indicate the presence of the mutation as a guanine (G) is substituted with an adenine (A) and the A residue could not be detected via BESS T analysis.

Figure 4.8: Autoradiograph of BESS T analysis generated for detection of the Gly248Arg mutation within exon nine of the RYR1 gene

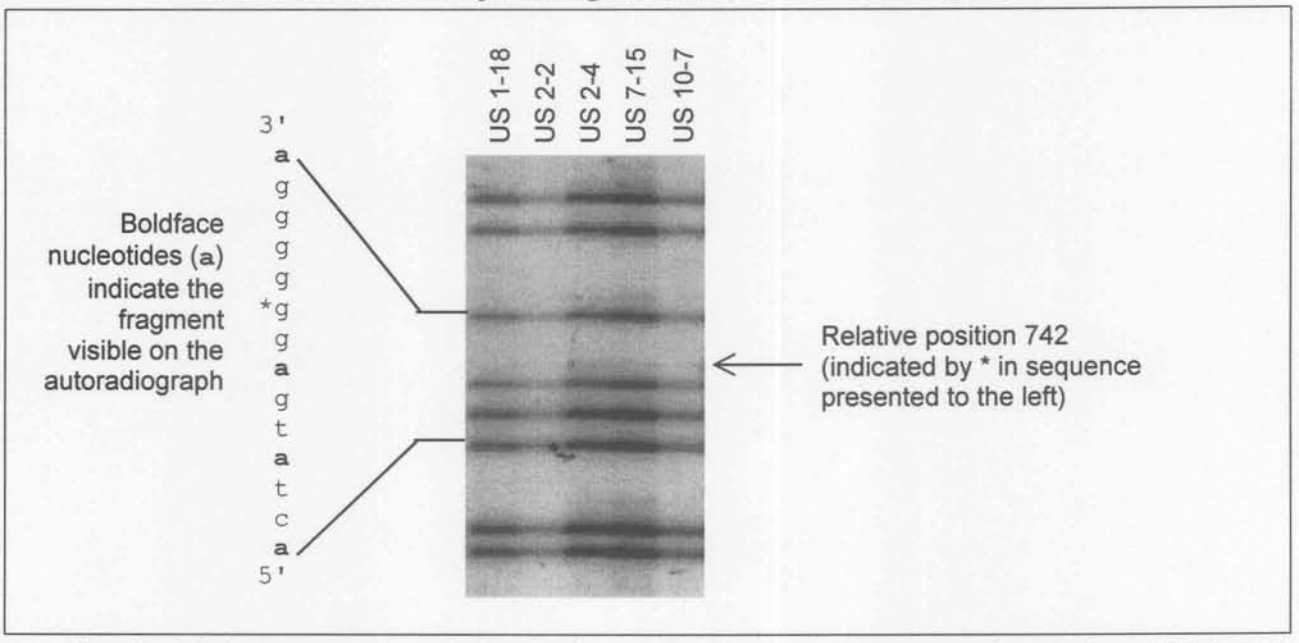


A = adenine; C = cytosine; G = guanine and T = thymidine. Autoradiograph of BESS T scan gel where the reverse primer was end labelled with γ^{32}P-dATP. The gel was electrophoresed for 1 hour 30 min at 60 W.

However, if only the single lane of interest, in this case A, was sequenced with the forward primer an additional fragment at position 742 would indicate the presence of the Gly248Arg mutation. In addition the rest of the fragment could be analysed for the

presence of possible known and new polymorphisms. The results generated for the selected individuals investigated via single lane sequencing of the A lane are presented in Figure 4.9.

Figure 4.9: Autoradiograph of single lane sequence analysis generated for detection of the Gly248Arg mutation in the RYR1 gene



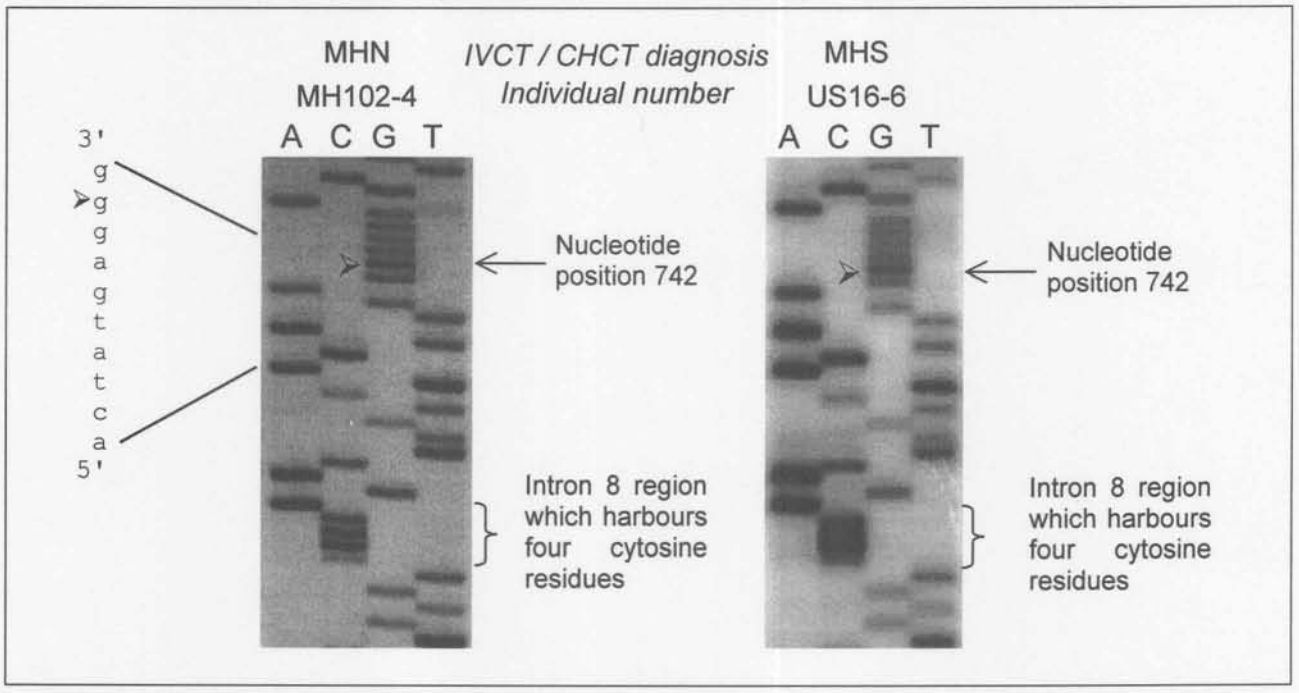
Autoradiograph of single lane sequencing gel where the forward primer was selected to sequence the samples and labelled with α^{32}P-dCTP. Gels were electrophoresed for 3 hours at 60 W. Arrow shows the nucleotide position 742 where an additional fragment at this position would indicate the G to A substitution responsible for the Gly248Arg mutation.

No additional fragments were observed in the region of interest in the selected individuals that were investigated, as depicted in Figure 4.9. Results obtained for the single lane sequencing analyses for the presence of the Gly248Arg mutation are listed in Table 4.3 (page 128). This mutation is absent in the sample population of the seventeen families investigated in this study.

In addition to the single lane sequencing, individuals were sequenced in full, confirming the absence of the mutation. None of the DNA samples sequenced from selected individuals displayed the G to A nucleotide substitution, as indicated in Figure 4.10. Upon comparison of sequence data from Genbank (accession number U48453) and the sequence data generated for the selected individuals included in this study a discrepancy was observed. The 228 bp PCR fragment was sequenced in both the forward and reverse direction to verify the observed discrepancy. In the sequence data obtained from Genbank (accession number U48453) five cytosine nucleotides are present in intron 8 of the gene at positions 719 to 723. However, only four of these cytosine residues were observed when the sequence data were analysed. Three possibilities may explain this discrepancy: a new polymorphism within intron 8, an unreported sequencing error or a typing error.

Ten individuals were sequenced and all of these individuals displayed only four cytosine residues. This sample size is too small to verify whether the discrepancy observed is due to a new polymorphism. A larger number of normal chromosomes need to be investigated to determine if the discrepancy is a polymorphism. It is most likely that this observation is an unreported sequencing error, as all of the individuals sequenced displayed only four C nucleotides and other sequencing errors have been reported previously (Phillip *et al.*, 1996). Although a printing error could be responsible for the difference observed in the reported and the generated sequence, it seems unlikely as no correction has ever been made to the Genbank sequence (accession number U48453). The most plausible explanation therefore seems to be that the anomalies observed are due to an unreported sequencing error.

Figure 4.10: Autoradiographs of chain termination sequencing utilised for detection of the Gly248Arg mutation in the RYR1 gene



MHN = MH normal; MHS = MH susceptible; CHCT = caffeine halothane contracture test; IVCT = *in vitro* contracture test; A = adenine; C = cytosine; G = guanine and T = thymidine. The forward primer was selected as the sequencing primer. The samples were labelled with α^{35}S-dATP and α^{32}P-dCTP for individual MH102-4 and US16-6 respectively. Gels were electrophoresed for 3 hours at 60 W. An arrowhead indicates nucleotide position 742.

The Gly248Arg mutation has only been described in one MHS family that included five family members of whom two were diagnosed to be MH positive with the IVCT. These two individuals also inherited the mutation (Gillard *et al.*, 1992). Tong *et al.* (1997) suggested that the Gly248Arg mutation is a very rare polymorphism rather than a causal mutation as the mutation was not observed in any of the other families studied worldwide. The family in which this mutation was identified also did not have strong linkage to the region on chromosome 19q13.1 (Gillard *et al.*, 1992). However, transfection studies indicated that

the mutation increased the sensitivity of the mutant protein to the effects of caffeine and halothane (Tong *et al.*, 1997). Gillard *et al.* (1992) did not analyse normal chromosomes for the presence of this mutation. The absence of the Gly248Arg mutation in the many MHS families studied worldwide does, however, indicate that this mutation is not a commonly occurring polymorphism. The presence or absence of this mutation should however, be investigated in the normal population to verify that the mutation is not a rare polymorphism. The nucleotide substitution responsible for this mutation may be a recent event that originated in the single MHS family in which it was reported. It may, therefore be a rare causal mutation that is population or even family specific explaining the absence of the mutation in other populations.

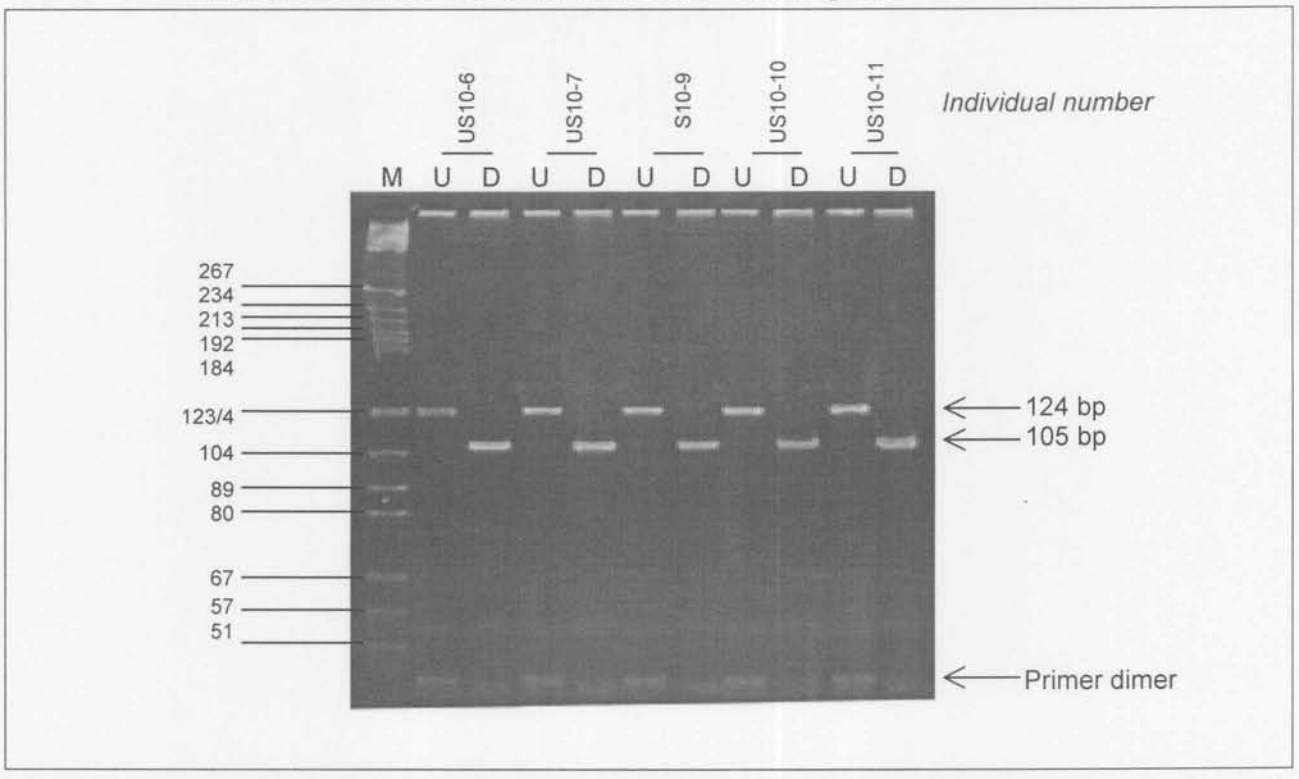
4.4 Gly341Arg mutation in the skeletal muscle ryanodine receptor (RYR1) gene

This mutation was originally detected via SSCP as described by Quane *et al.* (1994). The Gly341Arg mutation does not result in the loss or gain of a restriction enzyme site. Subsequently Alestrøm *et al.* (1995) developed a less labour intensive, more accurate and sensitive detection technique utilising an amplification created restriction site (ACRS) assay. This technique involves the designing of a primer, in this case the forward primer, which introduces a restriction enzyme site upon amplification - allowing for the detection of the mutation via RFLP (Haliassos *et al.*, 1989).

A 124 bp product was amplified utilising a standardised PCR protocol with an annealing temperature of 64°C as discussed in paragraph 3.4. In a normal individual the PCR amplified product contains an *Msp I* recognition site (CCGG) and will be cleaved upon restriction, yielding two fragments (19 bp and 105 bp). In homozygous affected individuals the restriction site will be abolished (CCAG) and the fragment will not be cleaved during *Msp I* digestion. This implies that three fragments (124 bp, 105 bp and 19 bp) should therefore be present in a heterozygous affected individual. Results generated for the selected individuals from the seventeen families included in this study are listed in Table 4.3 (page 128).

The 124 bp fragment was not observed in any of the individuals investigated in this study. It was therefore concluded that Gly341Arg mutation is not present in these individuals. Family US10 was utilised to illustrate the results generated upon screening of the selected individuals for this mutation as presented in Figure 4.11.

Figure 4.11: Photographic representation generated for detection of the Gly341Arg mutation within exon eleven of the RYR1 gene



RFLP fragments were separated on a 10% polyacrylamide gel. This gel was electrophoresed at 250 V for two hours and subsequently stained with SYBRGold for 30 min. "M" indicates the molecular weight marker (pBR322/*Hae III*) used to size the fragments. "U" indicates the undigested PCR product, and "D" the PCR product digested with 10 units of *Msp I*.

Primer dimers can also be observed migrating at the bottom of the gel. Two possible primer dimers that could be formed by the primer pair are presented in Figure 4.12. One primer dimer results from two of the forward primers annealing with each other and the other cross dimer when annealing occurs between the forward and reverse primers. No primer dimers could be formed between different copies of the reverse primer, as no region of complementarity was present. Of the two possible primer dimers the cross dimer "b" in Figure 4.12 is the most stable with a ΔG value of –7.07 kcal.mol^{-1}.

Figure 4.12: Secondary structures formed by the G341RF1 and G341RR primers

Self-dimers

G341RF1 forward primer dimers

(a)
```
5' gccccctgagatcaagtcc 3'
                ||||
    3' cctgaactagagtccccccg 5'
```
ΔG = -4.62 kcal.mol^{-1}

Cross dimers

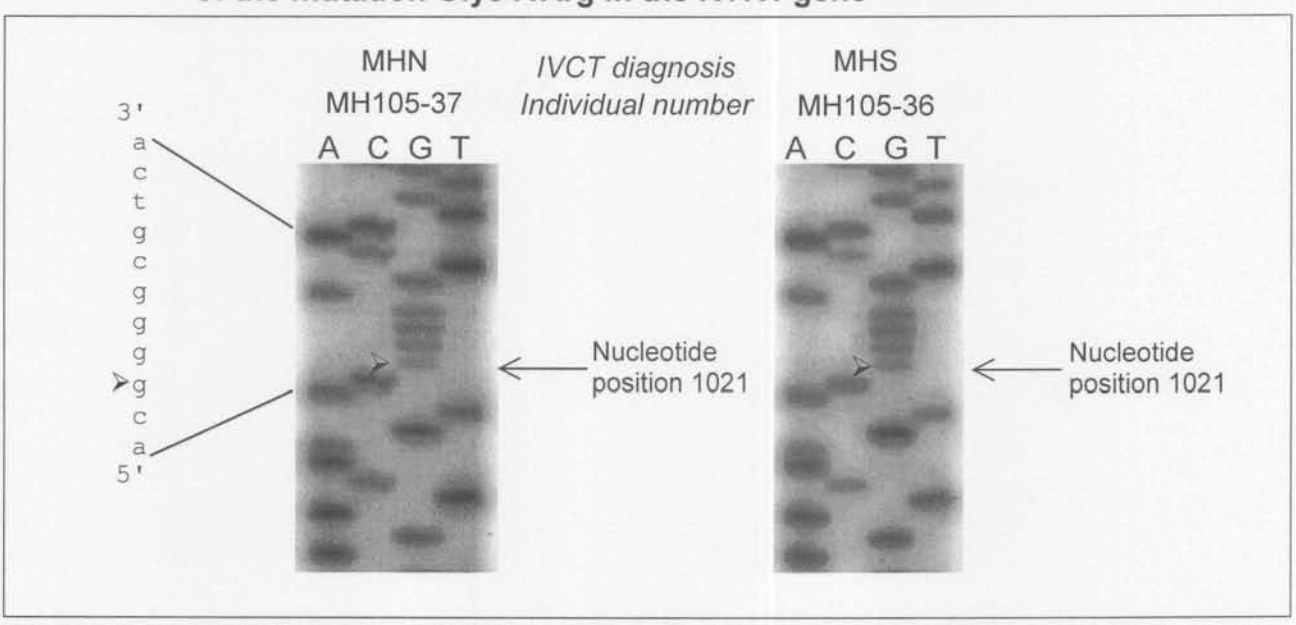
G341RF1 and G341RR primer dimers

(b)
```
5' gccccctgagatcaagtcc 3'
            : : |||||
  3' ccgcacgagttcttcttcca 5'
```
ΔG = -7.07 kcal.mol^{-1}
Most stable dimer

Primer sequences are listed in Table 3.3. ΔG indicates the free energy.

