mtDNA landscape in South African paediatric patients clinically diagnosed with suspected mitochondrial disorders

BY

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mtDNA landskap in Suid-Afrikaanse pediatriese pasiënte klinies gediagnoseer met vermeende mitochondriale siektetoestande

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This thesis is dedicated to my Son, Dan

ABSTRACT

The relevance of haplogroups in mitochondrial disease was investigated in this study. Twenty-seven paediatric patients with clinically suspected mitochondrial disorders from diverse ethnic origins were investigated via an automated sequencing strategy. Previous studies in a similar population identified only the A3243G reported causative mutation in a single patient, in addition to a number of reported and unreported polymorphisms. It suggested that the aetiology of mitochondrial disorders in Africa, specifically South Africa, might be different from that of other continents.

Comparison between these sequences and the Revised Cambridge Reference Sequence revealed 37 polymorphic sites (7 novel changes, 30 reported alterations). No previously reported causative mutation was detected. The findings support the hypothesis that the aetiology of mitochondrial disorders in Africa is unique.

Sixty-three percent of patients in the current study belonged to haplogroup L3 (48% in L3b), 22% to L0, 11% to L2a, and 4% to M. No patients with an L1 haplogroup were observed in this study. The above-mentioned observations have important implications. There was distinct clustering of affected patients in macrohaplogroup L. In this patient cohort, certain sub-haplogroups may play a susceptibility or protective role with regard to mitochondrial dysfunction. Results generated in this study suggested that differential haplogroup-tissue-specific reliance on mitochondrial ATP may culminate in specific phenotypic consequences.

OPSOMMING

Die belang van haplogroepe in mitochondriale siektetoestande is ondersoek in hierdie studie. Sewe-en-twintig pediatriese pasiënte met klinies verdagte mitochondriale afwykings uit verskillende etniese groepe, is bestudeer via 'n outomatiese volgordebepalingstrategie. Vorige studies in 'n soortgelyke bevolking het slegs die A3243G gerapporteerde veroorsakende mutasie geïdentifiseer in 'n enkele pasiënt, maar 'n aantal gerapporteerde en ongerapporteerde polimorfismes is ook waargeneem. Dit dui daarop dat die etiologie van mitochondriale afwykings in Afrika, spesifiek Suid-Afrika, moontlik kan verskil van dié op ander kontinente.

Vergelyking van hierdie volgordes met die Hersiene Cambridge Verwysingsvolgorde het 37 polimorfiese posisies (7 ongerapporteerde veranderinge, 30 bekende veranderinge) blootgelê. Geen voorheen gerapporteerde veroorsakende mutasie is opgespoor nie. Die bevindinge ondersteun die hipotese dat die etiologie van mitochondriale afwykings in Afrika uniek is.

Drie-en-sestig persent van die pasiënte in hierdie studie het aan haplogroep L3 (48% in L3b) behoort, 22% aan L0, 11% aan L2a, en 4% aan M. Geen pasiënte met 'n L1 haplogroep is waargeneem in hierdie studie nie. Hierdie waarnemings het belangrike implikasies. Dit toon aan dat daar 'n bepaalde groepering van aangetaste pasiënte in die makrohaplogroep L voorkom. In hierdie pasïentegroep is dit moontlik dat sekere sub-haplogroepe 'n vatbaarheids- of beskermende rol kan speel ten opsigte van mitochondriale disfunksie. Resultate uit hierdie studie dui daarop dat verskillende haplogroep-weefselspesifieke afhanklikheid op mitochondriale ATP moontlik kan uitloop op spesifieke fenotipiese gevolge.

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations and symbols are listed in alphabetical order.

List of symbols

#	number
β	beta
°C	degrees Centigrade
%	percent
μ	micro: 10^{-6}
12S	12S ribosomal RNA
16S	16S ribosomal RNA

List of abbreviations

A or a	adenine
A_{260}/A_{280}	ratio of absorbance at 260 nm to 280 nm
AA	amino acid
Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartate
ATP	adenosine triphosphate
ATPase 6	gene encoding ATP synthase subunit 6
ATPase 8	gene encoding ATP synthase subunit 8
avg	average
BAT	brown adipose tissue
bp	base pairs
C	cysteine
C or c	cytosine
ca.	circa: approximately
CIPO	chronic intestinal pseudo-obstruction with myopathy and ophthalmoplegia
cm	centimetre
CNS	central nervous system
CO I – III	cytochrome oxidase subunits I to III
CoQ	coenzyme Q
COX	cytochrome oxidase
CPEO	chronic progressive external ophthalmoplegia
CRS	Cambridge reference sequence
Cys	cysteine
cyt b	cytochrome b
D	aspartic acid
ddH_2O	double distilled water
DEAF	deafness
D-loop	displacement loop
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates

N	any of the four bases in DNA sequence
N	asparagine
NADH	nicotinamide adenine dinucleotide (reduced form)
NADH-Q	NADH coenzyme Q reductase complex
Na_2EDTA	di-sodium ethylenediamine tetraacetic acid
NARP	neurologic muscle weakness, ataxia, retinitis pigmentosum
ND1-6	NADH-Q reductase subunits 1 to 6
nDNA	nuclear DNA
NEG	negative control
ng	nanogram
nm	nanometre
np	nucleotide position
O_H	heavy strand origin of replication
O_L	light strand origin of replication
P	proline
PCR	polymerase chain reaction
PDH	pyruvate dehydrogenase complex
pH	potential of hydrogen ions
Q	glutamine
Q	ubiquinone (coenzyme Q or CoQ)
R	arginine
R	reverse primer
RC	respiratory chain
RCRS	revised Cambridge reference sequence
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
s	seconds
S	Svedberg unit or serine
$S^{(AGY)}$	transfer RNA for serine recognising codon AGY
SD	standard deviation
$S^{(UCN)}$	transfer RNA for serine recognising codon UCN
SNP	single nucleotide polymorphism
Syn	synonymous
T	threonine
T or t	thymine
T_a	annealing temperature
Taq	thermostable enzyme isolated from *Thermus aquaticus* BM, recombinant (*Escherichia coli*)
TBE	89.15 mM Tris®[1] (pH 8.0), 88.95 mM boric acid, 2.498 mM Na_2EDTA
Thr	threonine
T_m	melting temperature
Tris®	tris(hydroxymethyl)aminomethane:2-amino-2-(hydroxymethyl)-1,3-propanediol: $C_4H_{11}NO_3$
tRNA	transfer RNA
$tRNA^{Ala}$	transfer RNA for alanine
$tRNA^{Arg}$	transfer RNA for arginine
$tRNA^{Asn}$	transfer RNA for asparagine
$tRNA^{Gly}$	transfer RNA for glycine
$tRNA^{Leu(CUN)}$	transfer RNA for leucine specifically recognising the codon CUN
$tRNA^{Leu(UUR)}$	transfer RNA for leucine specifically recognising the codon UUR

[1] Tris® is the registered trademark of the United States Biochemical Corporation, Cleveland, OH, USA.

$tRNA^{Lys}$	transfer RNA for lysine
$tRNA^{Met}$	transfer RNA for methionine
$tRNA^{Thr}$	transfer RNA for threonine
Trp	tryptophan
U	uracil
U.S.A.	United States of America
UV	ultraviolet light
V	valine
V	volts
Val	valine
W	tryptophan
w/v	weight per volume
Y	tyrosine
YBP	years before present

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CHAPTER ONE

INTRODUCTION

The mitochondria are the energy factories of the cell. The process of glycolysis and the conversion of pyruvate into acetyl coenzyme A produce reduced nicotinamide adenine dinucleotide (NADH), the process of β-oxidation of fatty acids and the tricarboxylic acid cycle produce both NADH and reduced flavin adenine dinucleotide ($FADH_2$) that are used by the mitochondria to manufacture adenosine triphosphate (ATP) via oxidative phosphorylation (Holt, 2003). The mitochondrial system for energy transduction is very vulnerable to damage by genetic and environmental factors (Scholte, 1988). Defects in the mitochondria are observed mostly in the high-energy-dependent tissues of the body (Shoffner *et al.*, 1995). Heteroplasmy and heterogeneity offer one plausible explanation for the widely varying phenotypes in patients with mitochondrial disorders (Larsson and Clayton, 1995).

Mitochondria are inherited exclusively through the maternal line, and since they lack the sophisticated replication proofreading machinery of the nucleus, mutations accumulate over time, leading to disease (Wallace *et al.*, 1999). Mutations that sufficiently compromise energy production within the mitochondria are generally lost but the non-deleterious mutations are not lost and it is indeed these mutations or polymorphisms that accumulate over time (Wallace *et al.*, 1999). Some of these polymorphisms occurred after certain populations split from one another, and are today used to divide populations in the world into haplogroups (Brown *et al.*, 1998).

Human body size and body proportions are interpreted as markers of ethnicity, adaptation to temperature, nutritional history and socioeconomic status (Bogin and Rios, 2003). Geographical distribution and similarities in languages have also been used to infer phylogenetic relationships among humans (Excoffier *et al.*, 1991). Deoxyribonucleic acid (DNA) sequence is valuable because it provides the most detailed anatomy possible for any organism – the instructions on how each working part was assembled and operates (Page and Homes, 1998).