Sequencing of the fragment generated for the ACRS was problematic as compressions resulting from secondary structures due to the GC rich template were observed on the gel. In an effort to eliminate secondary structures a second primer was selected that amplified a larger fragment which was 174 bp in length. Even with the larger fragment a compression formed at nucleotide position 1021 when sequencing was performed with the reverse primer. Unambiguous sequence could only be obtained with the forward primer. Sequence generated for selected individuals confirmed the results obtained via the ACRS as no G to A substitution at position 1021 could be observed. A representation of the results is presented in Figure 4.13.

Figure 4.13: Autoradiographs of chain termination sequencing utilised for detection of the mutation Gly341Arg in the RYR1 gene



MHN = MH normal; MHS = MH susceptible; IVCT = *in vitro* contracture test; A = adenine; C = cytosine; G = guanine and T = thymidine. The forward primer was selected as the sequencing primer. The samples were labelled with α^{32}P-dCTP. Gels were electrophoresed for 1 hour 40 min at 60 W. An arrowhead indicates nucleotide position 1021.

Quane *et al.* (1994) estimated that the Gly341Arg mutation accounts for approximately 10% of Caucasian MHS individuals. Manning *et al.* (1998a) reported the estimated incidence of the Gly341Arg mutation to be 6% of MHS families. This mutation was reported by Barone *et al.* (1999) in the Italian population and by Quane *et al.* (1994a) in the Irish, Belgian and French populations. Monsieurs *et al.* (1998) identified three Belgium families harbouring the Gly341Arg mutation. These authors also suggested that the Gly341Arg mutation might be responsible for chronically evaluated serum CK activity in asymptomatic individuals. Even though this mutation is thought to be one of the most common mutations found to date (Manning *et al.*, 1999a) it has not been observed in the North American (Stewart *et al.*, 1998), German, Austrian and Swedish MH populations

(Brandt *et al.*, 1999). The mutation was also absent in one Danish and two Swiss families (Brandt *et al.*, 1999).

Segregation analysis suggested that this mutation is not the causative mutation in a large British family and that a second mutation or gene is responsible for the MHS phenotype in this particular family (Adeokun *et al.*, 1997). It is possible that the Gly341Arg mutation is a rare polymorphism observed in this particular family. Five hundred normal chromosomes were analysed for the aberrant SSCP conformer, and it was not observed (Quane *et al.*, 1994a). Even though the aberrant conformer was not observed it is not conclusive evidence that the Gly341Arg mutation is not absent from the general population. As discussed in paragraph 4.3, SSCP is not specific enough to determine the presence or absence of a particular mutation. However, transfection studies performed by Tong *et al.* (1997) provided biochemical evidence that the Gly341Arg mutation is a causative mutation for MH. The absence of this mutation in the different populations listed above provides further evidence of the causative nature of the Gly341Arg mutation.

Adeokun *et al.* (1997) reported that the distribution of the Gly341Arg mutation is not limited to specific geographical regions. In contrast to the statement by Adeokun *et al.* (1997), Stewart *et al.* (1998) suggested that the Gly341Arg gene pool is restricted to European families as the Gly341Arg mutation was not present in the North American population investigated by these authors. Results generated for this thesis supports the theory that this mutation is geographically restricted to the European continent as it has only been observed in European families and was not present in the two sample populations included in this study.

Only four South African families were investigated in this study. It is therefore possible that the sample size of this study is too small to conclusively determine whether the Gly341Arg mutation is absent from the entire South African MHS population. The results generated for the North American families included in this study contribute to the results obtained by Stewart *et al.* (1998) as discussed above.

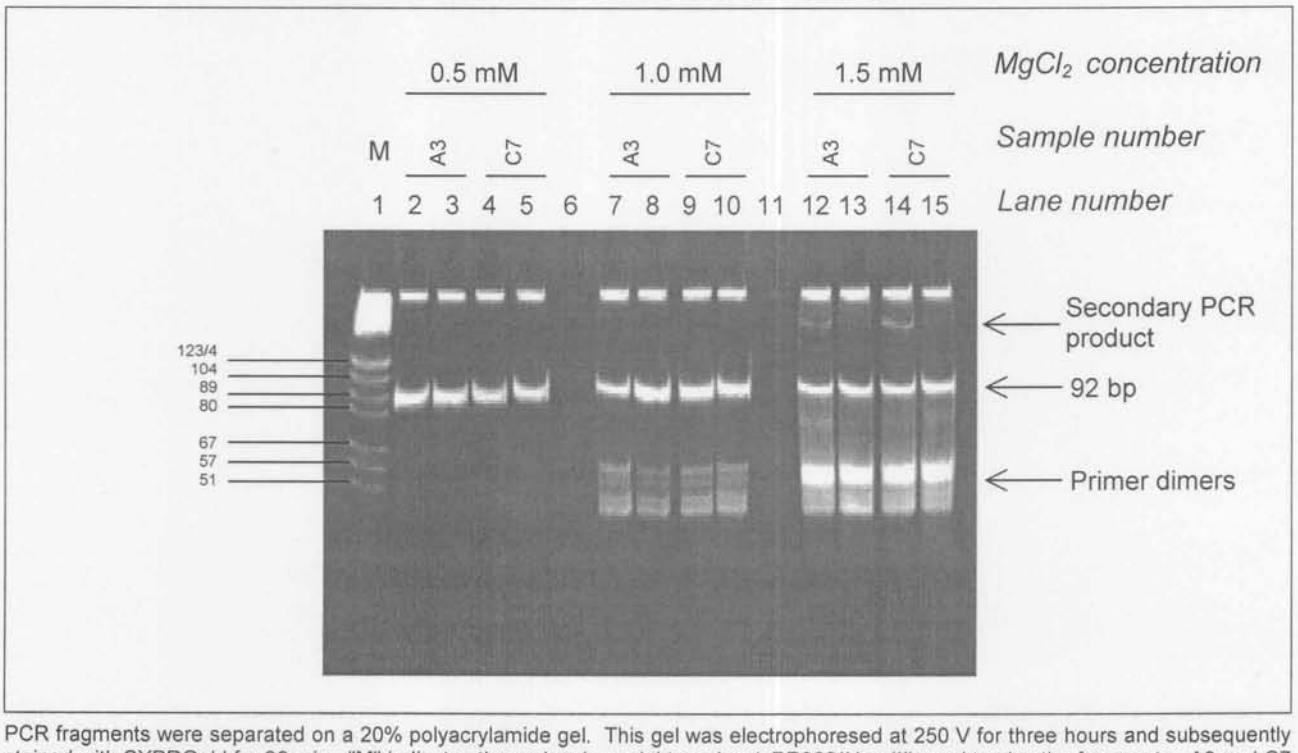
Since the South African and North American populations are from European descent the Gly341Arg mutation might be present in these populations. If the families included in this study are representative of the two populations then an explanation for the absence of the mutation in these two populations could be the fact that this mutation represents a recent genetic change. It is possible that the mutational event occurred after the emigration of

the European population to the North American and African continents. It is also possible that independent mutational events were responsible for the presence of this mutation in the different European populations. This hypothesis is supported by the fact that the Gly341Arg mutation has not been associated with a common haplotype (Quane *et al.*, 1994a). Haplotype analysis of six distinct RYR1 haplotypes by Quane *et al.* (1994) indicated that this mutation did not spread through a founder effect.

4.5 Ile403Met mutation in the skeletal muscle ryanodine receptor (RYR1) gene

Amplification of the gDNA was performed using the standard PCR program at an annealing temperature of 64°C as described in paragraph 3.4. The PCR reaction was optimised and the best results were achieved by reducing the $MgCl_2$ concentration to 0.5 mM. In Figure 4.14 the various $MgCl_2$ concentrations utilised for the optimisation are indicated. Very little or no primer dimers were observed when a concentration of 0.5 mM $MgCl_2$ was utilised. Additional fragments could be observed in one sample of both A3 (lane 12) and C7 (lane 14) when a concentration of 1.5 mM $MgCl_2$ was used. These secondary PCR amplified products are eliminated at lower concentrations of $MgCl_2$.

Figure 4.14: Influence of $MgCl_2$ concentration on the amplification of a fragment utilised for detection of the Ile403Met mutation



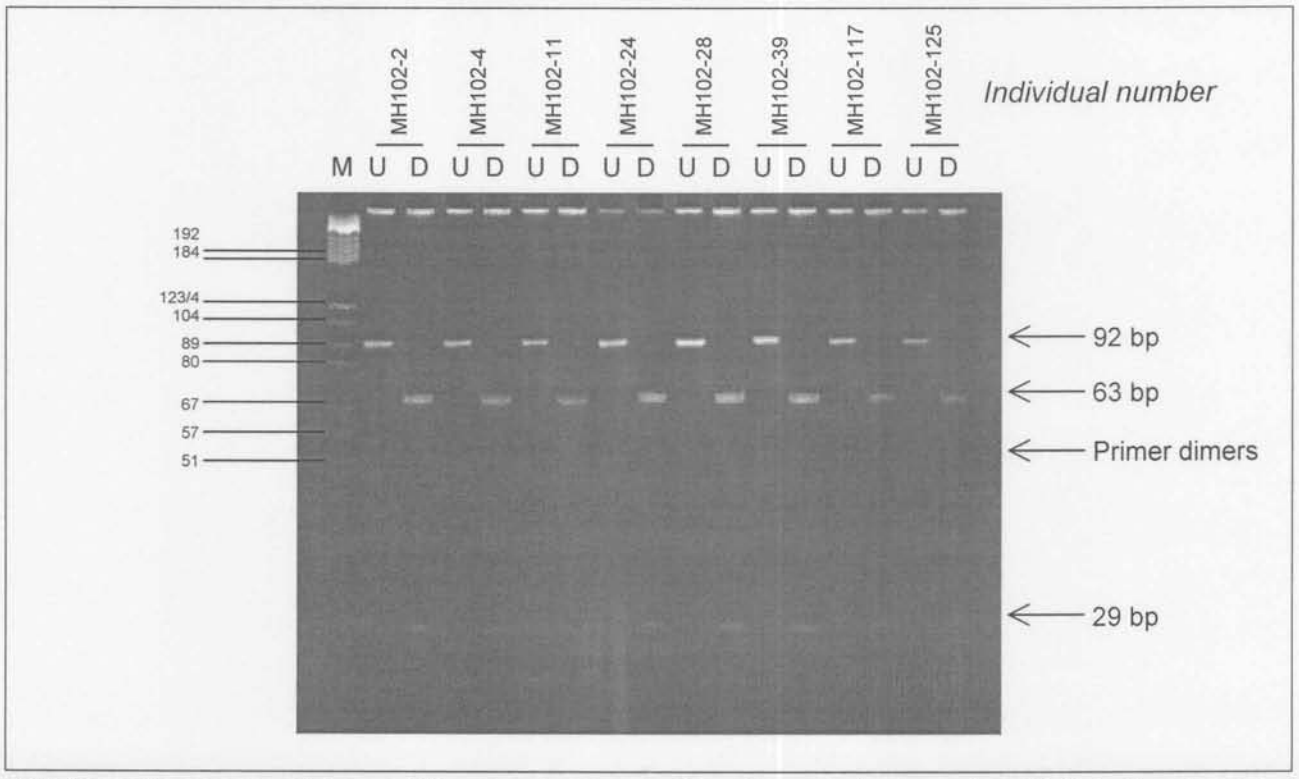
PCR fragments were separated on a 20% polyacrylamide gel. This gel was electrophoresed at 250 V for three hours and subsequently stained with SYBRGold for 30 min. "M" indicates the molecular weight marker (pBR322/*Hae III*) used to size the fragments. A3 and C7 are control DNA samples utilised for the optimisation of the PCR. No samples were loaded in lane 6 and lane 11.

The diagnostic assay described by Quane *et al.* (1993) was utilised to investigate the seventeen families in this study for the presence of the Ile403Met mutation. The diagnostic assay involves the amplification of a 92 bp fragment, which was subsequently digested by the *Mbo I* restriction enzyme. The C to G mutation at nucleotide position 1209 results in the abolishment of a *Mbo I* restriction site.

For diagnostic purposes the samples were digested with *Mbo I* after the amplification of the region encompassing the mutation. The 92 bp amplified product was cleaved into a 63 bp and 29 bp fragment in normal individuals. In affected individuals three (92 bp, 63 bp and 29 bp) or one (92 bp) fragment will be observed depending on whether the individuals are heterozygous or homozygous individuals respectively. Results of RFLP analyses of selected individuals for detection of the Ile403Met mutation are listed in Table 4.3 (page 128).

The 92 bp fragment was not present in any of the individuals investigated from the seventeen families screened in this study. Results obtained for individuals from South African MH family MH102 are presented in Figure 4.15. This family is representative of all the families included in this study.

Figure 4.15: Photographic representation generated for detection of the Ile403Met mutation within exon twelve of the RYR1 gene



RFLP fragments were separated on a 20% polyacrylamide gel. This gel was electrophoresed at 250 V for three hours and subsequently stained with EtBr for 30 min. "M" indicates the molecular weight marker (pBR322/*Hae III*) used to size the fragments. "U" indicates the undigested PCR product and "D" the PCR product digested with 10 units of *Mbo I*.

Primer dimers were observed in both the undigested and digested samples of individuals MH102-24 and MH102-39 as indicated in Figure 4.15. A total of nine primer dimer structures are possible. Five ("a"-"e" in Figure 4.16) are due to self-dimerisation between copies of the forward primer. Copies of the reverse primer could possibly form two secondary structures listed as "f" and "g" in Figure 4.16. The remaining two possible primer dimers are cross dimers that could form between the forward and reverse primers. These are indicated as structure "h" and "i" in Figure 4.16. The most stable primer dimers and 3' dimers are also indicated in Figure 4.16.

Figure 4.16: Secondary structures formed by the I403MF and I403MR primers

Self-dimers

I403MF forward primer dimers

(a)
```
            5' ccacatggacgacgactgtc 3'
                        ||||
3' ctgtcagcagcaggtacacc 5'
```
ΔG = -5.38 kcal.mol^{-1}
3rd Most stable dimer

(b)
```
5' ccacatggacgacgactgtc 3'
   |||             ::::
 3' ctgtcagcagcaggtacacc 5'
```
3' Dimer
ΔG = -4.29 kcal.mol^{-1}

(c)
```
5' ccacatggacgacgactgtc 3'
        |||         ::::
    3' ctgtcagcagcaggtacacc 5'
```
3' Dimer
ΔG = -3.91 kcal.mol^{-1}

(d)
```
5' ccacatggacgacgactgtc 3'
            |||     ::::
          3' ctgtcagcagcaggtacacc 5'
```
3' Dimer
ΔG = -3.91 kcal.mol^{-1}

(e)
```
5' ccacatggacgacgactgtc 3'
                |||  ::::
              3' ctgtcagcagcaggtacacc 5'
```
3' Dimer
ΔG = -3.91 kcal.mol^{-1}

I403MR reverse primer dimers

(f)
```
5' ggttgtataggccattggtgct 3'
    :    :  ||||  :    :
3' tcgtggttaccggatatgttgg 5'
```
ΔG = -9.27 kcal.mol^{-1}
Most stable dimer

(g)
```
       5' ggttgtataggccattggtgct 3'
            :   ||||   :
3' tcgtggttaccggatatgttgg 5'
```
ΔG = -3.39 kcal.mol^{-1}

Cross dimers

I403MF and I403MR primer dimers

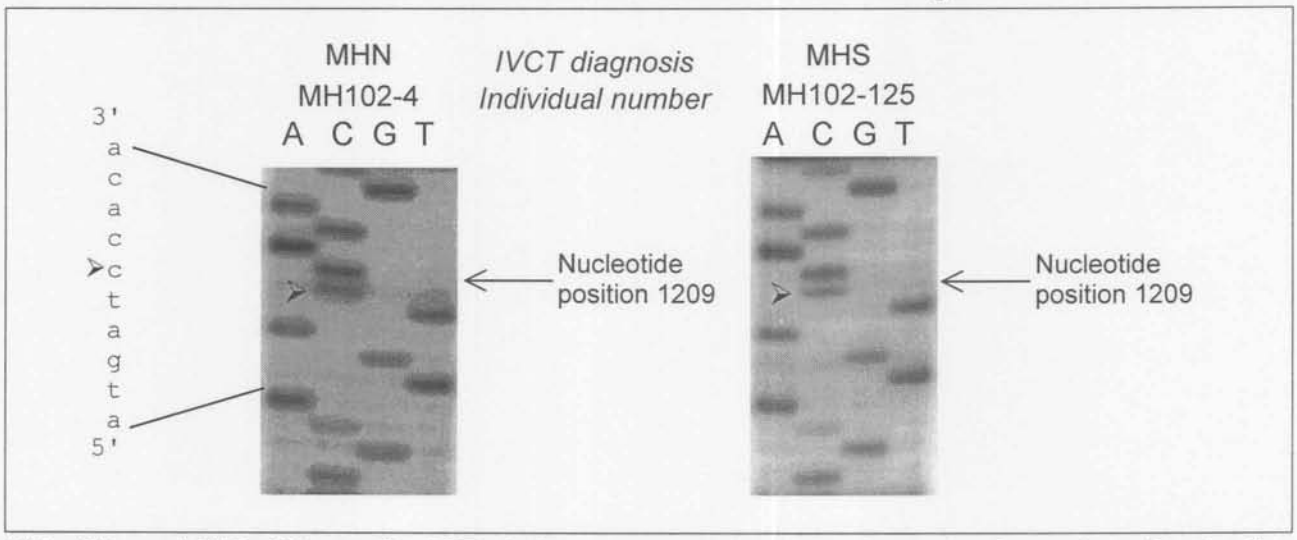
(h)
```
    5' ccacatggacgacgactgtc 3'
        :  ||||  :  :
3' tcgtggttaccggatatgttgg 5'
```
ΔG = -6.49 kcal.mol^{-1}
2nd Most stable dimer

(i)
```
 5' ccacatggacgacgactgtc 3'
    |||                 :
3' tcgtggttaccggatatgttgg 5'
```
3' Dimer
ΔG = -4.29 kcal.mol^{-1}

Primer sequences are listed in Table 3.3. ΔG indicates the free energy.