Analysis of population-specific mitochondrial DNA (mtDNA) polymorphisms permitted the reconstruction of human pre-history and demonstrated that some mtDNA diseases show a strong continental and sometimes a population-specific bias. Thus various mtDNA lineages are qualitatively different, and hence can be differentially acted on by selection (Wallace *et al.*, 1999). For example, the expressivity of the mitochondrial ND6 LHON14484C mutation shows that European haplogroup J mtDNAs are biochemically different from those of other population-specific mtDNA lineages, and that some population-specific mtDNA polymorphisms have adaptive significance (Wallace *et al.*, 1999). It is important to know the ultimate molecular basis of mitochondrial defects, not only for an understanding of the general paradigm of mtDNA-based disorders, but to enable the development of genetic rescue strategies that might eventually prove beneficial in patient care (Larsson and Clayton, 1995).

In this study, mtDNA extracted from South African paediatric patients clinically diagnosed with mitochondrial disorders was investigated. Previous attempts made to trace causative mutations in these patients found unreported polymorphisms except for one individual who harboured the A3243G polymorphism (Van Brummelen, 2003). In an attempt to find solutions and reach logical conclusions to the paradox, the patients were haplogrouped.

Chapter Two contains a review of the literature related to significance of haplogroups in mitochondrial disease. The patients' mtDNA was analysed via automated cycle sequencing as presented in Chapter Three. The single nucleotide polymorphisms presented in Figure 3.1 were used to infer that the patients included in this study belonged to particular haplogroups. Results from the haplogroup analyses are presented in Chapter Four, Section 4.2, and the correlation of haplogroups with clinical phenotypes is presented in Sections 4.3 to 4.4. Conclusions drawn from the data generated in this study are presented in Chapter Five.

CHAPTER TWO

THE SIGNIFICANCE OF HAPLOGROUPS IN MITOCHONDRIAL DISEASE

The original migration out of Africa created widely separated subpopulations of humans with distinct collections of gene variants (Wallace *et al.*, 1999). As humans evolved, and as our bodies interacted with different climates and diets on each of the continents, there was purifying and adaptive selection for these naturally occurring variants (Bogin and Rios, 2003; Mishmar *et al.*, 2003; Ruiz-Pesini *et al.*, 2004). Thus not only genetic drift but also natural selection greatly shaped regional mtDNA variation (Mishmar *et al.*, 2003; Ruiz-Pesini *et al.*, 2004). These variations or polymorphisms are important not only in the context of human evolution and origins (Wallace *et al.*, 1999; Adcock *et al.*, 2001) but also in the context of the global human phylogenetic tree (Wallace *et al.*, 1999; Chen *et al.*, 2000; Salas *et al.*, 2002) and in the context of human disease (Brown *et al.*, 1992; Wallace, 1995; Coskun *et al.*, 2003). The mtDNA sequence observed today in different populations can be used to reconstruct the history of the maternal line of a population (Richards *et al.*, 2003; Salas *et al.*, 2004) while Y-chromosome data have been used to do the same for the paternal line (Tarazona-Santos *et al.*, 2001; Cruciani *et al.*, 2002). Combined mtDNA and Y-chromosome genetic data have been used to estimate the relative contribution of females and males in shaping the history of humans (Kalaydjieva *et al.*, 2001; Wilson *et al.*, 2001).

In this study, South African paediatric patients clinically diagnosed with suspected mitochondrial disorders were haplogrouped using the revised Cambridge reference sequence (RCRS) of mtDNA (Andrews *et al.*, 1999) as the reference mtDNA sequence. It is important to know upon which haplogroup a particular mutation is expressed, as it can influence the phenotype and ultimate physiological course of the disease (Wallace *et al.*, 1999).

2.1 ORIGIN, STRUCTURE, FUNCTION AND DISTRIBUTION OF THE MITOCHONDRION

Mitochondria are double-membrane bound organelles (Borst, 1977; Bauer *et al.*, 1999; Chinenov, 2000) believed to have evolved from bacteria that lived symbiotically inside living cells (Wallace *et al.*, 1999). The matrix is encased by the inner mitochondrial membrane whereas the region between the two membranes is referred to as the intermembrane space. The outer mitochondrial membrane is porous due to the presence of porin, a protein that allows many molecules to traverse this membrane. However the inner mitochondrial membrane is intrinsically impermeable to nearly all ions and polar molecules. Specific protein carriers are required to transport molecules across the inner mitochondrial membrane, the inner side of which is highly folded into cristae in order to increase its surface area for metabolic activity. The four enzyme complexes (nicotinamide adenine dinucleotide coenzyme Q oxidoreductase (NADH-Q reductase), succinate coenzyme Q oxidoreductase (succinate-Q reductase), cytochrome reductase and cytochrome oxidase) that carry out electron transfer, the two mobile electron carriers, ubiquinone and cytochrome c, and the ATP synthesising complex are located in the inner mitochondrial membrane (Wallace, 1992; Wallace, 1994; Adams and Turnbull, 1996).

The conversion of pyruvate into lactate, the citric acid cycle, β-oxidation of fatty acids and oxidative phosphorylation reactions all take place in the mitochondria. The main function of the mitochondria is to produce ATP via oxidative phosphorylation (Scholte, 1988; Senior, 1988) but when the production of ATP is compromised, or when oxidative phosphorylation is uncoupled, the free energy generated from reduced cofactors is converted into heat (Wallace, 1992; Wallace, 1994) via thermogenesis. Thirty-six out of the 38 ATP, or 34 out of the 36 ATP molecules (depending on the shuttle system used to transport cytoplasmic NADH into the mitochondria for oxidation) obtained from complete oxidation of a molecule of glucose are produced in the mitochondria (Stryer, 1988). Thermogenesis is used by animals adapted to living in the cold, newborn mammals and hibernating animals on arousal to generate heat (Mortola and Naso, 1998; Rippe *et al.*, 2000; Zaninovich *et al.*, 2002). In newborn mammals, thermogenesis is used to adapt from the intrauterine life environment at constant body temperature to one of external cold stress. These animals are rich in brown adipose tissue (BAT) which is rich in mitochondria whose inner mitochondrial membrane is enriched with thermogenin, the uncoupling protein. Thermogenin generates heat by short-circuiting the mitochondrial proton battery (Stryer, 1988). In the Skunk cabbage *Symplocarpous foetidus*, thermogenesis generates heat that

melts snow around it and raises the ambient temperature (Minorsky, 2003), and in both *S. foetidus* and Lords and Ladies *Arum maculatum*, thermogenesis increases the evaporation of odoriferous molecules, thus attracting insects to pollinate their flowers (Wagner *et al.*, 1998; Ito *et al.*, 2004; Seymour, 2004). Mitochondria also serve as storage tanks for calcium ions and may act as sinks to buffer the effects of calcium overload (Shoffner *et al.*, 1995; Ichas *et al.*, 1997). Mitochondria also play a role in apoptosis (Kerr *et al.*, 1972; Susin *et al.*, 1998; Bauer *et al.*, 1999; Ferri *et al.*, 2000), glutamate-mediated excitotoxic neuronal injury, cellular proliferation, regulation of the cellular redox state, urea cycle, haem synthesis and steroid synthesis (Scholte, 1988; Bauer *et al.*, 1999).

The mitochondria are essentially abundant in tissues such as the flagellum, sperm (Díez-Sánchez *et al.*, 2003) and muscle (Naviaux, 1997). The flight muscle of birds and cardiac muscle are rich sources of mitochondria, similar to BAT (Stryer, 1988). Most of the nucleated cells in the human body contain 500 to 2,000 mitochondria, but the platelets have only two to six mitochondria, while mature red blood cells have none (Stryer, 1988; Naviaux, 1997).

2.2 MITOCHONDRIAL GENETICS

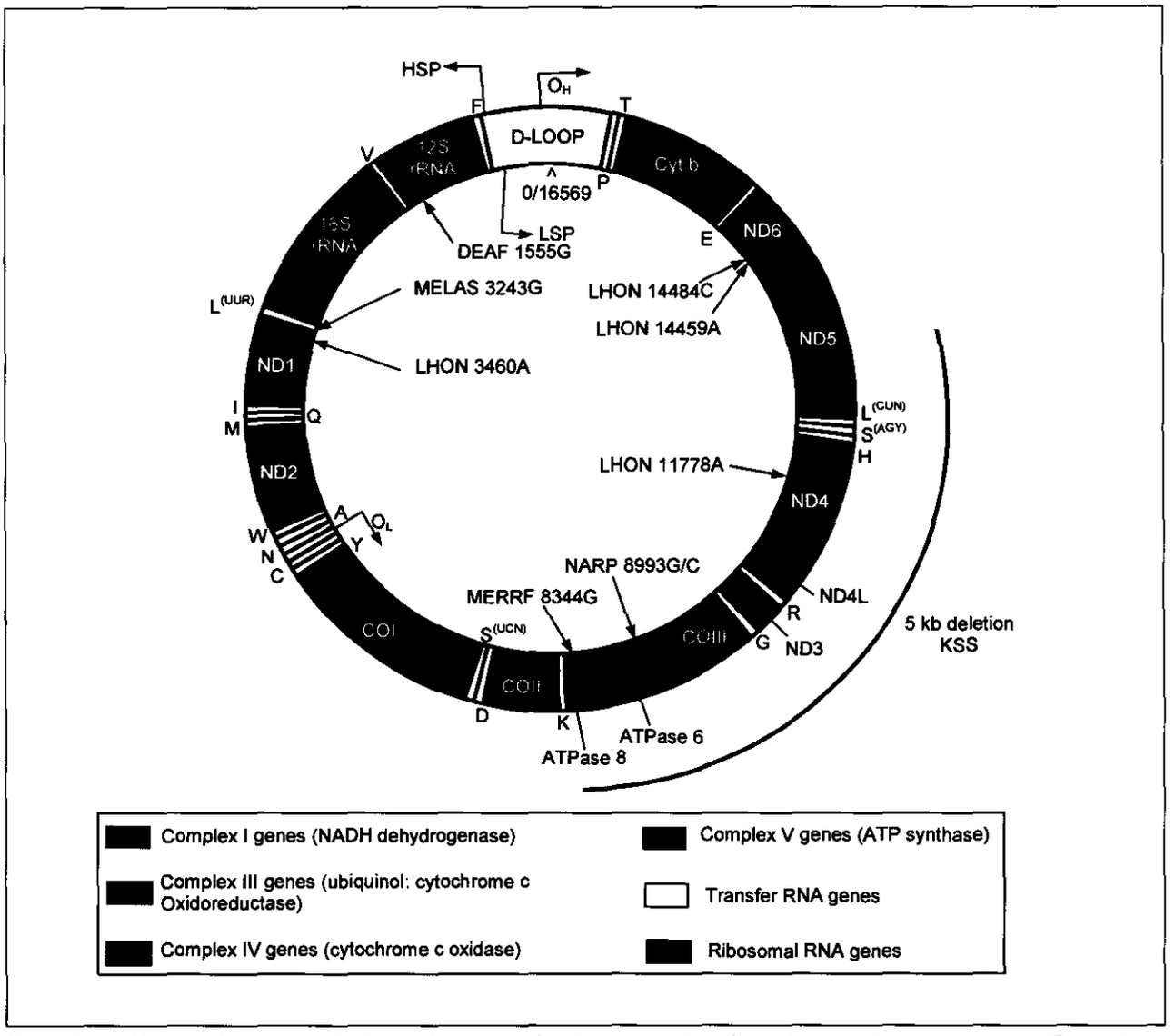
The segregation in a special environment, of a portion of the eukaryotic genome under the control of the nuclear genome, as is the case of the mitochondria, represents a unique situation in nature (Attardi, 1985). The determination of the complete sequence of the human mtDNA (Anderson *et al.*, 1981) and that of other mammals, the unravelling of the mitochondrial genetic code (Barell *et al.*, 1979; Barell *et al.*, 1980) and the parallel detailed description of the structural, mapping, and metabolic properties of the mtDNA transcripts have provided a large amount of information on the structure and function of the mitochondrial genome, which has no parallel in other genetic systems (Attardi, 1986).