The results obtained via RFLP analysis were confirmed via sequencing of selected individuals. As depicted in Figure 4.17, sequencing data revealed that the normal coding sequence was present in all the selected individuals. It was, therefore, concluded that the Ile403Met mutation was not present in this sample population.

Figure 4.17: Autoradiographs of the chain terminated sequencing utilised for detection of the Ile403Met mutation in the RYR1 gene



MHN = MH normal; MHS = MH susceptible; IVCT = *in vitro* contracture test; A = adenine; C = cytosine; G = guanine and T = thymidine. The forward primer was selected as the sequencing primer. The samples were labelled with α^{35}S-dATP. Gels were electrophoresed for 1 hour 30 min at 60 W. An arrowhead indicates nucleotide position 1209.

CCD and MH are phenotypically distinct allelic variants as mutations in the same gene are responsible for both these disorders (Zhang *et al.*, 1993; Quane *et al.*, 1993). To date, the I403M mutation has only been associated with CCD within one family consisting of four individuals (Quane *et al.*, 1993). Although the I403M mutation has only been observed in the Italian CCD family (Quane *et al.*, 1993) it may also play a role, or at least contribute to the MHS phenotype segregating in this family. It is unclear how mutations within the RYR1 gene can cause both CCD and MH. This mutation may be associated only with CCD and may not contribute to the MH phenotype. However, other mutations such as Arg163Cys, Tyr522Ser, Arg2163His and Arg2436His have been identified in families affected with both CCD and MH (Quane *et al.*, 1993; Quane *et al.*, 1994b; Manning *et al.*, 1998a; Zhang *et al.*, 1993). Interaction between the different mutations and their contribution to either the CCD or the MH phenotype is strongly suggested by families such as these. None of the families included in this study had a history of CCD. The presence of the Ile403Met mutation would not be expected in the families investigated here if this mutation is indeed only associated with CCD, and not MH.

The absence of the mutation may also be due to the fact that the Ile403Met mutation may be a recent mutational event which might have originated in the Italian family in which it

has been identified. The Italian population might not have had sufficient time to migrate to other regions thus spreading the mutation. The Ile403Met mutation could therefore be population specific or even family specific and the absence of this mutation in other populations studied worldwide supports this theory.

4.6 Tyr522Ser mutation in the skeletal muscle ryanodine receptor (RYR1) gene

This substitution does not result in the alteration of a restriction site and was therefore detected via SSCP (Quane *et al.* 1994b). However, as previously discussed in paragraph 4.3 the SSCP analysis of PCR products for the detection of a mutation is not ideal. The low specificity and sensitivity of the SSCP technique eliminates it as a molecular diagnostic tool.

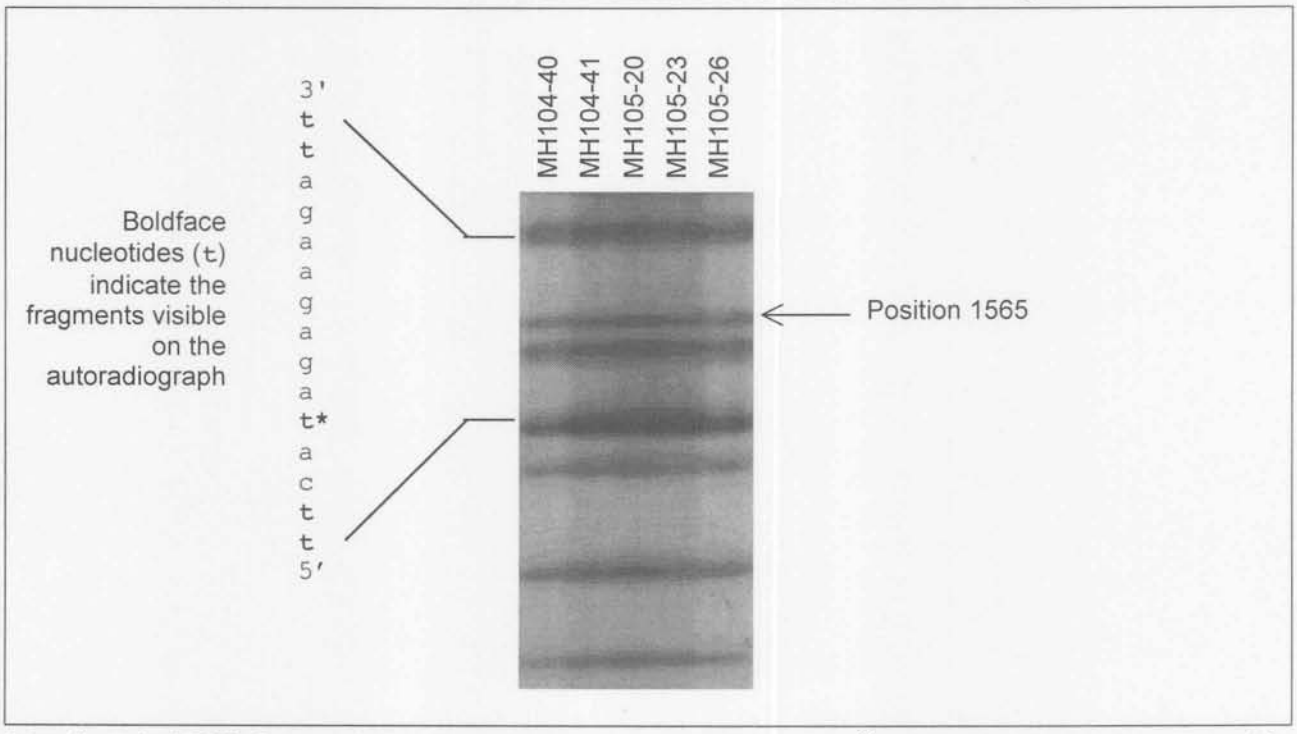
Another method of detecting this mutation was required in order to examine the possibility that the Tyr522Ser mutation might segregate with the MH phenotype in the families included in this study. As this substitution did not result in the alteration of a restriction site BESS T scan and chain termination sequencing seemed the most viable options. Manual sequencing of all the individuals is time consuming and very costly. Therefore, BESS T analysis seemed the best course of action.

However, Sei *et al.* (1998) developed a RFLP assay to detect the Tyr522Ser mutation by means of the ACRS technique as discussed in paragraph 4.3 and 4.4 for the Gly248Arg and Gly341Arg mutations respectively. A modified forward primer was designed which resulted in the amplification of a PCR fragment where the Tyr522Ser mutation would create a *BsmA I* restriction site. The detection of the mutation via an RFLP assay is preferred, as this detection protocol is highly specific and sensitive for specific mutations. Furthermore the method is rapid, cost effective and no radioactivity is required. However, as the samples were already investigated via BESS T analysis and single lane sequencing when the RFLP method was published, it was decided not to change the method of detection in the study reported here. The RFLP assay would, however, be the method of choice when performing analyses for molecular diagnostic purposes. Moreover, the RFLP analysis protocol can discriminate between heterozygous and homozygous MH individuals.

The Tyr522Ser mutation involves an adenine to a cytosine nucleotide substitution. Therefore, the reverse primer needed to be labelled in order to detect the thymidine

alteration of interest, or any other alterations involving a T. Representative results obtained utilising this technique are displayed in Figure 4.18. The absence of the T fragment or a change in intensity, at position 1565 would indicate the presence of the mutation in homozygous MHS and heterozygous MHS individuals respectively.

Figure 4.18: Autoradiograph of BESS T analysis generated for detection of the Tyr522Ser mutation within exon fourteen of the RYR1 gene



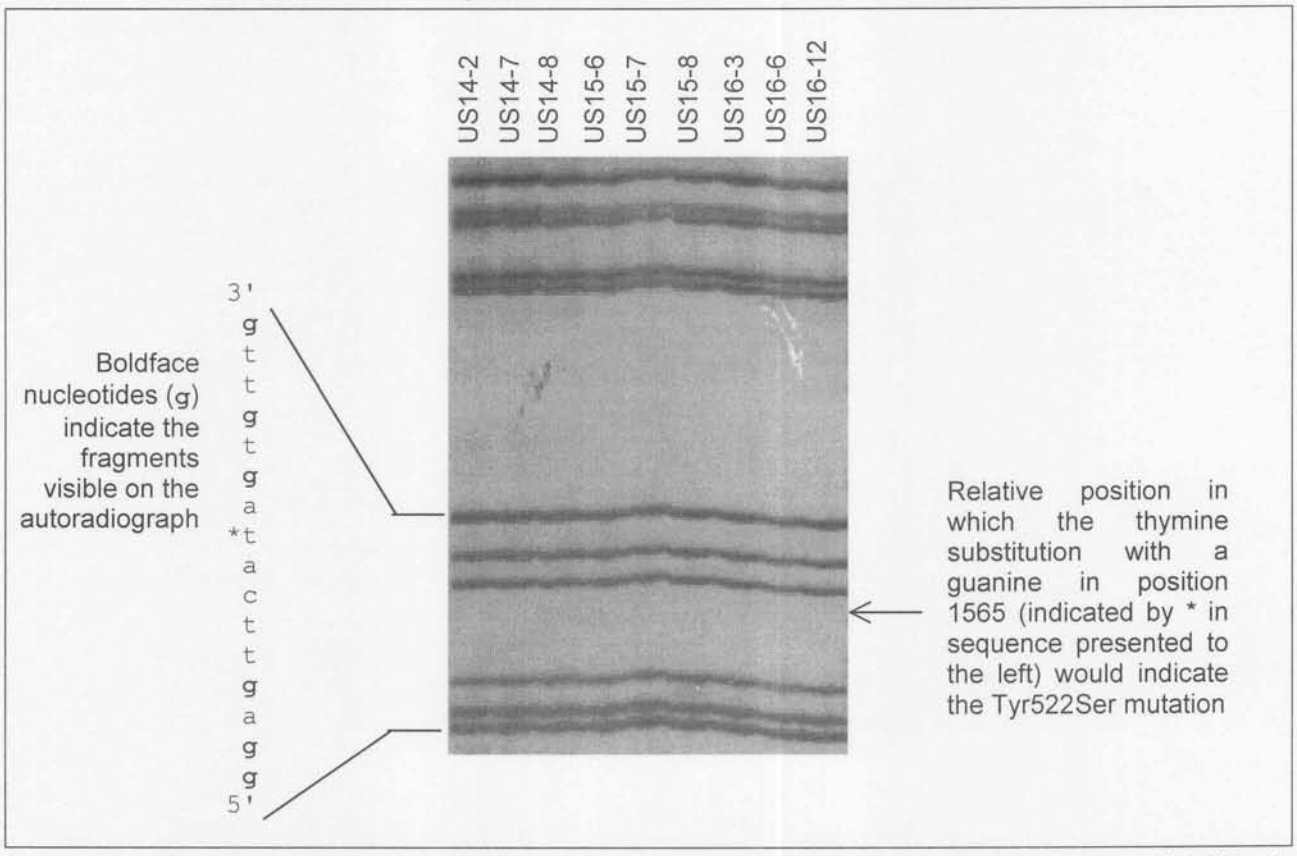
Autoradiograph of BESS T scan gel where the reverse primer was end labelled with γ^{32}P-dATP. The gel was electrophoresed for 2 hours at 60 W.

Analysis of the results indicated that the T fragment in position 1565 was not absent in any of the individuals. However, this did not imply that the mutation was not present in the population investigated, as the individuals could be heterozygous for the mutation. Furthermore, for an individual to be heterozygous for the mutation only one copy of the T fragment needs to be substituted, thus the other copy of the T fragment would still be present. The presence of the mutation would therefore be observed as the change in the *intensity* of the T fragment. Ascertaining whether there is a change in intensity is difficult and depends on the judgement of the individual analysing the data. The use of positive controls and unbiased detection systems, such as densitometry, should be implemented in the diagnostic environment. No positive controls were available and the presence of the mutation could, therefore, not be verified unless samples were sequenced.

Investigation of the opposite strand would not indicate the presence of the mutation as an adenine is substituted with a guanine and neither the absence of the adenine residue or

the gain of a guanine would be detectable via BESS T analysis. As with the Gly248Arg mutation, a single lane was therefore sequenced to detect an additional fragment at position 1565 in the region amplified to verify the presence of the mutation. The best results were achieved when the G lane was sequenced with the reverse primer, as the presence of an additional G fragment would indicate the presence of the mutation. The results obtained for the families investigated via single lane sequencing are presented in Table 4.3 (page 128). The Tyr522Ser mutation was not observed in any of the families investigated. The autoradiograph presented in Figure 4.19 is representative of the results obtained for all the individuals investigated in this study.

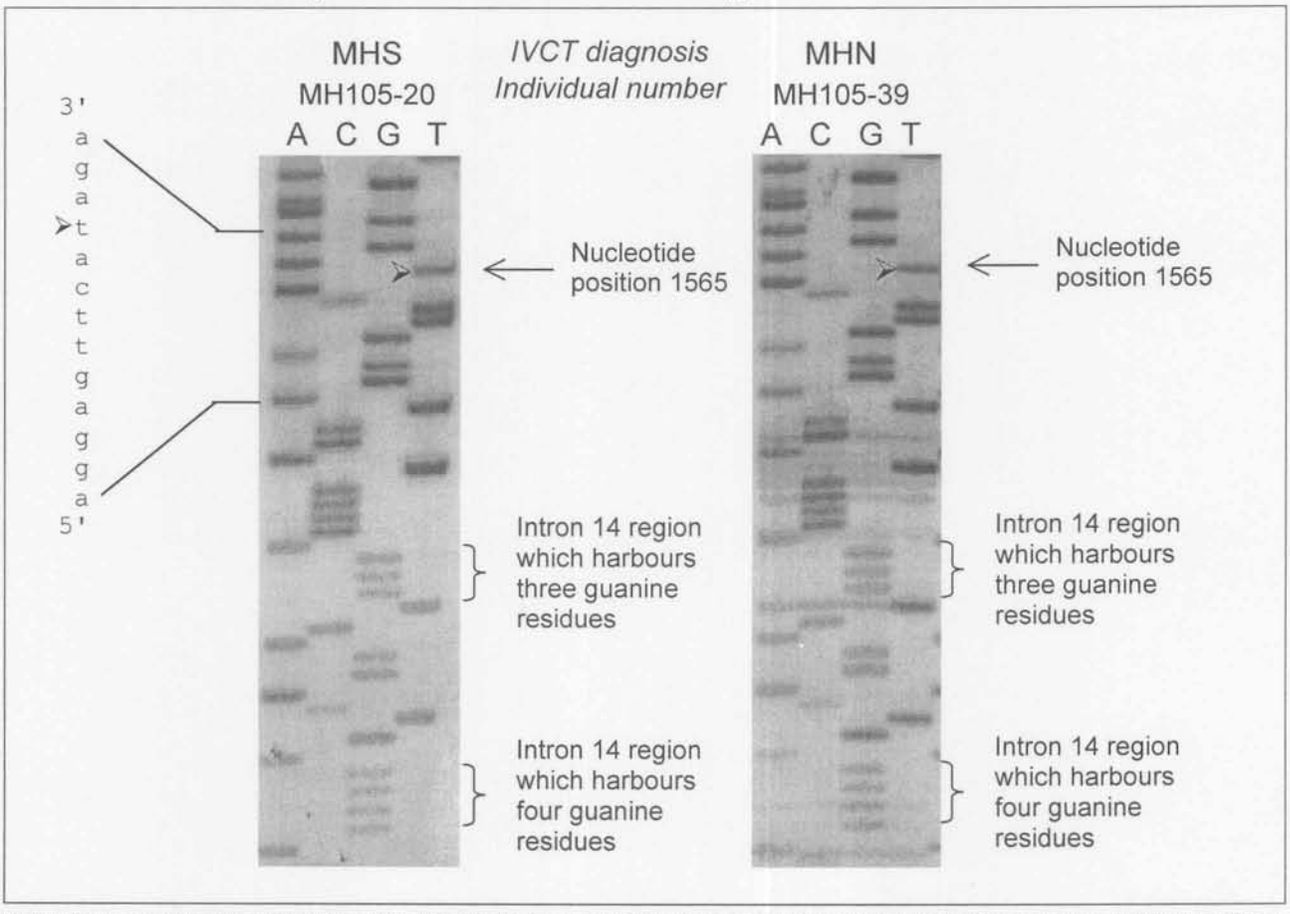
Figure 4.19: Autoradiograph of single lane sequence analysis generated for detection of the Tyr522Ser mutation in the RYR1 gene



Autoradiograph of single lane sequencing gel where the reverse primer was selected to sequence the samples and labelled with α^{32}P-dCTP. Gels were electrophoresed for 2 hours at 60 W.

Figure 4.20 displays the sequence generated for individuals MH105-20 and MH105-39 in addition to the single lane sequencing performed and presented in Figure 4.19. The T to G nucleotide substitution at position 1565 also was not observed in any of the selected samples sequenced. This verified the results obtained via SLS.

Figure 4.20: Autoradiographs of chain termination sequencing utilised for detection of the Tyr522Ser mutation in RYR1 gene



MHN= MH normal; MHS = MH susceptible; IVCT = *in vitro* contracture test; A = adenine; C = cytosine; G = guanine and T = thymidine. The reverse primer was selected as the sequencing primer and the samples were labelled with α^{32}P-dCTP. Gels were electrophoresed for 2 hours at 60 W. An arrowhead indicates nucleotide position 1565.

Inconsistencies were identified when the deposited sequence from Genbank (accession number U48456) was compared with the sequence generated for this mutation. In intron 14 the addition of a guanine in two different positions, between nucleotides 1585 and 1586 (reported sequence 3'-aggt-5' and observed sequence 3'-agggt-5') and between nucleotides 1597 and 1599 (reported sequence 3'-aggga-5' and observed sequence 3'-agggga-5'), were noted. The addition of two guanine bases in the intron region of the gene may be of no significance and may only be due to normal variation between individuals or populations. However, variation in this intronic region of the RYR1 gene has not been reported to date. This observed sequence anomaly may also be due to sequencing errors by the original investigators as sequence errors have been identified previously (Phillips *et al.*, 1996).

Even though only three individuals were investigated via sequencing the possibility that these are new polymorphisms can, however, not be excluded. Although the inconsistencies were observed in all three individuals the sample size in which the anomalies were observed is too small to draw a meaningful conclusion. The differences

observed between the reported RYR1 sequence and the generated sequence should be investigated in additional normal chromosomes. Analysis of these normal chromosomes for the presence of the additional two guanine nucleotides between 1585 to 1586 and 1597 to 1599 would confirm whether the observation reflects new polymorphisms or unreported sequencing errors.