2.2.1 Mitochondrial genes

The mitochondrion and the nucleus are the only cellular organelles that contain DNA. The mtDNA as indicated in Figure 2.1 is a circular molecule of 16,569 base pairs (bp) and encodes 13 polypeptides, two ribosomal ribonucleic acid molecules (rRNA) and 22 transfer ribonucleic acid molecules (tRNA) [Anderson *et al.*, 1981; Wallace, 1995; Andreas *et al.*, 1997]. The mitochondrion has a different genetic code (Barrell *et al.*, 1979; Barell *et al.*,

1980; Anderson *et al.*, 1981) and its genome exhibits high economy. Its genes are closely packed (some genes actually overlap) and in most cases lack introns (Anderson *et al.*, 1981).

Figure 2.1: Map of the human mitochondrial genome



Outer circle = H strand, inner circle = L strand, O_H = origin of H-strand replication, O_L = origin of L-strand replication, HSP = H-strand promoter, LSP = L-strand promoter, rRNA = ribosomal RNA, ND1 - 6 = genes encoding subunits 1 to 6 of NADH dehydrogenase, CO I - III = genes encoding subunits I to III of cytochrome c oxidase, ATPase 6 and 8 = genes encoding subunits 6 and 8 of ATP synthase, Cyt b = gene encoding cytochrome b, D-loop = displacement loop, DEAF = deafness, MELAS = mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes, LHON =Leber's hereditary optic neuropathy, MERRF = myoclonic epilepsy and ragged-red muscle fibres and NARP = neuropathy, ataxia and retinitis pigmentosa. The following letter symbols of amino acids represent the tRNA for that amino acid: A = alanine, C = cysteine, D = aspartic acid, E = glutamic acid, F = phenylalanine, G = glycine, H = histidine, I = isoleucine, K = lysine, L = leucine, M = methionine, N = asparagine, P = proline, Q = glutamine, R = arginine, S = serine, T = threonine, V = valine, W = tryptophan, and Y = tyrosine. The two tRNA genes for leucine are differentiated as $L^{(UUR)}$ and $L^{(CUN)}$ and the two tRNA genes for serine as $S^{(UCN)}$ and $S^{(AGY)}$. Adapted from Mitomap (2004).

2.2.2 Inheritance pattern

The mitochondria are maternally inherited (Giles *et al.*, 1980; Schwartz and Vissing, 2002). The bias in parental genotype is established at, or soon after, the formation of the zygote. It is due to the ovum providing many more mitochondria than the sperm, or the

mitochondria provided by the father not surviving (Kaneda *et al.*, 1995; Adams and Turnbull, 1996; Andreas *et al.*, 1997). The sperm mitochondria are tagged by the recycling marker ubiquitin for selective destruction (Sutovsky *et al.*, 1999). A woman will transmit her mtDNA to all her children, males as well as females, but only the daughters will in turn transmit it to their progeny (Shanske *et al.*, 2001).

2.2.3 Replication, transcription and translation

Replication of mammalian mtDNA proceeds by initiation of heavy (H) strand synthesis at a specific origin resulting in the formation of a displacement (D) loop (Shadel and Clayton, 1993) with a newly synthesized H strand of about 680 bases, the 7 short (7S) DNA (Clayton, 1991). Initiation of light (L) strand synthesis is at a specific origin (Wallace, 1992; Taanman, 1999) and does not occur until this region has been exposed by H strand synthesis (Attardi, 1985; Clayton, 1991). The termination codons are created post-transcriptionally by polyadenylation of the mRNAs (Anderson *et al.*, 1981). The mitochondrial apparatus for protein synthesis is assembled from RNA synthesised in the mitochondrion and proteins imported from the cytoplasm. The human mitochondrion uses 22 tRNAs for translation and has four codons different from the universal code of nuclear DNA (nDNA) as indicated in Table 2.1. AGA and AGG, which normally encode arginine, are stop codons; AUA codes for methionine instead of isoleucine and UGA codes for tryptophan rather than being a stop codon (Barrell *et al.*, 1979; Barrell *et al.*, 1980; Anderson *et al.*, 1981).

Table 2.1: Differences between the genetic code of the mitochondrial genome and the universal code of nuclear DNA

Codon	Universal code	Mitochondrial code
UGA	Stop	Trp
AUA	Ile	Met
AGA	Arg	Stop
AGG	Arg	Stop

A = adenine, G = guanine, U = uracil, Arg = arginine, Ile = isoleucine, Met = methionine, Trp = tryptophan.

2.2.4 Interaction between mitochondrial and nuclear DNA

Mammalian mtDNA relies on nuclear-encoded proteins for its maintenance and propagation (Holt, 2003). The nuclear genes encode the majority of the respiratory chain (RC) subunits, all the proteins required for replication and transcription of mtDNA, and

processing and translation of mtDNA transcripts, as well as all proteins required for mitochondrial protein import (Larsson and Clayton, 1995). The genes are transcribed and translated in the nucleus before acquiring a signal sequence for targeting the protein to the mitochondrion (Blanchard and Lynch, 2000). Because of this interaction, some defects in nDNA can lead to dysfunction of the mitochondrion (Wallace, 1992; Taanman, 1999; Carrieri *et al.*, 2001). The subunits encoded by mtDNA are indicated in Table 2.2. mtDNA also codes for 12S rRNA, 16S rRNA and 22 tRNAs (Wallace *et al.*, 1999).

Table 2.2: Enzyme complexes of the respiratory chain

Complex	Enzyme	Number of subunits	Subunits encoded by mitochondrial genes	Reference
I	NADH-Q reductase	46	ND1, ND2, ND3, ND4, ND4L, ND5 and ND6	Carroll *et al.*, 2002
II	Succinate-Q reductase	4	None	Adams and Turnbull, 1996
III	Cytochrome reductase	11	Cyt b	Adams and Turnbull, 1996
IV	Cytochrome oxidase	13	COI, COII, COIII	Campbell and Smith, 1993
V	ATP synthase	16	ATPase 6 and ATPase 8	Walker *et al.*, 1991

ND1-ND6 = NADH-Q reductase subunits 1 to 6, Cyt b = cytochrome b, COI-III = cytochrome oxidase subunits I to III, ATPase 6 and 8 = ATP synthase subunit 6 and 8.

2.2.5 Mutation rate of mitochondrial DNA

The mtDNA mutation rate is ca.10-17 times higher than that of nDNA (Brown *et al.*, 1979; Wallace, 1994). This higher mitochondrial mutation rate is due to the lack of a sophisticated proofreading mechanism (Larsson and Clayton, 1995; Andreas *et al.*, 1997) and oxidation by reactive oxygen radicals generated in the respiratory chain (Ames *et al.*, 1993). Because of the high mutation rate, mtDNA is an extremely useful molecule to employ for high-resolution analysis of the evolutionary process (Brown *et al.*, 1979).

2.3 HUMAN ORIGINS, MIGRATIONS AND ADAPTATIONS

The study of mtDNA has helped to demonstrate the African origin of the human species (Hagelberg, 2003). The delineation of human mtDNA variation and genetics has provided unique and often startling new insights into human evolution, degenerative diseases and aging (Wallace, 1995). Data from mtDNA analysis have been used to establish the time and route of major events in human history, such as the expansion of Neolithic farmers into Europe, and the settlement of the Pacific and the New World (Hagelberg, 2003).

mtDNA can be used to partially explain the variable expressivity and penetrance of human genetic diseases, delayed onset of symptoms, variable rates of aging (Wallace, 1995) and adaptation to specific environments (Ruiz-Pesini *et al.*, 2004).

2.3.1 Human origin

The "Eve" hypothesis postulates that all mtDNA variation found in modern humans is derived from a single female ancestor (Cann *et al.*, 1987). Since mtDNA is maternally inherited in primates, this implies that all copies of human mtDNA can be traced to a common female ancestor (Cann *et al.*, 1987), and all other primates have their own mitochondrial "Eve". This common female ancestor lived in Africa, around 200,000 years ago (Cann *et al.*, 1987; Tishkoff and Williams, 2002). The regional continuity evolution hypothesis, derived from the Eve hypothesis, postulates that not only do all mtDNAs in modern humans but indeed all modern humans trace back to the common female ancestor, and are derived from the same geographical population containing that common ancestor. This implies that anatomically modern humans arose in Africa, spread throughout the Old World about 100,000 years ago, and drove the earlier *Hominid* populations to extinction without genetic introgression - the "out-of-Africa replacement hypothesis". The multiregional evolution hypothesis holds that transformation of archaic to anatomically modern humans occurred in parallel in different parts of the Old World (Tishkoff and Williams, 2002). Of *Homo sapiens* remains discovered so far, the oldest that match the bones of living humans date from around the time that the mitochondrial Eve lived. Creationists accept the existence of an Eve, but some do not accept the dates, and such creationists emphasise that the evidence is inconclusive (Wikipedia, 2004). Fossil evidence supports the "out-of-Africa" hypothesis, but neither the "out-of-Africa" nor the "regional continuity" or the "multiregional evolution" hypotheses for the origin of modern humans, in their extreme forms, are fully consistent with the known fossil record for human evolution in the middle and late Pleistocene (Stringer and Andrews, 1988; Excoffier, 1990; Aiello, 1993; Templeton, 1993). Analysis of mtDNA variation enabled reconstruction of the ancient migrations of women and supported the "out-of-Africa" hypothesis (Wallace *et al.*, 1999).

Analysis of mtDNA from ancient Australian human remains in Lake Mungo 3 with morphologically gracile individuals, Holocene deposits at Wallandra Lakes with morphologically gracile individuals and Pleistocene (early Holocene) from Kow Swamp with individuals having robust morphologies outside the skeletal range of contemporary

indigenous Australians provided a perspective on the origin of modern humans and the relationship between molecular and morphological variation (Adcock *et al.*, 2001). mtDNA from remains at Lake Mungo 3 belonged to a lineage that only survives as a segment inserted into chromosome 11 of the nuclear genome, which is now wide-spread among human populations (Adcock *et al.*, 2001). This lineage probably diverged before the most recent common ancestor of contemporary human mitochondrial genomes. This timing of divergence implies that the deepest known mtDNA lineage from anatomically modern humans occurred in Australia and these humans were present in Australia before the complete fixation of the mtDNA lineage now found in all living people. Alternatively analysis restricted to living humans places the deepest branches in East Africa (Adcock *et al.*, 2001).

Analysis of African, Asian, European and American mtDNA confirmed that mtDNA variation correlated highly with the ethnic and geographic origin of the individual, that there was a single mtDNA tree and that the greatest variation and deepest root of the tree was in Africa, consistent with an African origin of humans (Wallace *et al.*, 1999). A survey of 147 mtDNAs, including 34 Asians, 21 Australian Aborigines, 26 aboriginal New Guineans, 46 Caucasians and 20 Africans (18 of whom were African Americans), also revealed that there was a single mtDNA tree, that the deepest root occurred in Africa, and that Africa harboured the greatest sequence diversity. Hence Africa is the origin of modern *Homo sapiens* (Cann *et al.*, 1987; Wallace *et al.*, 1999). The !Kung, Khwe and Biaka pygmies have one of the most ancient sub-lineages observed in African mtDNA and thus are possibly the most ancient African population and could represent one of the oldest populations in the world (Chen *et al.*, 2000). Analysis of Y-chromosome sequences have corroborated the evidence that mtDNA has provided for an African origin for hominids (Kalaydjieva *et al.*, 2001; Wilson *et al.*, 2001).

2.3.2 Human migration

mtDNA provides a simple system for reconstructing ancient human migrations (Wallace, 1995, Torroni *et al.*, 1996). Humans arose out of Africa about 150,000 years before present (YBP), migrated into Asia about 60,000 to 70,000 YBP and into Europe about 40,000 to 50,000 YBP. They migrated from Asia and possibly Europe into America around 20,000 to 30,000 YBP (Wallace *et al.*, 1999). There were also return migrations to Africa from India as detected by the presence of haplogroup M in Northeast Africa and a subclade of haplogroup U in Northwest Africa (Maca-Meyer *et al.*, 2001).

2.3.2.1 Human migrations from and into Africa

After coming out of Africa ca. 59,000 - 69,000 YBP, modern humans first spread to Asia following two main routes. The southern route is represented by haplogroup M and related clades that are present in India and Eastern and Western Asia in abundance. In Africa, this expansion did not replace, but rather mixed with older lineages that are today detectable only in Africa (Maca-Meyer *et al.*, 2001). Eurasian mtDNA sequences are derived from haplogroup L3, which bifurcated early from African macrohaplogroup L* dating 60,000 - 80,000 YBP (Watson *et al.*, 1997). The northern migration gave rise to lineages A and B, which are now prominent in North and East Asia (Maca-Meyer *et al.*, 2001). Around 39,000 - 52,000 YBP, the Western Asian branch spread radially, bringing Caucasians to North Africa and Europe, also reaching India and expanding to North and East Asia (Maca-Meyer *et al.*, 2001). Portugal, in agreement with mtDNA sequence data, is a region with known historical gene flow from Northern Africa and was a centre for the importation of slaves (Salas *et al.*, 2004).

2.3.2.2 Human migrations into and from Asia

Migrations into Asia were mainly from Africa (see Section 2.3.2.1) and from Asia humans migrated into Europe and America. There were also back migrations from Asia into Africa as detected by the presence of derivatives of haplogroup M in Northeast Africa, and the existence of Caucasoids of haplogroup U confined mainly to Northwest Africa (Maca-Meyer *et al.*, 2001).

Comparison of mtDNA variation of populations from the Near East and Africa found a very high frequency of African lineages present in the Yemen Hadramawt, with more than a third being of clear sub-Saharan origin (Richards *et al.*, 2003). Arab populations carried ca. 10% of the lineages of sub-Saharan origin, whereas non-Arab Near Eastern populations carried few or no such lineages, suggesting that gene flow has been preferentially into Arab populations. There was little evidence of male-mediated gene flow from sub-Saharan Africa in Y-chromosome haplotypes in Arab populations, including Hadramawt (Richards *et al.*, 2003). This study indicated the long-term effects of a particular socioeconomic system, based on slavery in this instance, on the gene pool of an entire region. The most likely explanation for the presence of predominantly female lineages of African origin in other parts of the Arab world trace back to women brought from Africa as part of the Arab

slave trade, and assimilated into the Arab population as a result of miscegenation and manumission (Richards *et al.*, 2003). Indeed, unlike the situation in the Americas, there are no substantial communities of African descent in the Near East today. The Arabs employed the majority of the male slaves in manual labour and military service or they were castrated and employed as eunuchs and therefore few left descendants. Women, by contrast, were imported specifically for the sexual gratification of elite males and for their reproductive potential (Cavalli-Sforza *et al.*, 1994; Richards *et al.*, 2003). Most of this gene flow probably occurred within the past 2,500 years (Richards *et al.*, 2003).

Central Asia is a region at the crossroads of different habitats, cultures and trade routes. Central Asian mtDNA sequences present features intermediate between European and Eastern Asian sequences. The most plausible explanation for the intermediate position of Central Asia involves extensive levels of admixture between Europeans and Eastern Asians in Central Asia, possibly enhanced during the Silk Road trade and clearly after the eastern and western Eurasian human groups had diverged (Comas *et al.*, 1998).

2.3.2.3 Human migrations into Australia

The mtDNA variation of the Walbiri tribe of the northern territories, Australia, appears to be unique to that of Asians although a few haplogroups appear to be sub-branches of larger clusters of Aboriginal Australians and/or Papua New Guinea haplotypes. The similarity of these haplotypes suggests that Aboriginal Australians and Papua New Guinea populations may have once shared an ancient ancestral population(s), and then rapidly diverged from each other once geographically separated. Overall, the mtDNA data corroborate the genetic uniqueness of Aboriginal Australian populations (Huoponen *et al.*, 2001) and thus the problem of the arrival of *Homo sapiens sapiens* in Australia is not completely understood (Cavalli-Sforza, 1994). A major problem of interest for the general history of world migrations is the possible similarity of some relic populations in South and Southeast Asia with Australian aborigines on one side and Africans on the other. These populations might be evidence of a southern route of migration from Africa to Australia. The genetic relationship between these "Australoid, Veddoid, Negritos, pre-Dravidian" populations as they are sometimes called indicates more similarity to African populations than their neighbours in India or Southeast Asia, as revealed by mtDNA and Y-chromosome data (Cavalli-Sforza *et al.*, 1994).

2.3.2.4 Human migrations into Europe

The earliest human occupants of Europe arrived during the Palaeolithic period, in the order of 40,000 - 50,000 YBP (Lell and Wallace, 2000) from the Near East (Richards *et al.*, 2000). Between 6,000 - 10,000 YBP Europe was greatly transformed by the entry of Neolithic farmers from the Middle East. The slow, gradual spread of the Middle Eastern farmers dramatically altered the genetic landscape of Europe, determining the most important and most regular multigenic gradient observed there (Cavalli-Sforza *et al.*, 1994).