To date, the Tyr522Ser mutation has only been identified in one French family (Quane *et al.*, 1994b). The mutation has also been associated with CCD as some of the individuals in this family were diagnosed with subclinical CCD after histological examination of their muscle biopsies. However, none of the individuals had shown clinical symptoms of CCD. The role of the Tyr522Ser mutation in the phenotypic expression of both MH and CCD remains unclear. It seems to cause MHS in all the individuals that carry the mutation and only result in subclinical CCD in some of the individuals harbouring the mutation (Quane *et al.*, 1994b). As none of the families included in this study had a history of CCD the absence of the Tyr522Ser mutation in the individuals investigated was not surprising. Since this mutation has only been observed in one family it is possible that the Tyr522Ser mutation is a relative new mutation which is specific to the particular family reported by Quane *et al.* (1994b). The absence of the Tyr522Ser mutation in the populations investigated in this study does, however, provide more evidence that the mutation is not present in all populations.

Quane *et al.* (1994b) utilised SSCP to analyse 264 normal chromosomes for the presence of the Tyr522Ser mutation in the normal population. However, as discussed in paragraph 4.3, SSCP is not specific or sensitive enough and is therefore not the preferred method of detection for the presence of the mutation in the unaffected population.

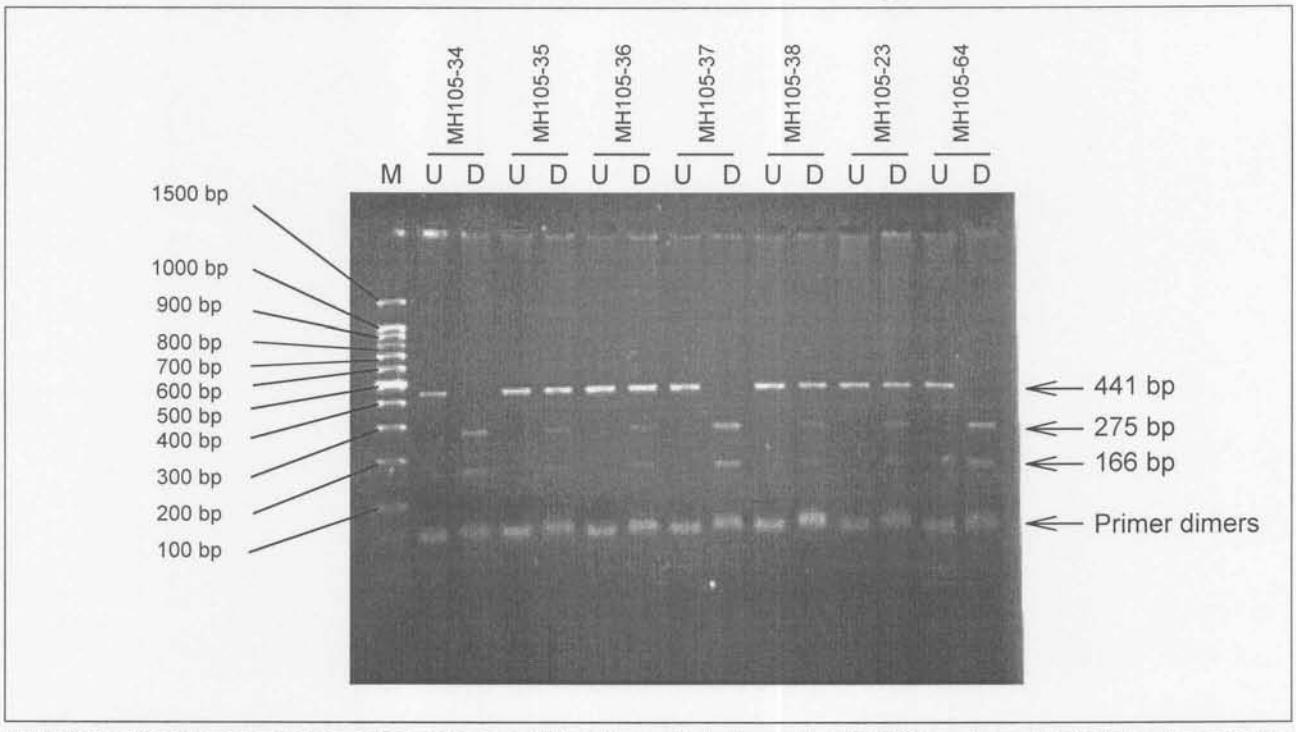
4.7 Arg614Cys mutation in the skeletal muscle ryanodine receptor (RYR1) gene

PCR amplification was performed with some adjustment to the standard PCR reaction cocktail and the PCR program described in paragraph 3.4. The region of interest which was amplified for RFLP analysis had a high GC content of 56%, which subsequently lead to poor amplification of the 441 bp product. Sarkar *et al.*, (1990) and Zhang *et al.*, (1991) reported that formamide could improve the specificity of PCR dramatically in templates with high GC content. Subsequent to the addition of formamide to the PCR reactions, to a final concentration of 1%, the amplification improved. The PCR program was also adjusted and the fragment was extended for 120 seconds, instead of 60 seconds, at 72°C.

The longer extension time also improved amplification of the 441 bp fragment from the GC rich template.

The C1840T nucleotide substitution results in the loss of a *Rsa I* restriction site, which can be detected via RFLP analysis (Otsu *et al.*, 1992). The amplified 441 bp PCR-product was digested with *Rsa I* and yielded three fragments (441 bp, 275 bp and 166 bp). The three fragments indicate that the individuals are heterozygous as only one *Rsa I* site was deleted by the C1840T substitution. In MHN individuals only two fragments (275 bp and 166 bp) were generated. Results of the restriction fragment length polymorphism analysed of selected individuals are presented in Figure 4.21.

Figure 4.21: Photographic representation generated for detection of the Arg614Cys mutation within exon seventeen of the RYR1 gene

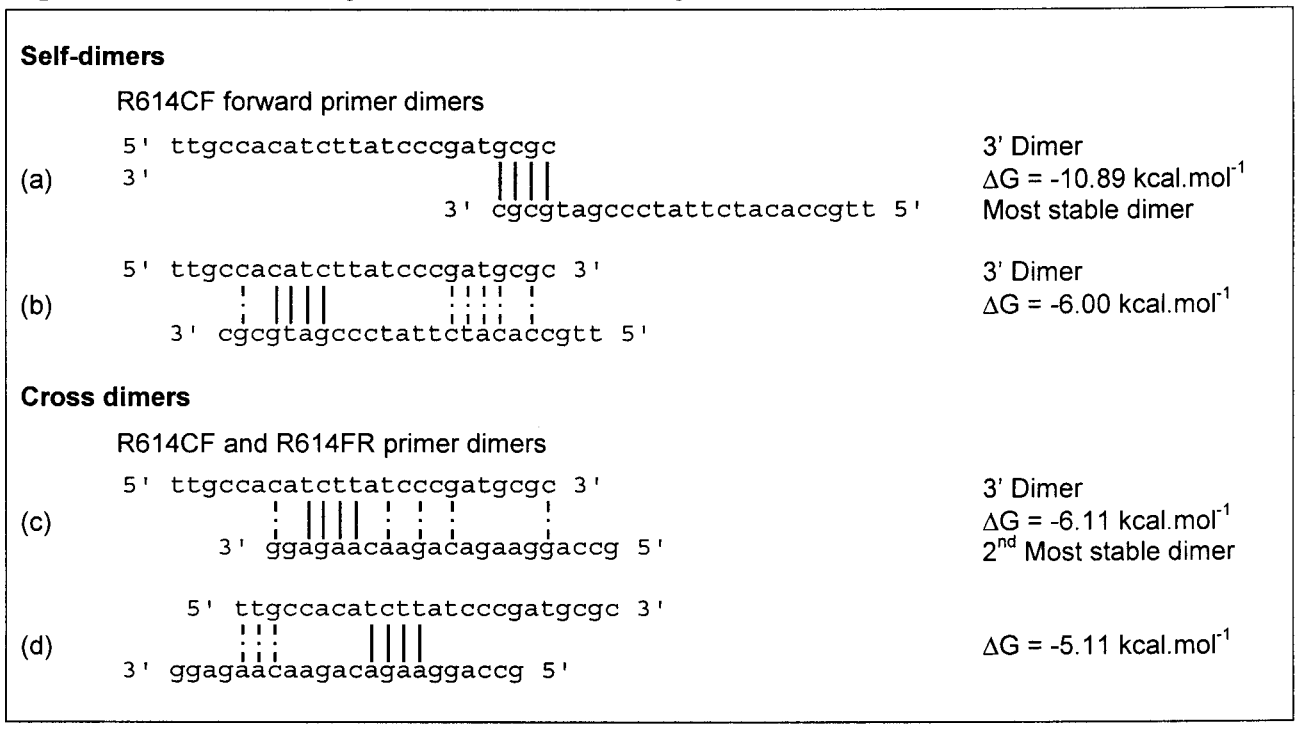


RFLP fragments were separated on a 2% agarose gel. This gel was electrophoresed at 200 V for an hour. "M" indicates the molecular weight marker used to size the fragments, 100 bp ladder. "U" indicates the undigested PCR product and "D" the PCR product digested with 10 units of *Rsa I*.

Primer dimers were identified on the 2% agarose gel as indicated in Figure 4.21. The primer dimers appear as a smear on the gel and not as sharp fragments. This might be due to the fact that there are several possible primer dimers which could form. Furthermore, not all of the possible bonds between the two primers may be formed, resulting in the primer dimers migrating through the gel at different molecular weights. All the possible primer dimers which could be formed by the two primers used to amplify the PCR product are indicated in Figure 4.22. Two self-primer dimers, "a" and "b" in Figure 4.22, and two cross dimers, "c" and "d' in Figure 4.22, could form between the

forward and reverse primers. ΔG values for the respective dimers, as well as the most stable dimers are also indicated. Three possible 3' dimers, (a), (b) and (c) in Figure 4.22, could lead to the formation of short products (44 bp – 27 bp) due to extension at the 3' end.

Figure 4.22: Secondary structures formed by the R614CF and R614CR primers



Primer sequences are listed in Table 3.3. ΔG indicates the free energy.

The Arg614Cys mutation was observed in one of the seventeen families investigated in this study, namely: South African family MH105. RFLP analysis results obtained for the selected individuals from the seventeen families included in this study are listed in Table 4.3 (page 128). Initially only the individuals selected for screening in this study were screened for the mutation. However, after it became apparent that this mutation may be causative within family MH105 the remaining individuals of whom DNA was available were also investigated.

Initially, 25 individuals were screened for the presence of the Arg614Cys mutation by Olckers *et al.* (1994). Upon confirmation of the results of Olckers *et al.* (1994) in this study an additional 14 individuals (see Table 4.1) from family MH105 were investigated for the presence of this mutation. In total 39 individuals from this family were thus screened for the presence of the Arg614Cys mutation. The results of the RFLP analysis for family MH105 are presented in Table 4.1 (page 114). Subsequently the Arg614Cys mutation was observed in twelve individuals in South African family MH105. Three of these

individuals, MH105-30, MH105-31 and MH105-60, were not phenotyped with the IVCT and for two individuals, MH15-30 and MH105-31, this study presented the first opportunity to identify their MH status. The RFLP result for individual MH105-60 was first reported by Olckers *et al.* (1994) and verified in this study. This individual can now be counselled accordingly.

Table 4.1: RFLP results of the Arg614Cys mutation for South African family MH105

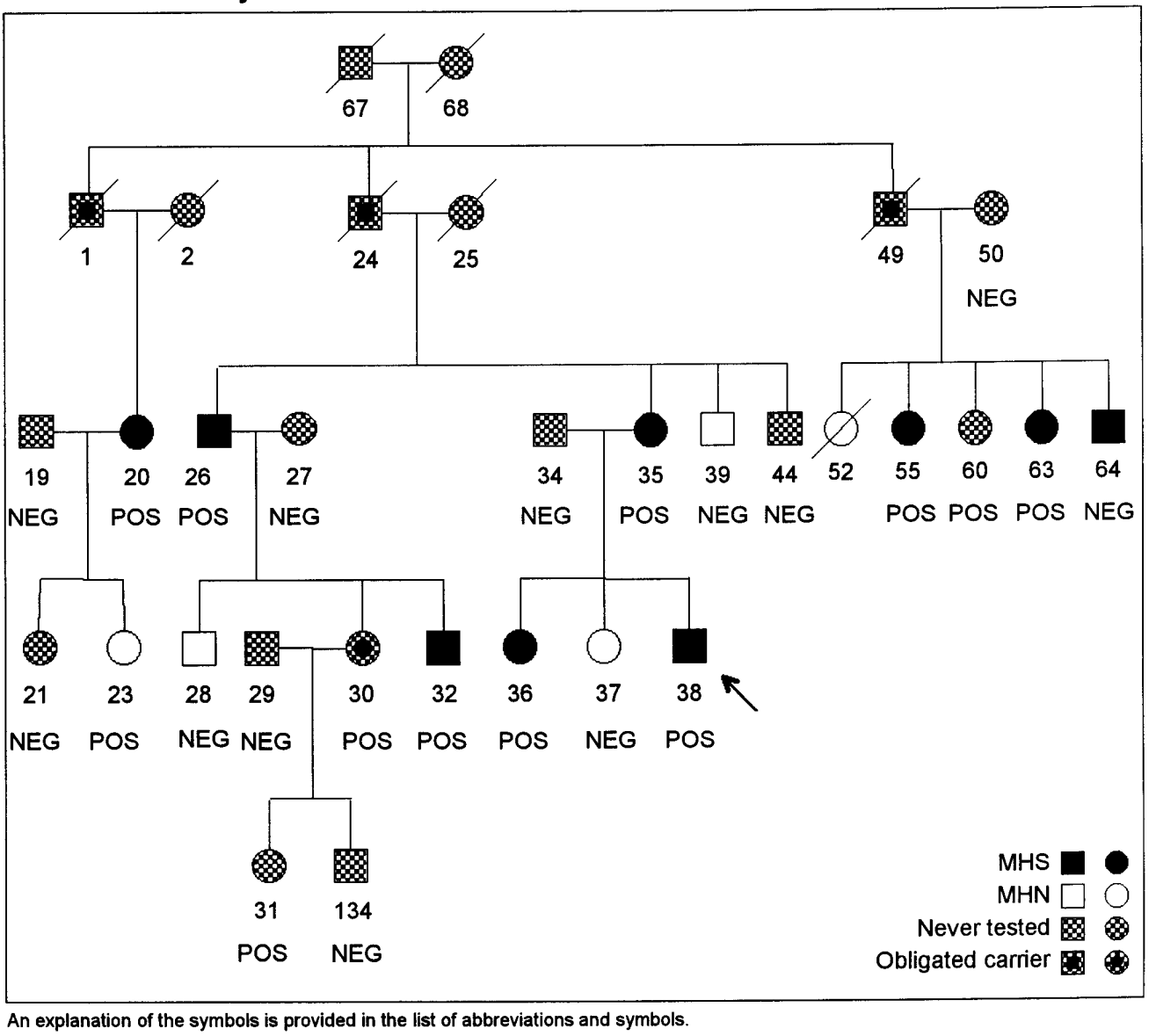
Family and individual number	MH Status via IVCT	RFLP results	MH Status via RFLP	Discordance between the IVCT and RFLP diagnoses
105-19	-	275 bp, 166 bp	MHN	-
105-20	MHS	275 bp, 166bp	MHN	NO
105-21*	-	275 bp, 166 bp	MHN	-
105-23	MHN	441 bp, 275 bp, 166 bp	MHS	**YES**
105-26	MHS	441 bp, 275 bp, 166 bp	MHS	NO
105-27	-	275 bp,166 bp	MHN	-
105-28	MHN	275 bp, 166 bp	MHN	NO
105-29*	-	275 bp, 166 bp	MHN	-
105-30*	-	441 bp, 275 bp, 166 bp	MHS	-
105-31*	-	441 bp, 275 bp, 166 bp	MHS	-
105-32	MHS	441 bp, 275 bp, 166 bp	MHS	NO
105-33*	-	275 bp, 166 bp	MHN	-
105-34	-	275 bp, 166 bp	MHN	-
105-35	MHS	441 bp, 275 bp, 166 bp	MHS	NO
105-36	MHS	441 bp, 275 bp, 166 bp	MHS	NO
105-37	MHN	275 bp, 166 bp	MHN	NO
105-38	MHS	441 bp, 275 bp, 166 bp	MHS	NO
105-39	MHN	275 bp, 166 bp	MHN	NO
105-44	-	275 bp, 166 bp	MHN	-
105-45*	-	275 bp, 166 bp	MHN	-
105-46*	-	275 bp, 166 bp	MHN	-
105-47*	-	275 bp, 166bp	MHN	-
105-50	-	275 bp, 166 bp	MHN	-
105-52*	MHN	275 bp, 166 bp	MHN	NO
105-54*	-	275 bp, 166 bp	MHN	-
105-55*	MHS	441 bp, 275 bp, 166 bp	MHS	NO
105-60	-	441 bp, 275 bp, 166 bp	MHS	-
105-63	MHS	441 bp, 275 bp, 166 bp	MHS	NO
105-64	MHS	275 bp, 166 bp	MHN	**YES**
105-70*	-	275 bp, 166 bp	MHN	-
105-81*	-	275 bp, 166 bp	MHN	-
105-83	MHN	275 bp, 166 bp	MHN	NO
105-88	MHN	275 bp, 166 bp	MHN	NO
105-98	MHN	275 bp, 166 bp	MHN	NO
105-104	MHN	275 bp, 166 bp	MHN	NO
105-115	-	275 bp, 166 bp	MHN	-
105-117	MHN	275 bp, 166 bp	MHN	NO
105-124	MHN	275 bp, 166 bp	MHN	NO
105-134*	-	275 bp, 166 bp	MHN	-

IVCT = *in vitro* contracture test; MHN = MH normal; MHS = MH susceptible; RFLP = Restriction fragment length polymorphism. A dash indicates that the individual was not phenotyped via the IVCT. An asterisk indicates individual genotyped for the first time in this study.

Individual MH105-30 has two siblings who have been diagnosed via the IVCT as MHN (MH105-28) and MHS (MH105-32) respectively. This female (MH105-30) has not been tested via the IVCT to determine her MH status although she was aware of her strong

family history of MHS. Upon screening the family for the presence of the mutation she was found to have inherited the Arg614Cys mutation segregating in this family. She has two young children, individuals MH105-31 (aged 8 years) and MH105-134 (aged 6 years) of whom the MH status is unknown as these children are too young to undergo the biopsy for the IVCT analysis. However, identification of the Arg614Cys mutation in this family allowed for the determination of the MH status of these two children. The daughter MH105-31 was subsequently found to have inherited the mutation from her mother. Her brother, individual MH105-134, was negative for the mutation as he inherited the normal allele from his mother. The Arg614Cys mutation segregating this family was absent in the father (MH105-29) of the two children. Individuals MH105-30 and MH105-31 both inherited the causative mutation and are both susceptible to MH. They should now be counselled accordingly.

Figure 4.23: Excerpt from the pedigree of the South African malignant hyperthermia family MH105

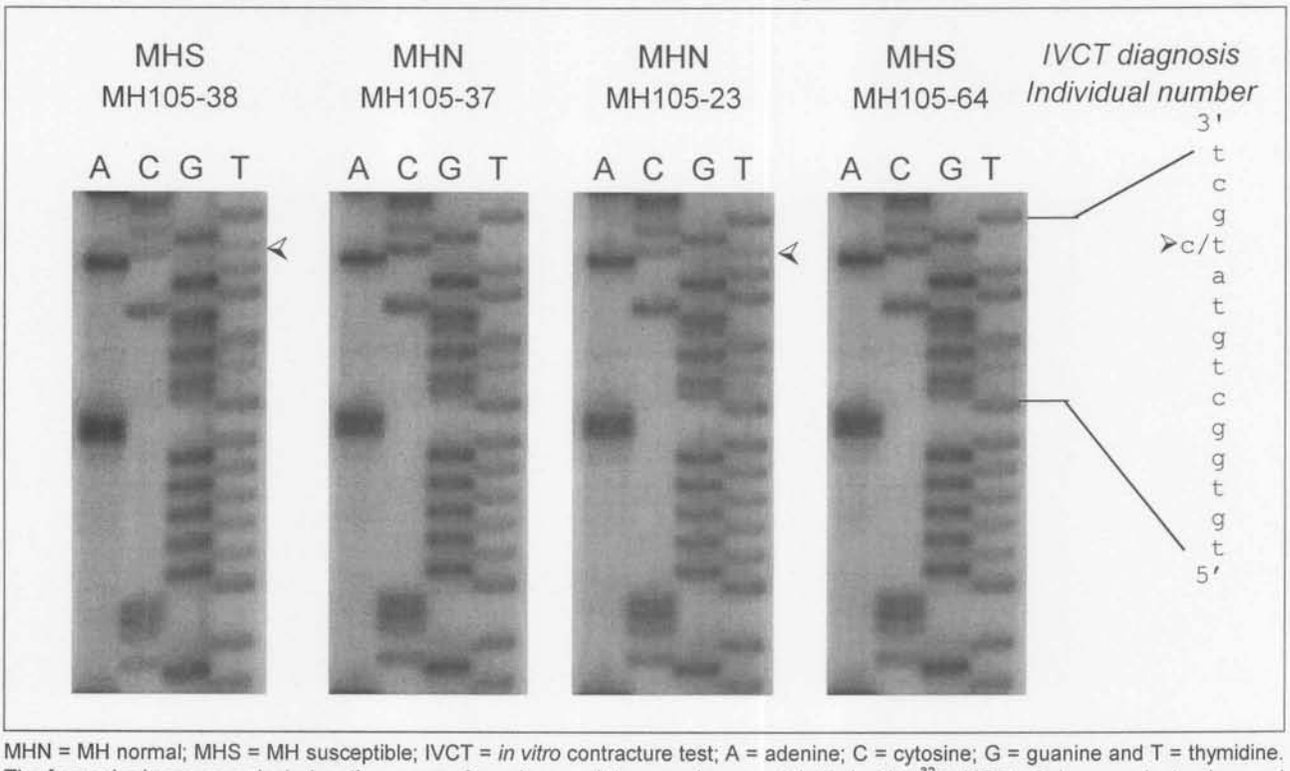


An explanation of the symbols is provided in the list of abbreviations and symbols.

Two individuals (MH105-23 and MH105-64) from this family displayed phenotype-genotype discordance as first reported by Olckers *et al.* (1994). Individual MH105-64 was diagnosed as MHS via the IVCT but did not inherit the mutation. In contrast, MHN individual MH105-23 was identified as harbouring the Arg614Cys mutation. The IVCT results and the RFLP results of this family are presented in Figure 4.23. The identification of the Arg614Cys mutation in family MH105 contributes towards establishing a limited molecular diagnostic service for individuals within this particular MH family.

Selected individuals were sequenced to verify the results obtained via RFLP analysis. These individuals were the proband MH105-38 that served as positive control, the sibling of the proband, individual MH105-37, which served as the negative control and the two discordant individuals MH105-23 and MH105-64. The C to T substitution was present in both the proband and individual MH105-23 whereas the other two individuals, MH105-37 and MH105-64, did not harbour the mutation as indicated in Figure 4.24.

Figure 4.24: Autoradiographs of chain termination sequencing utilised for detection of the Arg614Cys mutation in the RYR1 gene



MHN = MH normal; MHS = MH susceptible; IVCT = *in vitro* contracture test; A = adenine; C = cytosine; G = guanine and T = thymidine. The forward primer was selected as the sequencing primer and the samples were labelled with α^{32}P-dCTP. Gels were electrophoresed for 2 hours at 60 W. An arrowhead indicates nucleotide position 1840 where an additional fragment in the T lane of the sequence of individuals MH105-38 and MH105-23 indicates the presence of the Arg614Cys mutation.

The Arg614Cys mutation segregated with the IVCT results in this five generation South African MHS family with the exception of two individuals (MH105-23 and MH105-64). The possibility that this family might not display linkage to chromosome 19q can be rejected as linkage analysis in an earlier study by Olckers (1993) clearly indicated that the MH

phenotype segregating in this large South African family displayed linkage to the 19q13.1 region.

Individual MH105-23 was diagnosed as MHN with the IVCT and inherited the Arg614Cys mutation (Olckers *et al.*, 1994). This individual therefore displays phenotype-genotype discordance. Phenotype-genotype discordance similar to that observed by Olckers *et al.* (1994) has also been described by Deufel *et al.* (1995) for one individual from a German family and by Fortunato *et al.* (1999) for an individual from an Italian family. This type of discordance is of concern. The IVCT has been standardised to ensure that the sensitivity approaches 100% so that all possible MHS individuals are detected, and the possibility of false-negative diagnoses are eliminated (Ørding *et al.*, 1997). However, the estimated sensitivity is 99% leaving a remote chance that a false-negative diagnosis will occur (Ørding *et al.*, 1997). Isaacs and Badenhorts (1993) have reported four false-negative IVCT diagnoses and it is therefore not impossible for the IVCT diagnosis of individual MH105-23 to be false-negative.

Individual MH105-64 was diagnosed as MHS via the IVCT but did not harbour the Arg614Cys missense mutation. The specificity of the IVCT is reported to be 93.6% (Ørding *et al.*, 1997) and for this reason false-positive results can be anticipated. Individual MH105-64 could therefore have been diagnosed as false-positive via the IVCT. This is not the only case of phenotype-genotype discordance where the individual was diagnosed as MHS but did not inherit the mutation. Three other authors (Deufel *et al.*, 1995; Serfas *et al.*, 1996; Fagerlund *et al.*, 1997) have subsequently reported cases of false-positive IVCT results. If it was accepted that the IVCT results of individuals MH105-23 and MH105-64 are incorrect the Arg614Cys mutation co-segregates with the MHS phenotype within this family. The results obtained via the RFLP for individuals MH105-23 and MH105-64 were confirmed via sequencing analysis. A likely explanation for the discordance in these two individuals is therefore incorrect diagnosis with the IVCT.

The discordance may also be due to a modifying gene that might influence the penetrance of the Arg614Cys mutation in this family. Genetic heterogeneity has already been described in MH as linkage to six other loci on the human genome has been reported (Levitt *et al.*, 1992; Iles *et al.*, 1994; Sudbrak *et al.*, 1995; Olckers, 1997; Robinson *et al.*, 1997). Possible interaction between different genes involved in MH has not yet been investigated. It may well be that there is interaction between different genes that may modulate the function and expression of the gene or genes involved. This could result in

discordance between the MH phenotype and the genotype. However, if this theory is true more reports of phenotype-genotype discordance are expected, as the Arg614Cys mutation would then not be causative for MH. This would, however, contradict the current evidence in favour of this mutation being causative of MH.

A third explanation might be that the Arg614Cys mutation is not the causative mutation in this family. The discordant individuals are in different branches of the large family and another causative mutation may be responsible for the MHS phenotype in this particular family. The grandfathers (MH105-1 and MH105-24) of individual MH105-23 and the proband, MH105-38, were brothers. Individual MH105-30, the mother of individual MH105-23, was diagnosed as MHS via the IVCT and she harbours the Arg614Cys mutation. Individual MH105-23 could have inherited the Arg614Cys mutation from her mother. However, she most probably inherited the mutation from her father MH105-1 who is an obligate carrier of the Arg614Cys mutation. It is therefore unlikely that a different mutation is responsible for the discordance observed in this branch of the family. Individual MH105-64 and the mother of the proband, individual MH105-35, are first cousins. The MH status of individual MH105-49, the father of individual MH105-64, is unknown, as molecular studies could not be performed on him, because no DNA sample was available as he was deceased. However, he is also an obligated carrier of the Arg614Cys mutation, as well as individual MH105-24, his brother.

The Arg614Cys mutation is considered a causative MH mutation. This mutation complies with most of the requirements for a disease causing missense mutation. The requirements with which the Arg614Cys mutation comply are listed below:

1. The mutation is in the structural region of the RYR1 protein (Sorrentino and Volpe, 1993; Quane *et al.*, 1997).
2. The region is conserved across species and MH in porcine is an animal model for the Arg614Cys mutation (Fujii *et al.*, 1991; Gillard *et al.*, 1991).
3. The Arg614Cys mutation is absent from the normal population (Gillard *et al.*, 1991; Otsu *et al.*, 1992).
4. Segregation of the Arg614Cys mutation with the MH phenotype has been observed in several families worldwide (Steinfath *et al.*, 1995; Monori *et al.*, 1995)
5. Transfection studies concluded that the transfected cells containing the Arg614Cys mutation have abnormal Ca^{2+}-release due to the differences in the gating properties between the wild type and mutant RYR1 receptors (Otsu *et al.*, 1994; Treves *et al.* 1994; Tong *et al.*, 1997).

This type of result has major implications when patients are counselled. In this family the Arg614Cys mutation is considered as causative despite the discordance observed between the IVCT results and RFLP results. Three patients that did not undergo muscle biopsies were diagnosed here on a molecular level. These three individuals, MH105-30, MH105-31 and MH105-60 all inherited the Arg614Cys mutation and are considered as MH positive and should be counselled as such. Counselling of the two individuals that displayed discordance should be performed with care. Individual MH105-23 has considered herself as MHN and is now considered as MHS. She should therefore be counselled that she should consider herself to be positive. On the other hand individual MH105-64 has been considered as MHS due to the positive IVCT results. Although he has not inherited the Arg614Cys mutation he will be counselled that he should still consider himself MH positive since there might be another unidentified mutation that might be responsible for his positive IVCT result. This is in agreement with the general worldwide guidelines for counselling of MH patients. It was determined that an individual should clinically be considered as MHS when a mutation is identified within the individual or when the individual is diagnosed as MHS via the IVCT regardless of whether the IVCT and mutation results are in agreement (Healy *et al.*, 1996; Tong *et al.*, 1997).

4.8 Gly2435Arg mutation in the skeletal muscle ryanodine receptor (RYR1) gene

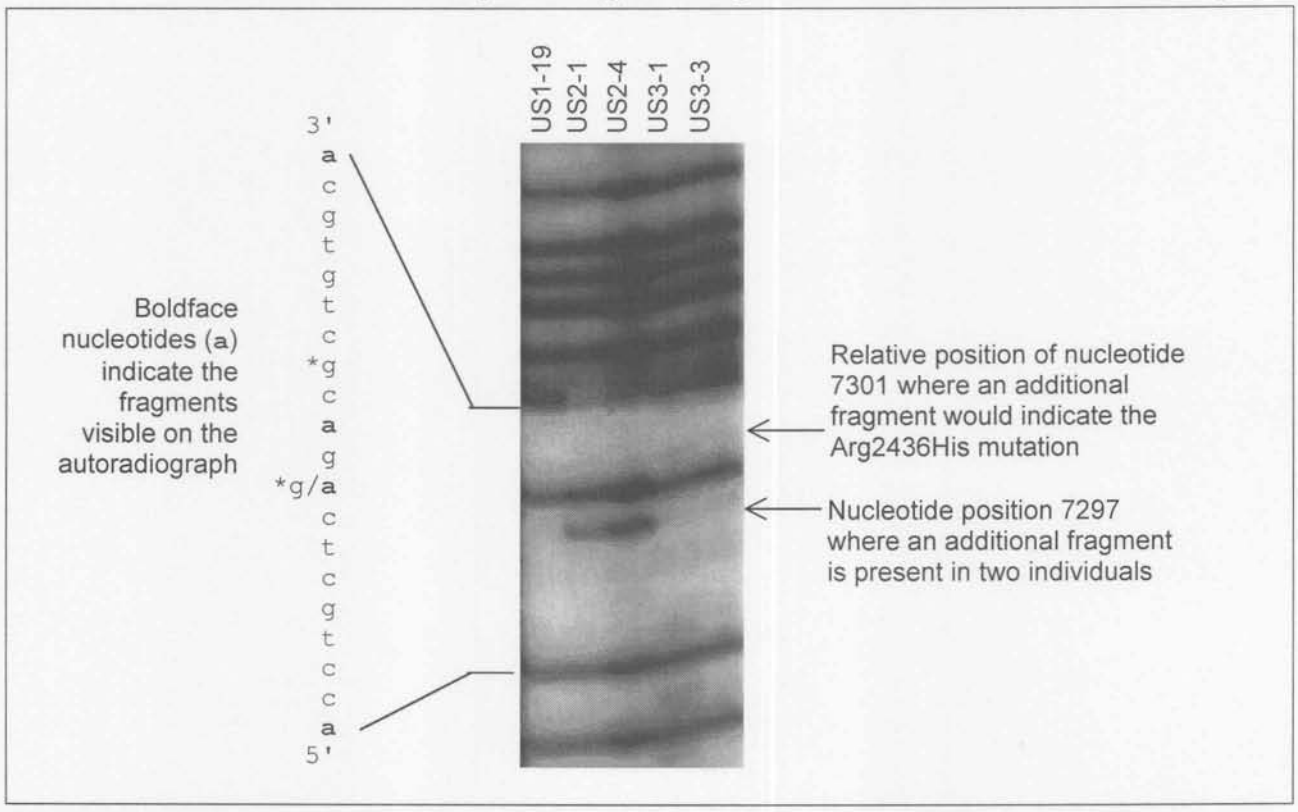
The region encompassing the Gly2435Arg and the Arg2436His mutations could be amplified using the same set of primers. PCR was performed using the standard PCR program discussed in paragraph 3.4 with an annealing temperature of 64°C. One percent formamide was added to the PCR reaction to improve amplification of the 187 bp fragment.

The *Hga I* restriction enzyme is expensive, and since the amplified region encompassed both the Gly2435Arg and the Arg2436His mutations, the detection of both these mutations simultaneously would be ideal. These mutations are also both caused by the substitution of a G with an A. Therefore, it was most cost effective and time efficient to examine individuals from the seventeen families by detecting these mutations via single lane sequencing of the A lane - the results of which are presented in Table 4.3 (page 128).

Representative results generated via SLS of lane A are presented in Figure 4.25. None of the individuals investigated exhibited an additional adenine fragment in position 7301. The Arg2436His mutation was, therefore, not present in the populations investigated in this

study. However, two individuals from family US2 did have an additional adenine fragment in position 7297 indicating the presence of the Gly2435Arg mutation.

Figure 4.25: Autoradiograph of single lane sequence analysis generated for detection of the Gly2435Arg and Arg2436His mutation in the RYR1 gene



Autoradiograph of single lane sequencing gel where the forward primer was selected to sequence the samples and labelled with α^{32}P-dCTP utilising 7-deaza-dGTP. Gels were electrophoresed for 2 hours at 60 W. An arrow indicates nucleotide position 7297 where an additional fragment indicates the presence of the Gly2435Arg mutation in individuals US2-1 and US2-4.

The Gly2435Arg mutation could be detected via RFLP as the mutation created a *Dde I* restriction enzyme site. It was more cost effective to screen the five individuals of family US2 for the Gly2435Arg mutation via RFLP than to repeat the SLS. Therefore, upon identification of the additional fragment at position 7297 in the two individuals from family US2, RFLP analysis for the Gly2435Arg mutation was performed on five individuals from this family. The results obtained for the RFLP analysis performed for family US2 are presented in Table 4.2.

Table 4.2: RFLP results of the Gly2435Arg mutation investigated in individuals from MHS families US2

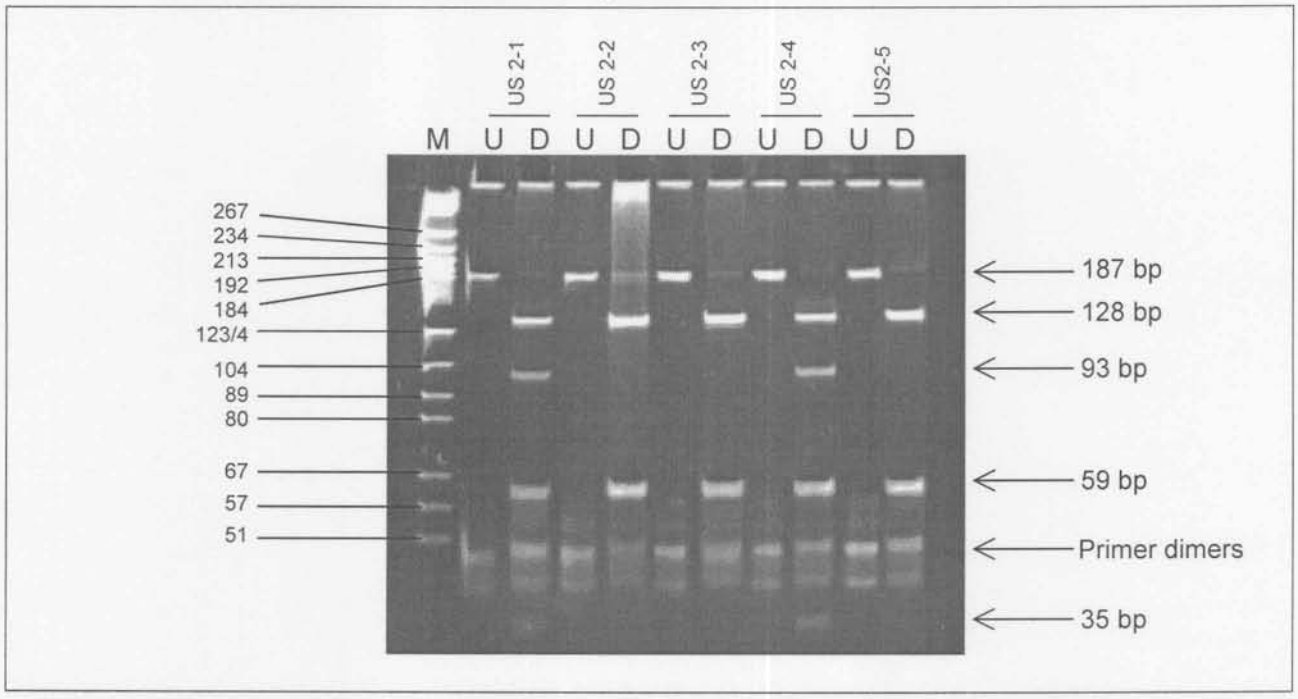
Family and individual number	MH Status via CHCT	Fragments observed	RFLP results
US2-1	MHS	128 bp, 93 bp, 59 bp, 35 bp fragments	***Positive***
US2-2	MHN	128 bp, 59 bp fragments	Negative
US2-3	MHN	128 bp, 59 bp fragments	Negative
US2-4	MHS	128 bp, 93 bp, 59 bp, 35 bp fragments	***Positive***
US2-5	MHN	128 bp, 59 bp fragments	Negative

MHN = MH normal; MHS = MH susceptible; CHCT = caffeine halothane contracture test; RFLP = Restriction fragment length polymorphism.