2.3.2.5 Human migrations into America

The prehistory of America is shorter than that of any other continent (Cavalli-Sforza *et al.*, 1994). The timing and number of prehistoric migrations involved in the settlement of the American continent is subject to intense debate. Reanalysis of Native American control region mtDNA accompanied by an appreciation of demographic factors made a better resolution of the issue (Forster *et al.*, 1996). mtDNA control region sequences of aboriginal Siberians and Native Americans together with linguistic, archaeological and climatic evidence confirm that the major wave of migration brought one population, ancestral to the Amerinds, from Northeastern Siberia to America 20,000 - 25,000 years ago, and a rapid expansion of a Beringian source population took place at the end of the Young Dryas glacial phase ca. 11,300 years ago, ancestral to present Inuit and Na-Dene populations (Torroni *et al.*, 1994; Forster *et al.*, 1996).

An investigation of the origins, diversity, and continental relationships via mtDNA analysis of haplogroup X showed an ancient link between Europe, Western Asia and North America supporting the possibility that some Native American founders were of Caucasian ancestry. Haplogroup X was found to represent a minor founding lineage in North Americans, the major lineages being haplogroups A, B, C and D (Brown *et al.*, 1998).

Between the 15th and 19th centuries Anno Domini, the Atlantic slave trade resulted in forced movement of ca. 13 million people from Africa, mainly to the Americas. In many cases, analysis of mtDNAs in America and Eurasia can be traced to broad geographical regions in Africa, largely in accordance with historical evidence (Salas *et al.*, 2004). Brazilians form one of the most heterogeneous populations in the world, the result of five centuries of interethnic crosses between the Portuguese (European colonisers), African

slaves and the autochthonous Amerindians. It is estimated that between 1551 and 1850 when the slave trade was abolished there were 3.5 million Africans in Brazil and between 1500 and 1808, 500,000 Portuguese immigrated into Brazil. Between 1500 and 1972, 58%, 40% and 2% of the immigrants who arrived in Brazil were Europeans, Africans and Asians respectively (Alves-Silva *et al.*, 2000). Analysis of 247 Brazilian mtDNAs for hypervariable segment (HVS)-1 and selected restriction fragment-length-polymorphism (RFLP) sites showed nearly equal amounts of Native American, African and European matrilineal genetic contribution, but with regional differences within Brazil (Alves-Silva *et al.*, 2000).

2.3.3 Human adaptation

Traditionally genetic drift, in addition to natural selection, shaped regional mtDNA variation, with some of the major selective influences being climate and diet (Mishmar *et al.*, 2003). Different human mtDNA lineages are functionally different. This differential functionality includes adaptation to colder climates in arctic populations (Ruiz-Pesini *et al.*, 2004), increased longevity in European haplogroup J individuals due to the C150T mutation that imparts resistance to stress (De Benedictis *et al.*, 1999; Coskun *et al.*, 2003; Mishmar *et al.*, 2003) and reduced sperm motility in European males belonging to haplogroup T (Ruiz-Pesini *et al.*, 2000) as indicated in Table 2.3. It also includes an increased possibility of haplogroup H individuals developing late-onset Alzheimer's disease if they have a mutation in the $tRNA^{Gln}$ gene at nucleotide position 4336 (Shoffner *et al.*, 1993) and of European haplogroup U males being susceptible to Alzheimer's disease (Van der Walt *et al.*, 2004) as well as a higher risk of individuals with mutations at nucleotide positions 5633, 7476 and 15812 of developing Alzheimer's disease, while position 709 (12S rRNA) and 15928 ($tRNA^{Thr}$) variants are protective against Alzheimer's disease (Chagnon *et al.*, 1999). mtDNA haplogroup U is a risk factor for occipital stroke among patients with migraine (Majamaa *et al.*, 1998) and there is an increased probability of becoming blind if an individual belonging to haplogroup J has Leber's hereditary optic neuropathy (Brown *et al.*, 1997; Brown *et al.*, 2002).

Given that mtDNA lineages are functionally different, it follows that the same variants that are advantageous in one climate and dietary environment might be maladaptive when these individuals are placed in a different environment. Hence, ancient beneficial mtDNA variants could be contributing to modern bioenergetic disorders such as obesity, diabetes, hypertension, cardiovascular disease and neurodegenerative diseases as people move to

new regions and adopt new lifestyles (Wallace, 1992; Mishmar *et al.*, 2003). Genome-wide association studies provide a powerful approach to implicate DNA variants and, by extension, the genomic regions they represent in the predisposition to complex diseases and in the genetic underpinnings of drug efficacy and adverse reactions. Such differences are expected to be found when genetically distinct population subgroups have a different prevalence of the target phenotype (Hinds *et al.*, 2004).

Table 2.3: Haplogroups with special adaptations

Haplogroup	Adaptation	Adaptive associate factor	Reference
H	Higher risk of Alzheimer's disease	Mutation at np 4336	Shoffner *et al.*, 1993
A, C, D, X	Living in cold climates	- - -	Coskun *et al.*, 2003; Ruizi-Pesini *et al.*, 2004
J	Increased longevity	C150T np mutation	Coskun *et al.*, 2003; Mishmar *et al.*, 2003
J	Higher risk of blindness	LHON	Brown *et al.*, 1997
J, K	Susceptibility to multiple sclerosis, protective against Parkinson's disease	- - -	Kalman and Ader, 1998; Van der Walt *et al.*, 2003
K	Increased longevity, protective against Alzheimer's disease	- - -	Ross *et al.*, 2001; De Benedicts *et al.*, 2000
T	Reduced sperm motility, protective against Alzheimer's disease	- - -	Ruiz-Pesini *et al.*, 2000, Chagnon *et al.*, 1999
U	Higher risk of occipital stroke	Migraine	Majamaa *et al.*, 1998

LHON = Leber's hereditary optic neuropathy, np = nucleotide position, - - - = no adaptive associate factor.

2.4 MITOCHONDRIAL PHYLOGENIES

Owing to a strict maternal mode of inheritance and a high mutation rate, the mtDNA sequence has evolved by the sequential accumulation of base substitutions along radiating maternal lineages, thus allowing for a phylogenetic study of *Homo sapiens sapiens* dispersals throughout the world from a female perspective (Maca-Meyer *et al.*, 2001). mtDNA does not undergo recombination (Eyre-Walker and Awadalla, 2001), and since the human mitochondrial genome is strictly maternally inherited, mtDNA lineages are clonal. As a result of this clonality, phylogenetic and population analyses based on mtDNA are free of complexities imposed by bi-parental recombination (Elson *et al.*, 2001).

Complete sequence data of the mtDNA yields a more reliable phylogenetic network and a more accurate classification of the haplogroups than one based on differences found in restriction fragment analysis of the coding region or in the sequence of the hypervariable

segment I. In population genetics, such networks may enable more detailed analyses of population history and mtDNA evolution whereas in medical genetics, such networks may help to distinguish between a rare polymorphism and a pathogenic mutation (Finnilä *et al.*, 2000). The common set of enzymes used in RFLP allows only a small proportion of the mtDNA sequence to be examined and therefore a number of polymorphisms may remain undetected (Wallace, 1994).

The phylogenetic relationship among mtDNA haplotypes is assessed by either the maximum parsimony tree or genetic distance/neighbour joining analysis and the reliability of the two techniques is obtained by subjecting the derived trees to bootstrap analysis (Chen *et al.*, 2000). The iterative maximum likelihood method of Nei and Tajima (1983) is used to calculate the intra and interpopulation genetic divergence, as well as divergence within specific haplogroups. The rate of human mtDNA divergence serves as a simple biological universal clock that can be used to time the major events of human evolution and geographical dispersal (Gibbons, 1998; Salemi and Vandamme, 2003).

2.5 MUTATIONS IN THE MITOCHONDRIAL GENOME

Normal respiratory function is dependent on an elaborate interplay between the mitochondrial and nuclear genomes (Munnich *et al.*, 1996). Loss or impaired function of one of the nuclear-encoded RC subunits usually leads to a deficiency of the corresponding enzyme complex (Munnich *et al.*, 1996; Holt, 2003). Mutations in mtDNA also lead to impairment of normal respiratory function (Holt, 2003). Although neurological diseases are the most common form of such respiratory dysfunction, virtually any tissue in the body can be affected (Larsson and Clayton, 1995; Holt, 2003). The ubiquitous nature of the mitochondrion and its unique genetic features contribute to the clinical, biochemical and genetic heterogeneity of mitochondrial diseases (Bauer *et al.*, 1999).

2.5.1 Causative mutations

The mtDNA mutations are divided into four broad categories – missense, protein synthesis, insertion-deletion and copy number mutations (Wallace, 1992). Majority of these mutations compromise energy production by the mitochondria and thus manifest themselves as defects in the high energy dependent tissues such as the skeletal muscles, the brain (Budd and Nicholls, 1998), the heart (Ozawa *et al.*, 1995), the liver, the nerves (Corral-Debrinski *et al.*, 1992), the eyes and the cochlea (Holt, 2003). These defects with

the most common causative mutations indicated in Table 2.4 generally manifest in clinical symptoms such as blindness, deafness, dementia, movement disorders, weakness, cardiac failure, diabetes, renal dysfunction and liver disease (Wallace, 1992). Although mtDNA was discovered more than 30 years ago, its importance in human pathology has become apparent only during the last 13 years, with pathogenic mutations of mtDNA being described in increasing number (Shanske *et al.*, 2001).

Table 2.4: Common mutations in the mitochondrial genome

Disorder	Gene	mtDNA mutation	Homoplasmy	Mode of inheritance	Reference
LHON	ND1	G3460A	+	Maternal	Wallace *et al.*, 1988a
LHON	ND2	G5244A	+	Maternal	Brown *et al.*, 1992
LHON	ND4	G11778A	+	Maternal	Wallace *et al.*, 1988a
LHON	ND5	G13708A	+	Maternal	Wallace *et al.*, 1999
LHON	ND6	G14459A	+	Maternal	Jun *et al.*, 1994
LHON	ND6	G14484C	+	Maternal	Johns *et al.*, 1992
LHON	Cytb	G15257A	+	Maternal	Brown *et al.*, 1992; Houponen *et al., 1993*
NARP	ATP6	T8993G	-	Maternal	Holt *et al.*, 1990
LS	ATP6	T8993G	-	Maternal	Leigh, 1951;Tatuch *et al.*, 1992; de Vries *et al.*, 1993
LS	ATP6	T9176C	-	Maternal	Campos *et al.*, 1997
MELAS	$tRNA^{Leu(UUR)}$	A3243G	-	Maternal	Goto *et al.*, 1990
MELAS	$tRNA^{Leu(UUR)}$	A3260G		Maternal	Nishino *et al.*, 1996
MELAS	$tRNA^{Leu(UUR)}$	T3271C	-	Maternal	Goto *et al.*, 1991
MELAS	$tRNA^{Leu(UUR)}$	T3291C	-	Maternal	Goto *et al.*, 1994
MMC	$tRNA^{Leu(UUR)}$	A3260G	-	Maternal	Zeviani *et al.*, 1991
MERRF	$tRNA^{Lys}$	A8344G	-	Maternal	Shoffner *et al.*, 1990
MERRF	$tRNA^{Lys}$	T8356C	-	Maternal	Silvestri *et al.*, 1992
MERRF	$tRNA^{Lys}$	G8363A	-	Maternal	Ozawa *et al.*, 1997
CIPO	$tRNA^{Gly}$	A10006G	+	Maternal	Wallace *et al.*, 1999
CIPO	$tRNA^{Leu(CUN)}$	A12308G	+	Maternal	Wallace *et al.*, 1999
CPEO	$tRNA^{Asn}$	A5692G	+	Maternal	Holt *et al.*, 2003
CPEO	$tRNA^{Leu(CUN)}$	G12315A	-	Sporadic	Fu *et al.*, 1996
Deafness	12S rRNA	A1555G	+	Maternal	Prezant *et al.*, 1993
Exercise intolerance	Cytb	G15059A	-	Sporadic	Andreu *et al.*, 1999

ATP6 = ATPase 6 subunit of ATP synthase complex, CIPO = chronic intestinal pseudo-obstruction with myopathy and ophthalmoplegia, CPEO = chronic progressive external ophthalmoplegia, Cytb = cytochrome b, LHON = Leber's hereditary optic neuropathy, MELAS = mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes, MERRF = myoclonic epilepsy and ragged-red fibres, MMC = maternally inherited myopathy and cardiomyopathy, ND1-6 = NADH dehydrogenase subunits, 12S rRNA = ribosomal RNA of 12 svedberg units, $tRNA^{Leu(CUN)}$ = transfer RNA for leucine (N = any of the four bases-U, A, C or G), $tRNA^{Leu(UUR)}$ = transfer RNA for leucine (R = any of the four bases-U, A, C or G), $tRNA^{Lys}$ = transfer RNA for lysine, $tRNA^{Gly}$ = transfer RNA for glycine, + = homoplasmy, - = heteroplasmy.

2.5.2 Haplogroups associated with specific mutations

As women migrated out of Africa into the different continents about 150,000 YBP they accumulated mtDNA mutations that today are seen as high frequency, continent-specific mtDNA sequence polymorphisms. These polymorphisms are associated with specific mtDNA haplotypes and haplogroups (Finnilä *et al.*, 2000), thus mtDNA variation correlates with the ethnic and geographical origin of an individual (Torroni *et al.*, 1993; Torroni and Wallace, 1994; Wallace, 1995; Alves-Silva *et al.* 2000) as indicated in Table 2.5. Most mitochondrial mutations occur in tRNA genes, and the existence of several polymorphic sites in tRNA gene regions may be helpful for defining haplogroups in different populations (Sternberg *et al.*, 1998).

Table 2.5: Global mtDNA haplogroup-specific polymorphisms

Haplogroup	Nucleotide substitution(s)	Haplogroup	Nucleotide substitution(s)
A	A663G	L3	3594C
B	9-bp deletion, T16519C	L3a	2349
C	A13263G	L3b	G8616A, A11002G
D	C2092T, C5178A, C8414T	L3c	10084
E	G7598A	L3d	T8618C
H	7028C, 14766C	L3e	T2352C
H1	G3010A	M	C10400T, T10873C
H2	1438A, 4769A	T	G709A, G1888A, A4917G, T10463C, G13368A, G14905A, A15607G, G15928A
I	G1719A, G8251A, T10238C		
J	T4216C, A12612G, G13708A	T1	C12633A
J1	G3010A	T2	A11812G, A14233G
J2	C7476T, G15257A	U	A12308G, G12372A
K	A1811G, G9055A, A12308G, G12372A	U2	A1811G, G9055A, A12308G, G12372A
		U4	A1811G, T4646C, C11332T
L	T10873C	U5	T3197C
L1	G2758A, C3594T	U5a	A7768G
L1a	C4312T	U5a1	A14793G
L1b	T2352C	U5b	A5656G
L1c	A9072G, A12810G	U6	G7805A, T14179C
L2	C3594T	V	G4580A, C15904T
L2a	A13803G	W	G709A, T1243C, G8251A, G8994A
L2b	A4158G	X	T6221C, G1719A, T14470C

bp = base pair. Adapted from Alves-Silva *et al.* (2000).

All African lineages belong to macrohaplogroup L* (Chen *et al.*, 1995; Chen *et al.*, 2000; Salas *et al.*, 2002; Salas *et al.*, 2004), which is subdivided into a number of haplogroups and subhaplogroups (Chen *et al.*, 2000). All European and Asian mtDNAs originate from L3. Half of the Asian mtDNAs fall into macrohaplogroup M (Quintana-Murci *et al.*, 1999; Lell and Wallace, 2000); the remaining Asian and European mtDNAs belong to macrohaplogroup N (Ballinger *et al.*, 1992; Quintana-Murci *et al.*, 1999; Salas *et al.*, 2002).

Asian-specific haplogroups belonging to macrohaplogroup M include C, D, G, E, Y and Z. Asian-specific lineages belonging to macrohaplogroup N include A, B and F and Western-Eurasian lineages H, I, J, K, R, T, U, V, W and X (Ballinger *et al.*, 1992; Quintana-Murci *et al.*, 1999; Lell and Wallace, 2000). The founding Americans belong to haplogroups A (A1 & A2), B, C, D (D1 & D2) and X (Forster *et al.*, 1996).

2.6 THE DIAGNOSIS AND MANAGEMENT OF MITOCHONDRIAL DISEASE

The prevalence of mtDNA disease in East England is 6.57 per 100,000 adults of working age (Chinnery *et al.*, 2000). This prevalence is comparable to that of amyotrophic lateral sclerosis (6.2 per 100,000 population) and Huntington's disease (6.4 per 100,000 population) but is more common than other inherited neuromuscular disorders such as Duchenne's muscular dystrophy (3.2 per 100,000 population) and myotonic dystrophy [5 per 100,000 population] (Chinnery *et al.*, 2000). Out of every 100,000 clinically unaffected adults, 7.59 were also identified as being at risk of developing mtDNA disease (Chinnery *et al.*, 2000). These findings have resource implications, particularly for supportive care and genetic counselling (Chinnery *et al.*, 2000).

The diagnosis of mitochondrial disorders is challenging. The difficulty arises when no known mtDNA defect can be found, or when the clinical abnormalities are complex and not easily matched to those of more common mitochondrial disorders, warranting a full mitochondrial evaluation (Gillis and Kaye, 2002). Since available information or data on the prevalence and/or incidence of Mendelian genetic disorders are widely dispersed, a central information repository (database) is urgently required. Such information is of importance in the planning of genetic services for patients of different ethno-geographic origin, for assessing health care priorities and for monitoring trends of disease prevalence (Al-Jader *et al.*, 2001).

Despite the clinical importance of mtDNA diseases, and despite the fact that the sequence, the genes, and the presumed function of the mitochondrial chromosome have been completely described for nearly two decades, the molecular mechanism leading from genotype to clinical phenotype has remained enigmatic. This has prevented useful counselling of patients and the search for therapeutic interventions (Shoffner *et al.*, 1995; Fischel-Godsian, 2000).

2.6.1 Primary and secondary mitochondrial disease

A primary mitochondrial disease is caused by a genetic defect in a mitochondrial enzyme or translocator. The mutation may occur in the primary transcript, in one of the enzymes that catalyses posttranslational modification, or in the translocators functioning in the import pathway. It may affect the catalytic activity, the import or export of a protein into the mitochondria or its degradation (Scholte, 1988).