In a normal individual the amplified product is cleaved into two fragments (128 and 59 bp). The inclusion of a *Dde I* digestion site in the 187 bp fragment functions as a built-in control for incomplete digestion. A homozygous MHS individual would display three fragments (93, 59 and 35 bp) and four fragments (128, 93, 59 and 35 bp) would be observed in a heterozygous affected individual.

RFLP analyses confirming the results obtained via single lane sequencing of family US2 are presented in Figure 4.26. The two individuals (US2-1 and US2-4) who were diagnosed as positive with the CHCT were both heterozygous for the Gly2435Arg mutation. The 128 bp and 59 bp fragments were observed in the rest of the individuals from this small North American family. This is consistent with the MH negative CHCT diagnosis for individual US2-2. A fragment that was lighter in intensity than the 128 bp and 59 bp fragments were observed in individuals US2-2, US2-3 and US2-5, indicating incomplete digestion. Individuals US2-3 and US2-5 have never been tested via the IVCT. Neither of them harboured the Gly2435Arg mutation and both could, therefore, be considered to be MH negative with regard to this mutation. The above results demonstrated that the Gly2435Arg mutation segregated with the MHS phenotype in this family.

Figure 4.26: Photographic representations of the Gly2435Arg mutation identified in North American MHS family US2



RFLP fragments were separated on a 10% polyacrylamide gel. This gel was electrophoresed at 250 V for two hours and subsequently stained with SYBRGold for 30 min. "M" indicates the molecular weight marker (pBR322/*Hae III*) used to size the fragments. "U" indicates the undigested PCR product and "D" the PCR product digested with 10 units of *DdeI*.

Additional fragments could be observed in the samples between the 59 bp and 35 bp fragments, appearing as a smear on the gel. This is due to the multiple number of possible primer dimers that could form, indicated in Figure 4.27. The 3' dimers and most stable primer dimers as determined by their ΔG values are also indicated in Figure 4.27.

Figure 4.27: Secondary structures formed by the G2435RF and R2436HR primers

Self-dimers

G2435RF forward primer dimers

(a)
```
5' ttccctgcagctttggtgaggaacc 3'
                  ||||||
3' ccaaggagtggtttcgacgtccctt 5'
```
ΔG = -10.24 kcal.mol^{-1}
Most stable dimer

(b)
```
  5' ttccctgcagctttggtgaggaacc 3'
     ||||    :       :    ||||
3' ccaaggagtggtttcgacgtccctt 5'
```
ΔG = -7.58 kcal.mol^{-1}

(c)
```
    5' ttccctgcagctttggtgaggaacc 3'
       ::  :   ||||   : ::
3' ccaaggagtggtttcgacgtccctt 5'
```
ΔG = -6.33 kcal.mol^{-1}

(d)
```
5' ttccctgcagctttggtgaggaacc 3'
                  |||     :::
           3' ccaaggagtggtttcgacgtccctt
```
3' Dimer
ΔG = -5.41 kcal.mol^{-1}

R2436HR reverse primer dimers

(e)
```
            5' ctgcatgaggcgttcaaag 3'
                  ||||
3' gaaacttgcggagtacgtc 5'
```
ΔG = -7.04 kcal.mol^{-1}

(f)
```
         5' ctgcatgaggcgttcaaag 3'
            :  ||||  :
3' gaaacttgcggagtacgtc 5'
```
ΔG = -5.38 kcal.mol^{-1}

(g)
```
5' ctgcatgaggcgttcaaag 3'
       :::     |||
  3' gaaacttgcggagtacgtc 5'
```
ΔG = -4.52 kcal.mol^{-1}

Cross dimers

G2435RF and R2436HR primer dimers

(h)
```
          5' ttccctgcagctttggtgaggaacc 3'
               :  ||||||
3' gaaacttgcggagtacgtc 5'
```
ΔG = -8.64 kcal.mol^{-1}
2nd Most stable dimer

(i)
```
5' ttccctgcagctttggtgaggaacc 3'
               ||||||
        3' gaaacttgcggagtacgtc 5'
```
3' Dimer
ΔG = -8.44 kcal.mol^{-1}
3rd Most stable dimer

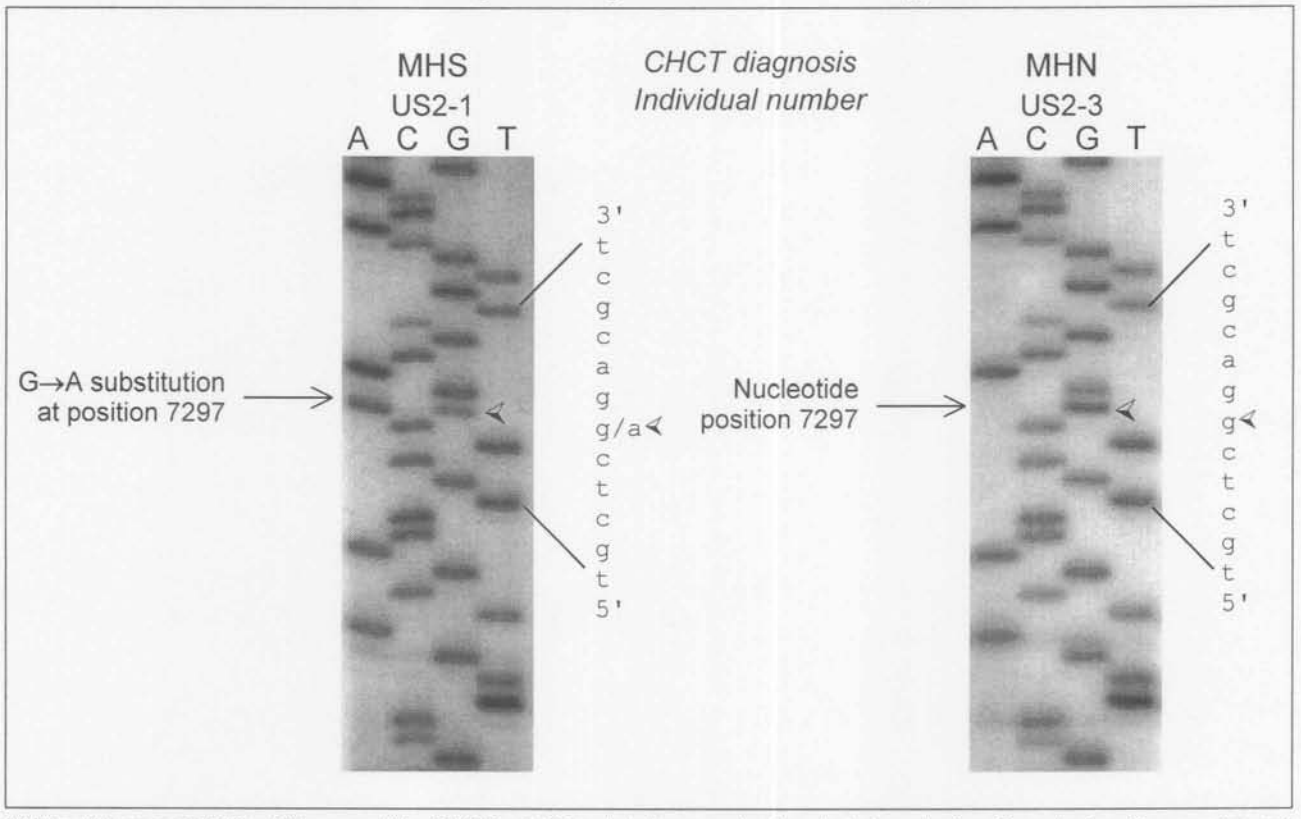
(j)
```
5' ttccctgcagctttggtgaggaacc 3'
                  ||||
           3' gaaacttgcggagtacgtc 5'
```
3' Dimer
ΔG = -5.86 kcal.mol^{-1}

(k)
```
5' ttccctgcagctttggtgaggaacc 3'
                  |||    :
         3' gaaacttgcggagtacgtc 5'
```
3' Dimer
ΔG = -4.52 kcal.mol^{-1}

Primer sequences are listed in Table 3.3. ΔG indicates the free energy.

The RFLP analysis was verified via sequencing of the PCR product amplified from gDNA of five individuals in family US2. The sequence generated for two of the individuals (US2-1 and US2-3) in family US2 are presented in Figure 4.28. The two MHS individuals US2-1 and US2-4 each had an additional fragment in the A lane at nucleotide position 7297 indicating that these individuals are positive for the Gly2435Arg mutation. None of the other three individuals, including the MHN individual US2-2, displayed an additional fragment in the A lane of the sequence. Upon analysis of the sequence data no discrepancies were observed when the sequence was compared with the sequence deposited in Genbank (accession number U48477).

Figure 4.28: Autoradiographs of chain termination sequencing utilised for the detection of the Gly2435Arg mutation in RYR1 gene



MHN = MH normal; MHS = MH susceptible; CHCT = Caffeine halothane contracture test; A = adenine; C = cytosine; G = guanine and T = thymidine. The forward primer was selected as the sequencing primer and the samples were labelled with α^{32}P-dCTP. Gels were electrophoresed for 2 hours at 60 W. An arrowhead indicates nucleotide position 7297 where an additional fragment in the A lane of the sequence of individual US2-1 indicates the presence of the Gly2435Arg.

This mutation was reported to have an estimated frequency of 4% in MHS families (Manning *et al.*, 1998a). Brandt *et al.* (1999) reported that the Gly2435Arg mutation was observed in 7% of the German MHS population investigated. Keating *et al.* (1994) described the presence of the Gly2435Arg mutation in four MHS individuals from different MHS populations, which included one Irish and two German MHS families. Phillips *et al.* (1994) also identified the Gly2435Arg mutation in four Canadian MHS pedigrees and Barone *et al.* (1999) described it in an Italian family. This is the first report of the

Gly2435Arg mutation in the Mid-Atlantic North American MH population. The absence of the mutation from the South African population is most likely due to the fact that only four South African families were investigated.

This mutation can be considered as the disease causing mutation in this family if no other mutations or other genetic factors, such as the genetic background of the individuals, possible epistatic interaction, or modifier genes, contribute to the MH phenotype. This mutation is thought to be a causative mutation as it was absent from the normal population (Keating *et al.*, 1994; Phillips *et al.*, 1994) and segregated with the MH phenotype in several MHS families (Keating *et al.*, 1994; Phillips *et al.*, 1994; Manning *et al.*, 1998a; Barone *et al.*, 1999; Brandt *et al.*, 1999). Furthermore, Gly2435 is a functionally important amino acid conserved across several species. Transfection studies performed by Tong *et al.* (1997) provide further evidence that the Gly2435Arg mutation is causative of MH. Identification of the Gly2435Arg mutation in family US2 contributes towards establishing a limited molecular diagnostic service for individuals within this particular MH family.

Though no discordance was observed in the North American MHS family which harbours the Gly2435Arg mutation, discordance has been reported by Phillips *et al.* (1994) and Keating *et al.* (1994). Keating *et al.* first described this mutation in 1994 and noted that the MHS phenotype co-segregated with all but one MHS pedigree in which one individual did not harbour the mutation but was diagnosed as MHS. Later that same year Phillips *et al.* (1994) also reported the mutation in four MHS families and found discordance in four individuals from two of the MHS families. One individual presented with a false-negative diagnosis and the other three individuals were reported to have false-positive diagnoses. Both authors concluded that the reason for the discordance was most likely the occurrence of a false-negative IVCT diagnosis and false-positive IVCT diagnoses (Phillip *et al.*, 1994; Keating *et al.*, 1994). Neither the European IVCT nor the North American CHCT is 100% sensitive and therefore false-negative diagnoses were to be expected. It is also well documented that the specificity of the two protocols are not 100% and therefore false-positive diagnoses are expected (Ørding *et al.*, 1997; Allen *et al.*, 1998).

Haplotype analysis performed by Phillips *et al.* (1994) indicated that the mutation has arisen at least twice as two different haplotypes were associated with the mutation and the MHS phenotype. The time when these different mutational events occurred is unknown. This mutation is also not population specific as it has been identified in at least five different populations to date; including the Irish, Canadian, German, Italian populations

(Keating *et al.*, 1994; Phillips *et al.*, 1994; Manning *et al.*, 1998a; Barone *et al.*, 1999; Brandt *et al.*, 1999) and now the Mid-Atlantic North American population.

4.9 Arg2436His mutation in the skeletal muscle ryanodine receptor (RYR1) gene

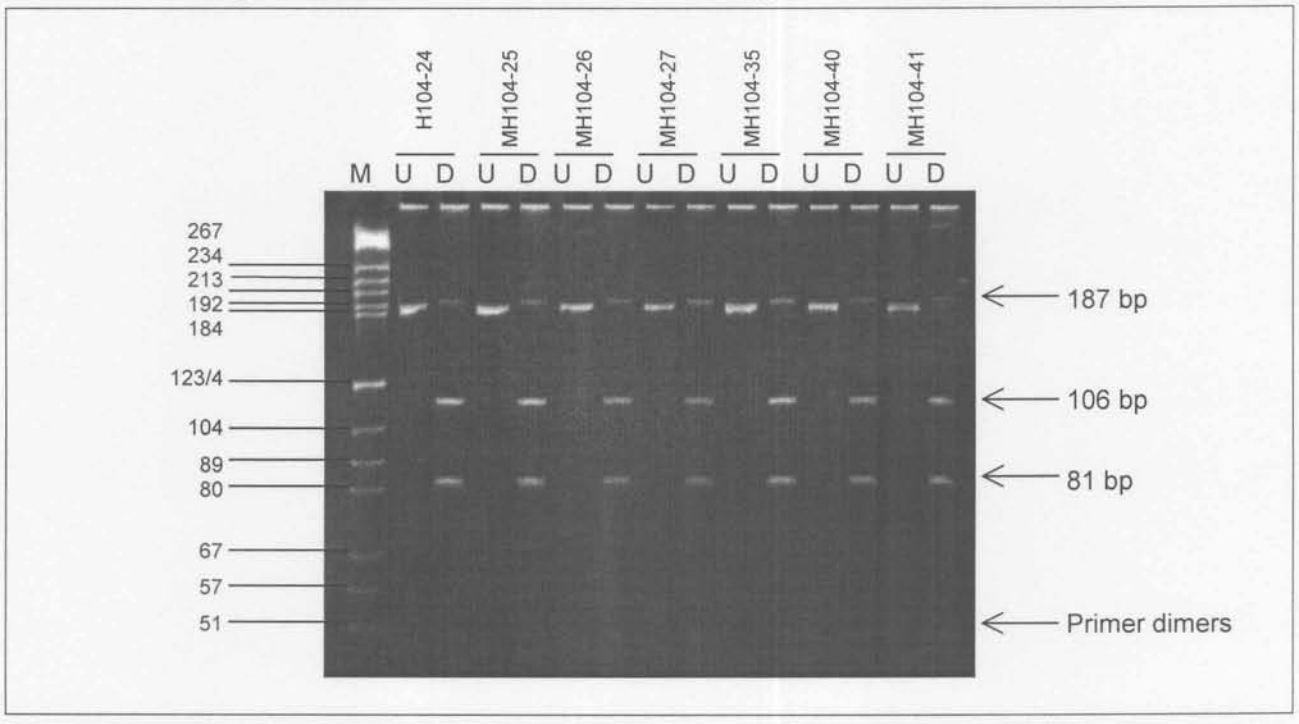
Zhang *et al.* (1993) detected the substitution of G to A at position 7301 via RFLP analysis. This change in nucleotide sequence abolished the *Hga I* restriction enzyme site, creating an RFLP which can be used to detect the mutation. Amplified gDNA was cut with *Hga I* resulting in the observation of two fragments (106 and 81 bp) in a MHN individual. However, when the mutation is present either one (187 bp) or three fragments (187, 106 and 81 bp) would be present in homozygous or heterozygous MHS individuals, respectively.

Members of South African family MH104 were analysed via RFLP to illustrate the RFLP protocol reported for the Arg2436His mutation as described in paragraph 3.5 and discussed in the previous paragraph. RFLP results of family MH104 concurred with the SLS results that are listed in Table 4.3 (page 128).

All the individuals displayed the 106 bp and 81 bp fragments when the PCR products were digested. However, they also displayed a 187 bp fragment which was lighter in intensity than the 106 bp and 81 bp fragments indicating possible incomplete digestion. Incomplete digestion was confirmed since individuals MH104-26 and MH104-27 were sequenced and no additional A fragment in position 7301 was present in these two individuals as depicted in Figure 4.30. Samples presented in Figure 4.29 were digested with 1 unit of the *Hga I* restriction enzyme. Optimisation of the RFLP protocol was attempted with various units (1U - 5U or 10U) of enzyme and incomplete digestion was still observed as indicated in Figure 4.29. The *Hga I* restriction enzyme is expensive and it was therefore not cost effective to optimise the RFLP protocol as the Arg2436His mutation can be detected together with the Gly2435Arg mutation via SLS.

Additional fragments could also be observed in the 51 bp region where the primer dimers are expected to migrate. The possible secondary structures formed by the set of primers used to amplify the 187 bp fragment are depicted in Figure 4.27.