Secondary mitochondrial defects are caused by lack of compounds that enable proper mitochondrial function or by inhibition of that function. This may result from malnutrition, circulatory or hormonal disturbances, viral infection, poisoning, or an extramitochondrial error of metabolism (Scholte, 1988). Malnutrition causes a depletion of components essential for mitochondrial functioning. Defects at the level of hormones and neurotransmitters may cause abnormalities in mitochondrial metabolism or its regulation. Viral infection is an important aetiological factor in acute childhood encephalopathies and in juvenile cardiomyopathies where there is massive disruption of heart mitochondria. e.g. Reye's syndrome (Scholte, 1988). Other factors are poisoning by exogenous compounds such as alcohol, anti-tumour drugs such as adriamycin, antibacterial drugs such as chloramphenicol and tetracycline, which inhibit mitochondrial protein synthesis, zidovudine (Lewis and Dalakas, 1995), diphtheria toxin, anticonvulsants such as barbiturates and valproate, antimycin, azide, bonkrekic acid, carbon monoxide, atractyloside, cyanide, fluoroacetate, fluorocitrate, lewisite, malonate, oligomycin and rotenone. Long-term zidovudine therapy in patients with human immunodeficiency virus (HIV) infection can cause a destructive mitochondrial myopathy with histological features of ragged-red fibres and proliferation of abnormal mitochondria with severely reduced amounts of mtDNA (Arnaudo *et al.*, 1991). Depletion of muscle mtDNA is probably due to zidovudine-induced inhibition of mtDNA replication by DNA polymerase gamma and is not a secondary effect of HIV infection (Vittecoq *et al.*, 2002).

There are inborn errors that cause formation of mitochondrial inhibitors. Phenylpyruvate production in Phenylketonuria inhibits the mitochondrial monocarboxylate translocator for pyruvate and ketone bodies (Scholte, 1988). In fructose intolerance there is depletion of phosphate that is essential for mitochondrial functioning. Accumulated protoporphyrin in porphyrias cause loose coupling of oxidative phosphorylation. In cystic fibrosis, there is a decreased affinity of cytochrome oxidase for cytochrome c in fibroblast mitochondria (Scholte, 1988).

2.6.2 Diagnosis of mitochondrial disorders

Mitochondrial disorders are clinically, biochemically and molecularly heterogeneous. Diagnosis of mitochondrial disorders is via histochemical, biochemical and molecular biology techniques. As there is no clear-cut correlation between genotype and phenotype, the diagnosis has to be based on the sum of clinical, morphological, biochemical and molecular genetic findings (Bauer *et al.*, 1999). The confirmation or exclusion of an RC disorder is therefore a common dilemma for clinicians (Bernier *et al.*, 2002). The diagnostic process starts with a careful clinical assessment and family history (Bauer *et al.*, 1999), of which female ancestry history is most important.

Histochemically, fresh-frozen sections of striated muscle are stained by Gomori's trichrome stain (Engel *et al.*, 1963). In normal muscles, the myofibrils are stained green with the A-bands being darker than the I-bands. The membranous intermyofibrillar material, consisting of mitochondria and sarcoplasmic reticulum, is stained red. Normal muscles are rounded and have generally convex borders (Engel *et al.*, 1963). In defective muscles, reddish blotches of abnormal deposits of mitochondria appear (Shanske *et al.*, 2001). The difficulty with mammalian skeletal muscle is that it is at times not easy to distinguish between mitochondria and sarcoplasmic reticulum with the light microscope on the basis of morphology. Biochemical tests involve testing for deficiencies in enzyme activities in the RC. Analysis via molecular biology techniques aims to trace mutations in mtDNA and nDNA that affect mitochondrial function (Shanske *et al.*, 2001).

Proposed criteria for diagnosis of RC disorders place patients into "definite", "probable", "possible", or "unlikely" categories (Walker *et al.*, 1996; Bernier *et al.*, 2002). A definitive diagnosis is made when two major criteria, or one major plus two minor criteria are met. The characteristics of a major and a minor criterion are presented in Table 2.6. A probable diagnosis is defined as one major plus one minor criterion or at least three minor criteria

being present. A possible diagnosis is due to either a major criterion or two minor criteria, one of which must be clinical. Evidence from at least two relatively independent types of investigation (i.e. clinical, biochemical, or molecular) is required to establish a definite diagnosis (Bernier *et al.*, 2002). Distinguishing between normal and abnormal values of RC enzyme activities remains a contentious issue, and the varied approaches used by different centres may make it impractical to develop consensus on specific cut-offs to delineate major and minor criteria (Bernier *et al.*, 2002).

Table 2.6: Characteristics of major and minor criteria

Major criteria	Minor criteria
Clinical Clinically complete RC encephalopathy or a mitochondrial cytopathy defined as fulfilling all of the following conditions: Unexplained combination of multisystemic symptoms that is essentially pathognomonic for an RC disorder. Symptoms must include at least three of the organs system presentations, namely neurologic, muscular, cardiac, renal, nutritional, hepatic, endocrine, hematologic, otologic, ophthalmologic and dermatologic or dysmorphic. A progressive clinical course with episodes of exacerbation or a family history that is strongly indicative of an mtDNA mutation (at least one maternal relative other than the proband whose presentation predicts a probable or definite RC disorder). Other possible metabolic or nonmetabolic disorders have been excluded by appropriate testing, which may include metabolite, enzyme, or mutational analyses, imaging, electrophysiological studies, and a histology **Histology** >2% ragged red fibres in skeletal muscle **Enzymology** >2% COX-negative fibres if <50 years of age >5% COX-negative fibres if >50 years of age <20% activity of any RC complex in a tissue <30% activity of the same RC complex activity in ≥ two tissues **Functional** Fibroblast ATP synthesis rates > 3SD below mean **Molecular** Identification of a nuclear or mtDNA mutation of undisputed pathogenicity	**Clinical** Symptoms compatible with an RC defect **Histology** 1%-2% ragged red fibres if aged 30 – 50 years Any ragged red fibres if <30 years of age >2% subsarcolemmal mitochondrial accumulations in a patient (16 years of age) Wide-spread electron microscopic abnormalities in any tissue **Enzymology** Antibody-based demonstration of a defect in RC complex 20%-30% activity of any RC complex in a tissue 30%-40% activity of any RC complex in a cell line 30%-40% activity of the same RC complex activity in ≥ two tissues **Functional** Fibroblast ATP synthesis rates 2-3 SD below mean Fibroblasts unable to grow on media with glucose replaced by galactose **Molecular** Identification of a nuclear or mtDNA mutation of probable pathogenicity **Metabolic** One or more metabolic indicators of impaired RC function

ATP = adenosine tiphosphate, COX = cytochrome oxidase complex, mtDNA = mitochondrial deoxyribonucleic acid, RC = respiratory chain, SD = standard deviation.

In an effort to facilitate and standardise diagnosis of respiratory chain disorders in infants and children, a new scoring system, the Mitochondrial Disease Criteria (MDC), was developed by Wolf and Smeitink (2002). The MDC comprises well-defined clinical symptoms, metabolic and imaging findings, and skeletal muscle morphology, together with results of biochemical investigations of the skeletal muscle. The scores as indicated in Table 2.7 range from 0 to 12 (Wolf and Smeitink, 2002).

Table 2.7: Evaluation of the mitochondrial disease criteria score

MDC score	RC disorder evaluation
1	Unlikely
2 – 4	Possible
5 – 7	Probable
8 – 12	Definite

MDC = mitochondrial disease criteria, RC = respiratory chain. Compiled from Neurology (2003).

2.6.3 Treatment and management of mitochondrial disorders

Treatment of patients with mitochondrial diseases is woefully inadequate on a global scale and is often limited to anecdotal cases or to trials that do not allow for definitive conclusions (Beal, 2003). Therapeutic approaches as indicated in Table 2.8 aim at the removal of noxious metabolites, supplementation of RC components and use of substrates that bypass defective RC enzyme complexes (Shanske *et al.*, 2001). The virtual lack of deleterious adverse effects has encouraged the use of coenzyme Q_{10} (CoQ_{10}) and administration of thiamine and riboflavin. Administration of cofactors of mitochondrial enzymes is also a common practice (Beal, 2003).

Table 2.8: Compounds that have therapeutic effects on mitochondrial disease

Compound	Defect remedied	Site of action	Reference
Coenzyme Q_{10}	MELAS, Parkinson's and Huntington's disease, Friedreich's ataxia	1st mobile electron carrier from complex I or II to III	Shoffner and Wallace, 1994; Beal, 2003; Abe *et al.*, 1991
Acetyl-L-carnitine	Carnitine deficiency	IMM	DiMauro, 1996
Lipoic acid	General	Citric acid cycle	Beal, 2003
Dichloroacetate	PDH deficiency	Mitochondrial matrix	Shanske *et al.*, 2001
Thiamine	Variety of mt enzymes	Mitochondria	Beal, 2003
Riboflavin	Variety of mt enzymes	Mitochondria	Beal, 2003
Ascorbate	Complex I, II & III defects	Complex IV	Eleff *et al.*, 1984
Succinate	Complex I defects	Complex II	Shoffner and Wallace, 1994
Galantamine	Alzheimer's disease	Presynaptic nicotinic receptors	Wilcock *et al.*, 2000; Rockwood *et al.*, 2001
NADH	MELAS	Complex I	Majamaa *et al.*, 1997

IMM = inner mitochondrial membrane, MELAS = myoclonic epilepsy, lactic acidosis and stroke-like episodes, NADH = reduced form of nicotinamide adenine dinucleotide, PDH = pyruvate dehydrogenase complex.

2.7 THE SIGNIFICANCE OF HAPLOGROUPS IN MITOCHONDRIAL DISEASE

Although much of the mtDNA variation that exists in modern populations may be selectively neutral, studies of the mildly deleterious mtDNA mutations causing LHON have demonstrated that some continent-specific mtDNA lineages are more prone to manifest the clinical symptoms of LHON than others. Hence, all mtDNA lineages are not equal, which may provide insights into the extreme environments that were encountered by our ancestors, and which may be of great importance in understanding the pathophysiology of mitochondrial disease (Wallace *et al.*,1988b; Wallace *et al.*,1999).