Figure 4.29: Photographic representation generated for the detection of the Arg2436His mutation within exon forty-five of the RYR1 gene

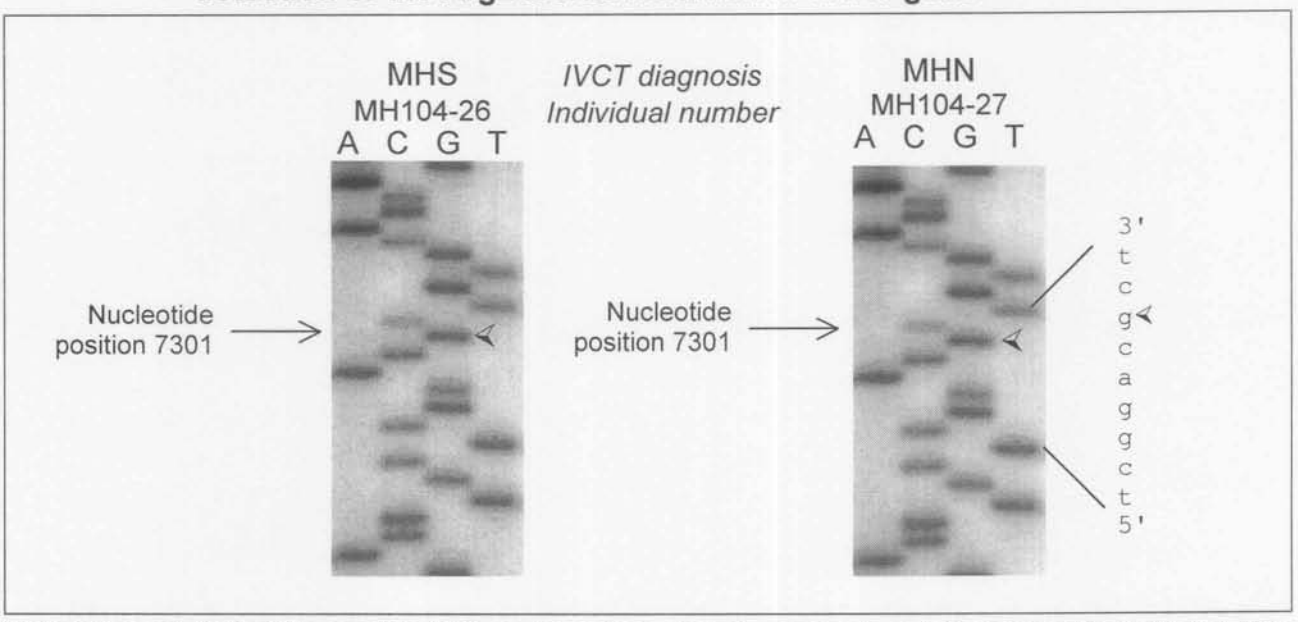


RFLP fragments were separated on a 10% polyacrylamide gel. This gel was electrophoresed at 250 V for two hours and subsequently stained with EtBr for 30 min. "M" indicates the molecular weight marker (pBR322/*Hae III)* used to size the fragments. "U" indicates the undigested PCR product and "D" the PCR product digested with 1 unit of *Hgal*.

Single lane sequencing analysis of the A lane for the Arg2436His mutation has been described in conjunction with the results for the Gly2435Arg mutation, in paragraph 4.8. Figure 4.25 depict the representative results for mutation Arg2436His obtained for the families investigated in this study and these results are summarised in Table 4.3 (page 128).

PCR amplified fragments from individuals MH104-26 and MH104-27 were selected for sequencing to verify the RFLP results obtained for both the *Dde I* and the *Hga I* restriction enzyme assays as both the restriction sites are located in the 187 bp fragment. Individuals MH104-26 and MH104-27 are the parents of the proband, MH104-38, who died due to an MH episode. The father of the proband, individual MH104-26, was diagnosed as MHS and individual MH104-27 (her mother) was diagnosed as MHN via the IVCT. Both individuals displayed the normal nucleotide sequence depicted in Figure 4.30, indicating that the RFLP results are a true reflection of the absence of the two mutations (Gly2435Arg and Arg2436His) in this family. It was therefore concluded that another mutation is responsible for the MH phenotype in these MHS individuals. No discrepancies were observed when the generated sequence was compared with the sequence submitted to Genbank (accession number U48477).

Figure 4.30: Autoradiographs of chain termination sequencing utilised for the detection of the Arg2436His mutation in RYR1 gene



MHN = MH normal; MHS = MH susceptible; IVCT = *in vitro* contracture test; A = adenine; C = cytosine; G = guanine and T = thymidine. The forward primer was selected as the sequencing primer and the samples were labelled with α^{32}P-dCTP. Gels were electrophoresed for 2 hours at 60 W. An arrowhead indicates nucleotide position 7301.

The Arg2436His mutation is adjacent to the Gly2435Arg mutation, which have been associated with 4% of MHS families (Phillip *et al.*, 1994; Keating *et al.*, 1994). To date the Arg2436His mutation has only been observed in a single large Canadian family (Zhang *et al.*, 1993). Seven members from this family have been diagnosed with both CCD and MH. In addition two members of the family had MH episodes but were not diagnosed with the IVCT. The role of the Arg2436His mutation in the phenotypic expression of both MH and CCD remains unclear. It appears to cause both MHS and CCD although the authors refer to the mutation as potentially leading to CCD (Zhang *et al.*, 1993). The Arg2436His mutation was identified prior to identification of the Gly2435Arg mutation (Keating *et al.*, 1994; Phillips *et al.*, 1994) and was the only mutation reported in the central region of the RYR1 gene. Zhang *et al.* (1993) suggested that the Arg2436His mutation and possibly other mutations within the central region may lead to CCD rather than MH as other mutations (Gly248Arg and Arg163Cys) reported in the N-terminal region of theRYR1 gene have been associated with MH or CCD or both. However, thirteen other mutations have since been reported in the central region of the RYR1 gene and these mutations have been associated with MHS. This mutation may, therefore, most likely cause both CCD and MH as discussed for the Arg163Cys mutation in paragraph 4.2.

Zhang *et al.* (1993) suggested that this mutation is family specific. To date, this theory seems plausible due to the absence of this mutation in multiple MHS and CCD families investigated worldwide. The mutation could be family specific because the mutation might

be a recent event that originated in this particular Canadian family. This would explain the absence of this mutation in other families, and therefore also the families included in this study. Another explanation for the absence of the Arg2436His mutation in the families included in this study could be that the mutation is associated with the CCD phenotype rather than the MHS phenotype. None of the families included in this study had a history of CCD and the mutation might therefore not be present in the families investigated here.

4.10 Summary of mutation analysis results

The results obtained for all nine of the missense mutations investigated in this study are listed in Table 4.3. Five of the mutations, Cys35Arg, Arg163Cys, Gly341Arg, Ile403Met and Arg614Cys, were investigated via RFLP analysis. The remaining four mutations, Gly248Arg, Tyr522Ser, Gly2435Arg and Arg2436His, were investigated via single lane sequencing. The molecular techniques utilised to detect these mutations have been standardised and the results are summarised below. The nine mutations investigated are discussed in detail in the previous sections of this chapter. A mutation screening service for MHS individuals can now be provided for the nine reported MH mutations investigated in this study. Individual US9-10 could only be screened for three mutations (Arg163Cys, Gly341Arg and Arg614Cys), as a limited amount of DNA was available. Similarly, only the Cys35Arg, Gly341Arg and Arg614Cys mutations could be investigated in individual US11-4 due to a limited amount of DNA available for investigation.

Table 4.3: Results of the mutation analyses for the MHS families investigated

Family and individual number	Cys35Arg	Arg163Cys	Gly248Arg	Gly341Arg	Ile403Met	Tyr522Ser	Arg614Cys	Gly2435Arg	Arg2436His
South African families									
MH101-6	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH101-10	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH101-12	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH102-2	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH102-4	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH102-11	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH102-24	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH102-28	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH102-39	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH102-48	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH102-96	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH102-117	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

continued ...

Table 4.3, continued ...

Family and individual number	Cys35Arg	Arg163Cys	Gly248Arg	Gly341Arg	Ile403Met	Tyr522Ser	Arg614Cys	Gly2435Arg	Arg2436His
MH102-125	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH104-24	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH104-25	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH104-26	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH104-27	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH104-35	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH104-40	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH104-41	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH105-20	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH105-23	Neg	Neg	Neg	Neg	Neg	Neg	***Positive***	Neg	Neg
MH105-26	Neg	Neg	Neg	Neg	Neg	Neg	***Positive***	Neg	Neg
MH105-28	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH105-32	Neg	Neg	Neg	Neg	Neg	Neg	***Positive***	Neg	Neg
MH105-35	Neg	Neg	Neg	Neg	Neg	Neg	***Positive***	Neg	Neg
MH105-36	Neg	Neg	Neg	Neg	Neg	Neg	***Positive***	Neg	Neg
MH105-37	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH105-38	Neg	Neg	Neg	Neg	Neg	Neg	***Positive***	Neg	Neg
MH105-39	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH105-55	Neg	Neg	Neg	Neg	Neg	Neg	***Positive***	Neg	Neg
MH105-63	Neg	Neg	Neg	Neg	Neg	Neg	***Positive***	Neg	Neg
MH105-64	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH105-83	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH105-88	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH105-98	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH105-104	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH105-117	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MH105-124	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
North American families									
US1-3	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US1-7	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US1-12	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US1-17	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US1-18	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US1-19	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US2-1	Neg	Neg	Neg	Neg	Neg	Neg	Neg	***Positive***	Neg
US2-2	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US2-4	Neg	Neg	Neg	Neg	Neg	Neg	Neg	***Positive***	Neg
US3-1	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US3-3	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US3-4	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US7-14	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US7-15	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US7-16	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US8-5	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US8-7	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US8-11	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

continued ...

Table 4.3, continued ...

Family and individual number	Cys35Arg	Arg163Cys	Gly248Arg	Gly341Arg	Ile403Met	Tyr522Ser	Arg614Cys	Gly2435Arg	Arg2436His
US8-12	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US8-19	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US9-6	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US9-8	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US9-10	NA	Neg	NA	Neg	NA	NA	Neg	NA	NA
US9-11	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US10-6	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US10-7	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US10-9	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US10-10	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US10-11	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US11-1	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US11-3	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US11-4	Neg	NA	NA	Neg	NA	NA	Neg	NA	NA
US12-2	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US12-4	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US12-7	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US13-5	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US13-6	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US13-9	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US13-10	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US13-11	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US13-12	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US14-2	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US14-7	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US14-8	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US15-6	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US15-7	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US15-8	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US16-3	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US16-6	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
US16-12	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Neg = Negative, NA = Not available.

Of the nine mutations investigated only two, Arg614Cys and Gly2435Arg, were identified in the group of families included in this study. The Arg614Cys mutation was identified in a large family (SA105) from the South African population. The Gly2435Arg mutation was identified in a family (US2) from the North American population. The MH phenotype of the selected individuals from these two MH families could be characterised at a molecular level. These two families can now be counselled appropriately according to their genotype and IVCT results, as discussed in paragraph 4.7. Moreover, family members not

previously included in this study can now be investigated for the specific mutation segregating in their family. This implies that a diagnostic service is available to individuals related to these two families.

Possible interaction between the nine mutations investigated can be excluded in the seventeen MH families included in this study. Results from this study do not support the hypothesis of an epistatic relationship between these MH mutations. However, the possibility that interaction between these nine mutations exists in *other* MH families or populations cannot be excluded. The possibility of interaction between other missense mutations in the RYR1 gene and the nine mutations investigated here can also not be excluded in the MH families investigated in this study.

CHAPTER FIVE

CONCLUSION

Since identification of the first MH mutation, Arg614Cys, in the human RYR1 gene in 1991 twenty-two other missense mutations have been identified. The presence of nine of these reported missense mutations were investigated in the four South African and thirteen North American MHS families included in this study. These nine mutations were: Cys35Arg, Arg163Cys, Gly248Arg, Gly341Arg, Ile403Met, Tyr522Ser, Arg614Cys, Gly2435Arg and Arg2436His.

Only two of the nine mutations investigated, Arg614Cys and Gly2435Arg, were observed in the group of families included in this study. Olckers *et al.* (1994) identified the Arg614Cys mutation in 25 selected individuals from South African family MH105. As a result of this observation an additional 14 members from South African family MH105 were subsequently screened for the presence of this mutation. Twelve individuals, three (MH105-30, MH105-31 and MH105-60) of whom were never tested via the IVCT, were identified as harbouring the Arg614Cys mutation. The phenotype-genotype discordance observed in two individuals from this family (MH105-23 and MH105-64) and reported earlier by Olckers *et al.* (1994), was confirmed in this study. One individual (MH105-64) was diagnosed as MHS via the IVCT but did not inherit the mutation. In contrast, an MHN individual (MH105-23) was identified as harbouring the Arg614Cys mutation. Identification of the Arg614Cys mutation in family MH105 contributes towards establishing a molecular diagnostic service for individuals within this particular MH family.

The Gly2435Arg missense mutation was observed in North American MHS family US2. This is the first report of the Gly2435Arg mutation in the U.S.A. MH population. Identification of this mutation in family US2 now allows for the provision of a molecular diagnostic service for individuals related to this family.

Haplotype analysis performed by Phillips *et al.* (1994) indicated that the Gly2435Arg mutation arose at least twice over time, as this mutation has been observed to segregate on two distinct haplotypes. It is not known exactly when these different mutational events occurred. Haplotype analysis was previously performed for North American family US2 (Levit *et al.*, 1991, unpublished data). Unfortunately, the haplotypes of US2 and those generated earlier for the Canadian families could not be compared, as Phillips *et al.* (1994)

did not publish their haplotype data. Haplotype analyses were not performed for the other families in which the Gly2435Arg mutation was identified (Keating *et al.*, 1994). The presence of this mutation in the North American population confirms that the mutation is not population specific as it has been identified in at least five different populations from three continents. Results from this study contribute towards the accurate estimation of the global mutation frequency of the Gly2435Arg mutation. The estimated mutation frequency of this mutation was reported to be 4% in the MH population (Manning *et al.*, 1998b).

The causative nature of both the Arg614Cys and Gly2435Arg missense mutation associated with MH have been verified (Treves *et al.*, 1994; Richter *et al.*, 1997). There is convincing biochemical evidence to suggest a causative nature for fourteen of the other missense mutations, with the exception of the Cys35Arg (Tong *et al.*, 1997) mutation and six mutations (Arg2163Pro, Thr2206Arg, Arg533His, Arg2436Leu, Arg2454Cys and Arg2454His) identified by Barone *et al.* (1999) and Brandt *et al.* (1999).

None of the other seven mutations (Cys35Arg, Arg163Cys, Gly248Arg, Gly341Arg, Ile403Met, Tyr522Ser and Arg2436His) were observed in the seventeen MH families investigated. If the families investigated in this study are truly representative of the South African and North American populations it is possible that these changes do not contribute to the MH phenotype segregating in these two populations.

The fact that seven missense mutations were not observed in the selected families investigated corroborates the suggestion that these mutations are population or even family specific (Quane *et al.*, 1993; Quane *et al.*, 1994b; Tong *et al.*, 1997). Five of these mutations (Cys35Arg, Gly248Arg, Ile403Met, Tyr522Ser and Arg2436His) have, to date, only been identified in a single family worldwide and, therefore, currently appears to be family specific. The Gly341Arg mutation seems to be restricted to the European continent as it has only been observed in European families and therefore appears to be population specific. If this is indeed true and future studies identify all the mutations that contribute to the MH phenotype, populations need not be screened for all the mutations but only those specific mutations observed in the population. However, such an assumption is currently premature and might only be valid when all the mutations contributing to the MH phenotype have been identified.

The origin and migration of populations in which MH mutations are present have not yet been investigated. It is possible that these mutations are recent events and is therefore population specific. Investigation of the origin and distribution of the mutations within populations would shed light on whether some of the mutations are indeed population or even family specific. Haplotype analysis utilising the same markers should be performed in all the families in which a specific mutation is identified. This type of analysis would indicate whether the mutation originated due to a single mutation or resulted from multiple distinct mutational events.

The seventeen families included in this study should also be investigated for the remaining fourteen reported mutations. Two mutation hotspots have been identified in the skeletal muscle ryanodine receptor gene (Zhang *et al.*, 1993; Zorzato *et al.*, 1997). These two regions that are prone to mutations should be investigated for possible novel mutations. The development of mutation detection techniques that allow for the highest sensitivity and specificity are essential for the screening of reported and unreported mutations. Identification of reported and novel mutations in any of these MH families will allow the establishment of a more comprehensive molecular diagnostic service in both North America and South Africa.

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APPENDIX A

FAMILY PEDIGREES OF SOUTH AFRICAN MALIGNANT HYPERTHERMIA FAMILIES

This appendix contains full pedigrees of South African MH families MH102, MH104 and MH105, which were not presented in chapter three. The pedigrees were constructed by Dr. A Olckers (Olckers, 1997).

Definitions of the symbols are as follows:


male/female: tested MH normal

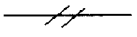

male/female: tested MH susceptible


male/female: MH equivocal


male/female: never tested for MH


male/female: deceased


sex unknown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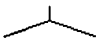
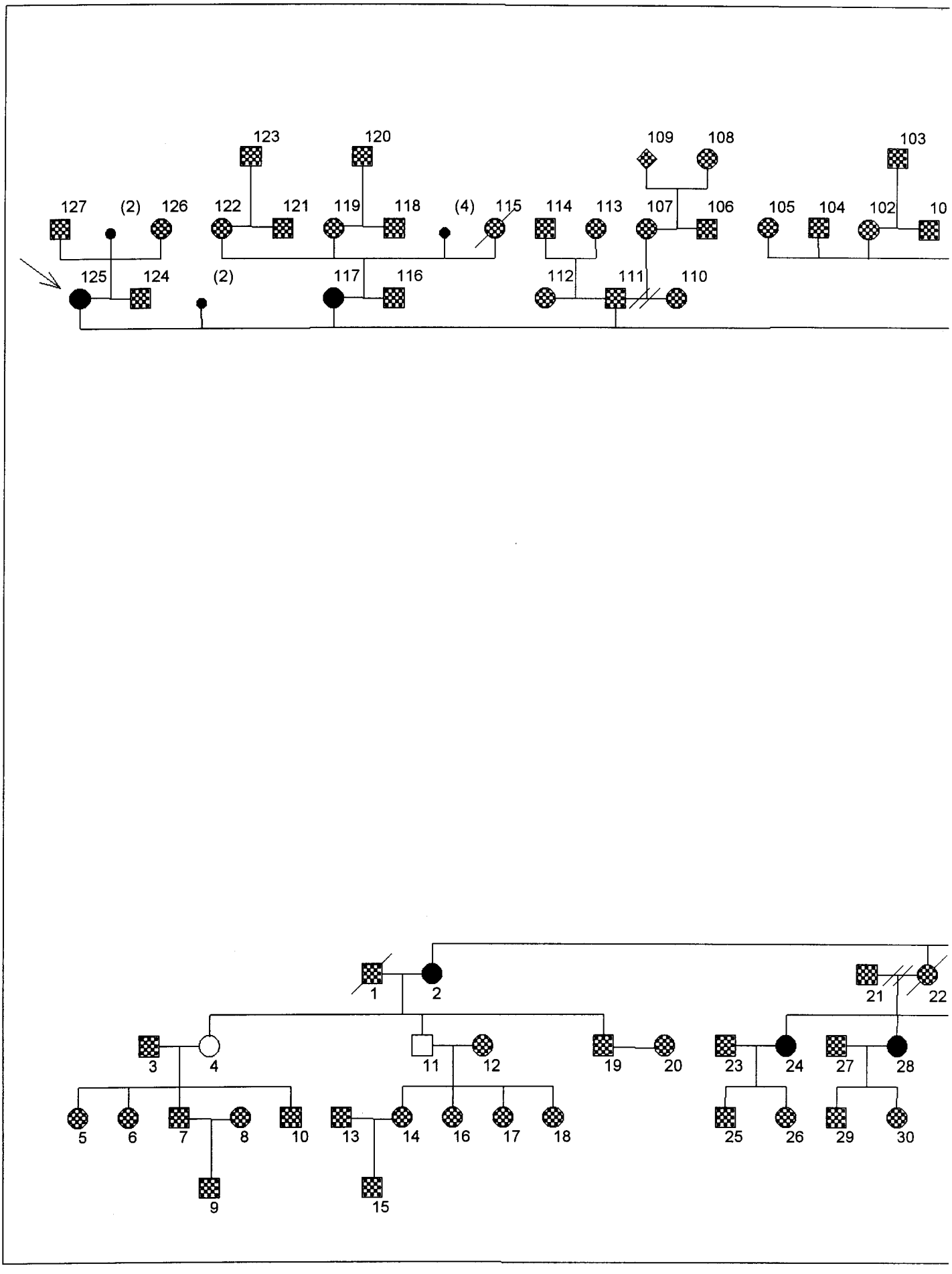

monozygotic twins

Figure A.1: Full pedigree of South African malignant hyperthermia family MH102



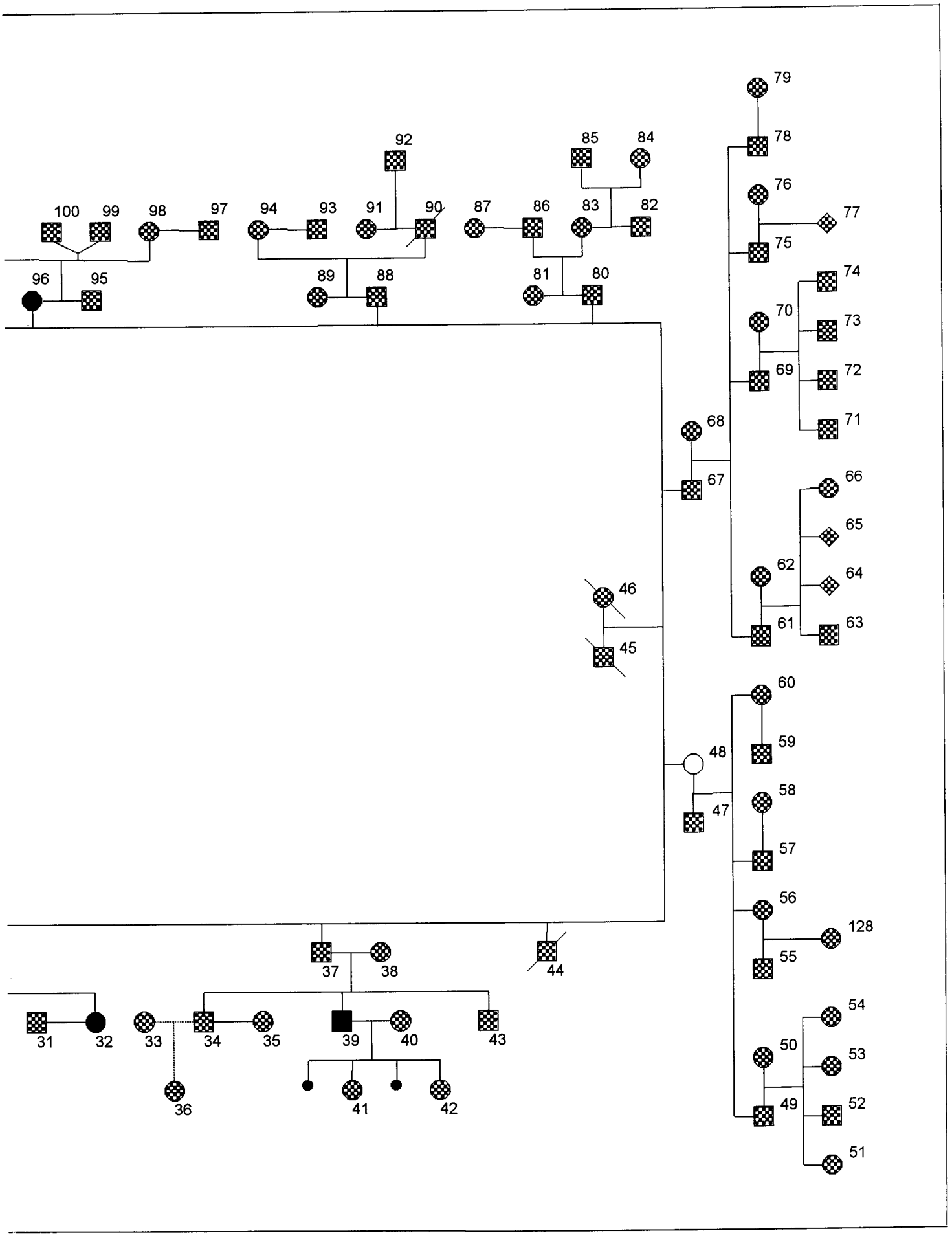

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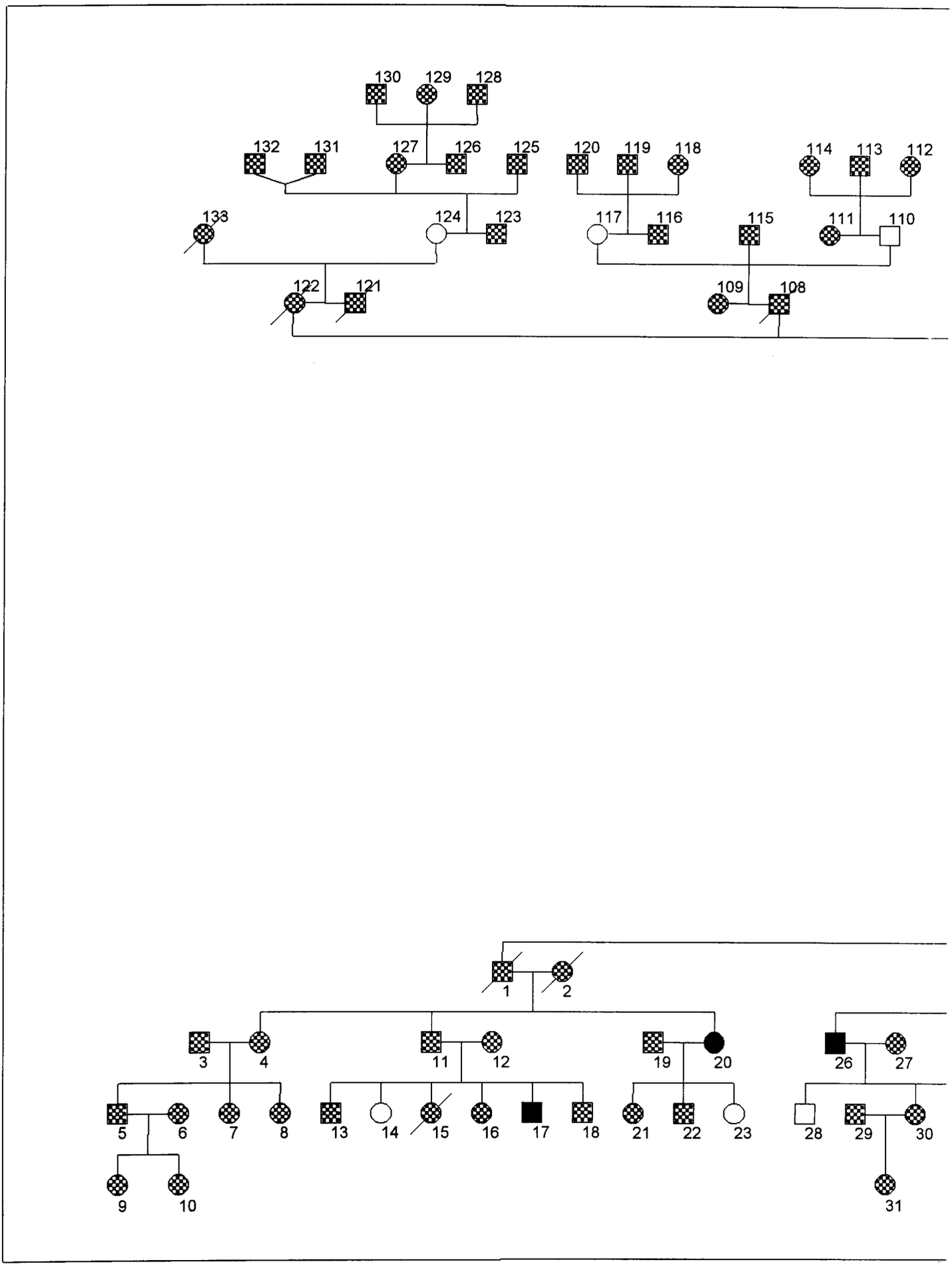
Figure A.2: Full pedigree of South African malignant hyperthermia family MH105



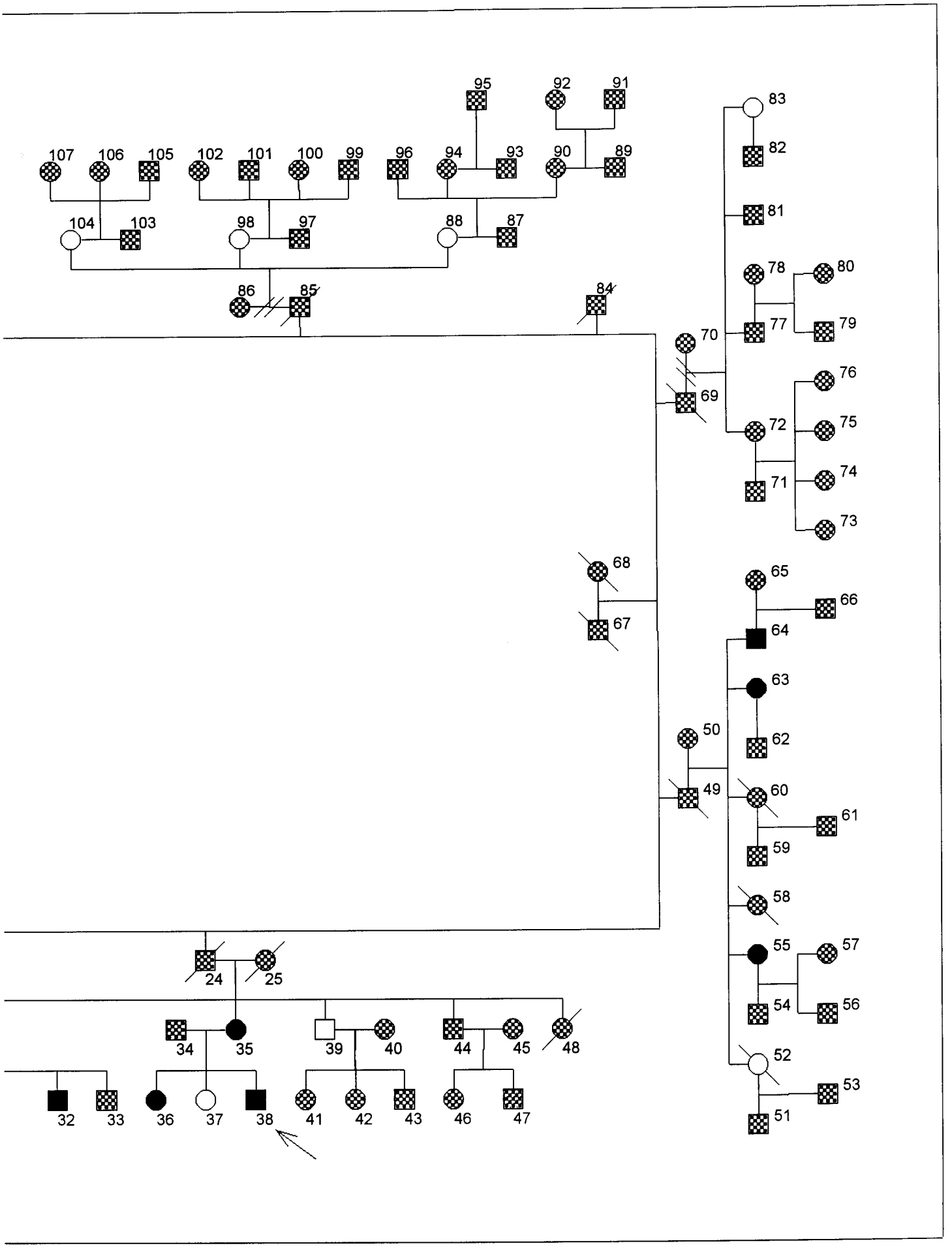

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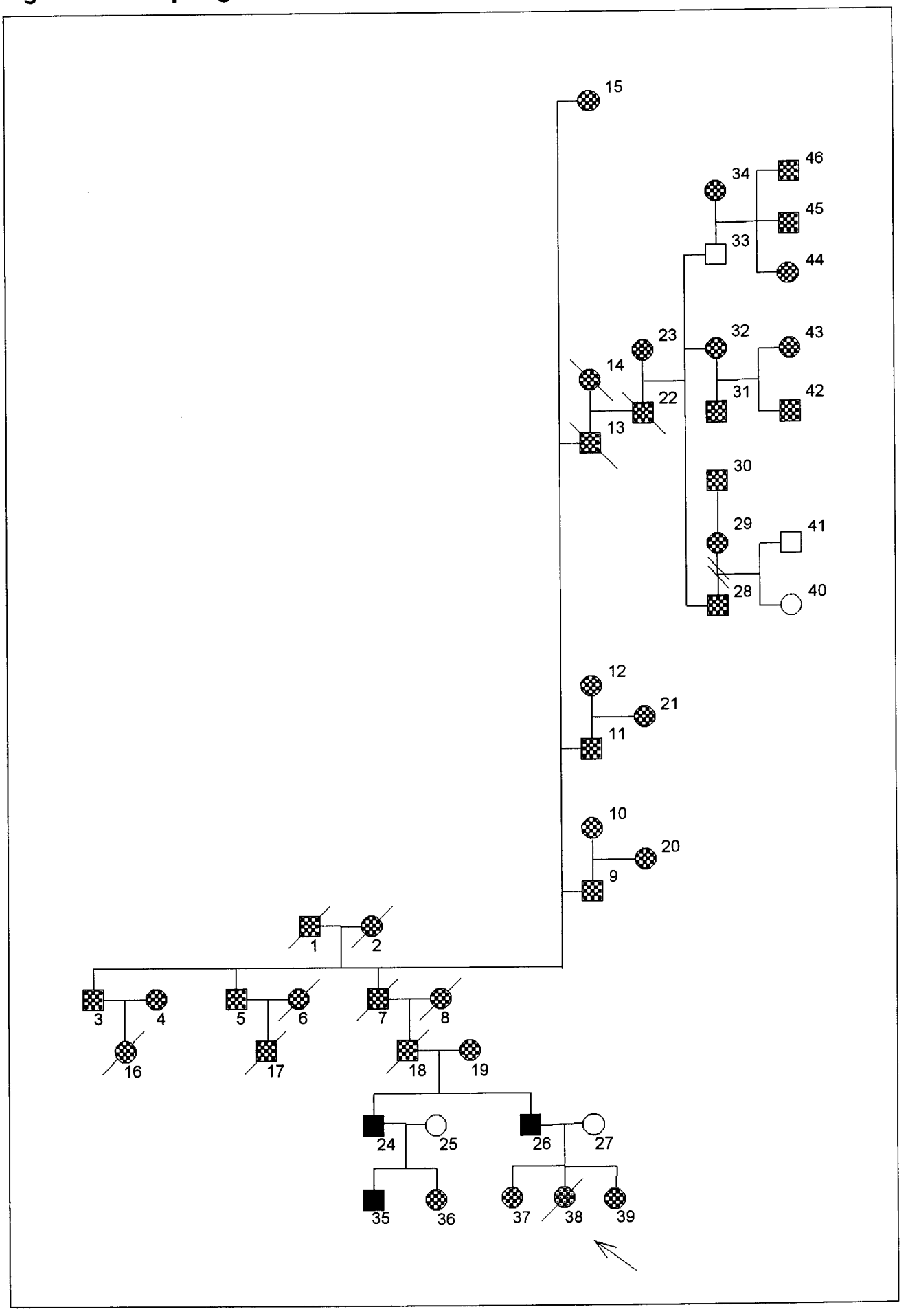
Figure A.3: Full pedigree of South African malignant hyperthermia family MH104



APPENDIX B

CONFERENCES AND MEETINGS AT WHICH RESEARCH WERE PRESENTED

Research results contained in this thesis were presented at the following national meetings. In each case the name of the presenting author is underlined.

B.1 Research presented at national conference

B1.1 **Eighth Biennial Southern African Society of Human Genetics Congress**: Gordon's Bay, South Africa, March 1999.

Havenga Y., Cawood D. and Olckers A. Malignant hyperthermia (MH): Does an epistatic relationship between different MH mutations contribute to the phenotype?

B.2 Research presented at national symposium

B2.1 **25th Anniversary of the Muscular Dystrophy Foundation of South Africa. One day symposium on muscular dystrophy in South Africa**: Cape Town, South Africa, March 1999.

Havenga Y., Cawood D. and Olckers A. An investigation of the relationship between the different malignant hyperthermia (MH) mutations and their possible epistatic contribution to the phenotype.