The 10663C mutation causing LHON appears to be restricted to haplogroup J for LHON expression (Brown *et al.*, 2002). Such a strict association not only supports the hypothesis that haplogroup J itself can contribute to LHON as a predisposing factor, but also demonstrates that mitochondrial disease can result from complex genetic interactions (Brown *et al.*, 2002).

In the current study, DNA from blood or muscle samples of patients clinically diagnosed with mitochondrial disorders were analysed via haplogroups as part of a clinical management plan. The failure to detect causative mutations in these patients by the attempts made so far presented a unique situation where genetic aetiology was of interest to investigate. If the patients are found to cluster in a particular haplogroup or haplogroups,

there is probably a susceptibility effect or protective role such a haplogroup plays in mitochondrial dysfunction.

2.8 OBJECTIVES OF THE STUDY

The major aim of this investigation was to identify on which haplogroups the clinical phenotypes of 27 South African paediatric patients clinically diagnosed with suspected mitochondrial disorders were expressed. Previous attempts to trace mtDNA mutations in these patients resulted in no reported causative mutations being detected except for one individual.

2.8.1 Specific objectives

2.8.1.1 Specific sets of single nucleotide polymorphisms (SNPs) were used to characterise patients to identify their haplogroups via automated sequencing strategy.

2.8.1.2 Haplogroups were correlated to the clinical phenotypes of the patients.

2.8.1.3 Haplogroups were correlated with clinical phenotypes to identify a possible role for haplogroups in to the expression of the clinical phenotypes.

CHAPTER THREE

MATERIALS AND METHODS

Only standardised protocols were utilised throughout this investigation. Chemicals and reagents of analar grade were utilised in this study. Unless otherwise stated, reagents were supplied by Promega®. DNA was extracted from blood or muscle prior to analysis via automated cycle sequencing.

3.1 ETHICAL APPROVAL

Ethical approval for this project was granted by the Ethics Committee of the North-West University in 2002 with the title "Mitochondrial DNA (mtDNA) mutations in patients with a suspected mitochondrial disorder in the South African context" with approval number 02M02. Informed consent was obtained from the patients or their guardians prior to the collection of blood or muscle samples from the patients.

3.2 SAMPLE POPULATION

All patients included in this study were black, Caucasian, coloured or Indian South African children who had been referred to the Paediatric Neurology Clinic of Pretoria Academic Hospital. They were diagnosed by Dr Izelle Smuts as having clinical mitochondrial disorder phenotypes. Five millilitres (ml) of blood was collected from each of 21 patients by venous puncture and stored in ethylenediamine tetraacetic acid (EDTA) vacutainer tubes at 4 degrees Centigrade (°C), for not more than three days prior to DNA extraction. Approximately 100 milligrams (mg) of muscle was obtained from each of other six patients via biopsy of the *Vastus Lateralis* muscle. The muscle samples were each put in a tube of saline or SET buffer (0.25 M sucrose, 2 mM EDTA, 10 mM Tris® at pH 7.4) and incubated on ice for not more than three hours before DNA was extracted. DNA from a total of 27 patients with an MDC score of 6, 7 or 8, and patients with striking clinical manifestations was used in this investigation. The investigated patient population had different ethnic origins.

3.3 EXTRACTION OF DNA

DNA was extracted[1] from the blood samples using the Wizard®[2] Genomic DNA purification kit (Section 3.3.1) and from muscle with the QIAamp®[3] DNA Mini Kit (Section 3.3.2). The principle of the Wizard® Genomic DNA purification kit technique involves lysis of red blood cells, and white blood cells with their nuclei in the cell lysis solution. The cellular proteins are removed by salt precipitation, genomic DNA (gDNA) concentrated and desalted by isopropanol. The principle of the QIAamp® DNA Mini Kit involves lysis of the tissue cells using proteinase K in a lysis buffer. The cellular proteins are removed by salt precipitation, where after gDNA is concentrated and desalted by ethanol.

3.3.1 Extraction of DNA from whole blood

Three hundred microlitres (µl) of blood were lysed with 900 µl cell lysis solution in a sterile 1.5 ml microcentrifuge tube. The reaction was incubated at room temperature for 10 minutes (min), while mixing by inversion. This step serves to lyse the red blood cells. The sample was centrifuged at 13,000 x gravity (x *g*) for 20 seconds (s) at room temperature, the supernatant discarded and the white pellet vortexed until the white blood cells were completely resuspended in the remaining supernatant.

The white cells were lysed by the addition of 300 µl nuclei lysis solution followed by incubation at 37°C for 30 min. The solution was pipetted six times to lyse the white blood cells. The sample was allowed to cool down to room temperature. The proteins were precipitated by the addition of 100 µl protein precipitation solution followed by vigorous vortexing. Afterwards the sample was centrifuged at 13,000 x *g* for 3 min.

DNA was precipitated by transferring the supernatant to a 1.5 ml microcentrifuge tube containing 300 µl 100% isopropanol, followed by inversion of the tube until the white thread-like strands of DNA became visible. The gDNA was recovered via centrifugation for 1 min at 13,000 x *g* and all the remaining salts were washed out with 300 µl 70% ethanol (EtOH). The sample was centrifuged again at 13,000 x *g* for 1 min, and the pellet was air-dried for 10 to 15 min. The DNA pellet was rehydrated in 100 µl DNA rehydration solution, followed by incubation overnight at 4°C. The isolated DNA was stored at -20°C.

[1] DNA extraction was performed by former students of the Centre for Genome Research.
[2] Wizard® is a registered trademark of the Promega Corporation, Madison, WI, U.S.A.
[3] QIAamp® is a registered trademark of QIAGEN, Clifton Hill, Victoria, Australia.

3.3.2 Extraction of DNA from muscle

One hundred and eighty µl of buffer ATL was added to 25.0 mg of homogenised muscle tissue, in 1.5 ml microcentrifuge tubes. Homogenisation of the muscle tissue facilitates efficient lysis. Twenty µl of a 20 $mg.ml^{-1}$ solution of proteinase K was subsequently added to each solution in the microcentrifuge tubes and mixed by vortexing. The contents were incubated at 56°C in a shaking water bath for ca. 3 hours, which served to lyse the cells. The microcentrifuge tubes were centrifuged for a few seconds to remove drops from the inside of the lids, prior to opening.

Two hundred µl of buffer AL was added to each sample, mixed by pulse-vortexing for 15 s, and incubated at 70°C for 10 min. Thereafter, 200 µl of 99.8% ethanol was added, and the samples were mixed thoroughly by vortexing for 15 s, to yield a homogeneous solution. This mixture, including the precipitate, was applied directly to the QIAamp spin column that was placed in a 2 ml collection tube. The columns were centrifuged at 6,000 x *g* for 1 min. The filtrate was discarded and the QIAamp spin columns placed in clean 2 ml collection tubes.

Five hundred µl of buffer AW1 was applied directly to the spin columns, and the columns were centrifuged for 1 min at 6,000 x g. The filtrates, together with the collection tubes, were discarded and the QIAamp spin columns placed in clean 2 ml collection tubes. Five hundred µl of buffer AW2 was applied directly to the spin columns, and the columns were centrifuged at 20,000 x *g* for 3 min. In order to ensure that there was no buffer AW2 left in the columns, the QIAamp spin columns were placed in new 2 ml collection tubes and centrifuged for 1 min at 20,000 x *g*. The collection tubes with the filtrates were discarded.

The QIAamp spin columns were placed in clean 1.5 ml microcentrifuge tubes, 200 µl buffer AE was added directly to them. They were then incubated at room temperature for 5 min and centrifuged at 6,000 x *g* for 1 min. This step served to elute gDNA from the columns. This final step was repeated for the purpose of increasing the DNA yield. The concentration of the isolated DNA was determined via spectrophotometry as described in Section 3.4.

3.4 DETERMINATION OF DNA CONCENTRATION

The concentration of the isolated DNA was estimated via spectrophotometry by measuring the absorbance at 260 nm (A_{260}) using an Eppendorf Biophotometer. This Biophotometer was calibrated to have one absorbance unit of double-stranded DNA (dsDNA) equivalent to 50 ng.μl^{-1}, and the DNA was diluted 1 in 10 to 1 in 20 before optical density was measured to achieve an absorbance value in the range of Beer-Lambert's law.

The absorbance at 280 nm (A_{280}) was also recorded to determine the level of protein contamination in the isolated DNA. The A_{260}/A_{280} ratio is a measure of the degree of protein contamination, which is acceptable for a value equal to or above 1.8. A DNA working dilution of 50 ng.μl^{-1} was prepared from the stock and stored at 4°C while the rest of the stock DNA was placed at -20°C for longer preservation.

3.5 DNA AMPLIFICATION

The DNA was amplified via the polymerase chain reaction (PCR) using primers and conditions listed in Table 3.3. The protocol utilised is a modified version of one described by Mullis *et al.* (1986). The reaction constituents included double distilled water (ddH_2O), 1 x PCR buffer [50 mM potassium chloride (KCl), 10 mM Tris®-hydrochloride of pH 9.0 and 0.1% Triton® X-100], 1.5 mM magnesium chloride ($MgCl_2$), 200 µM of each of the 2'-deoxyribonucleotide-5'-triphosphates (dNTPs), 0.4 picomol.μl^{-1} of each of the respective forward and reverse primers, 0.04 unit.μl^{-1} of *Thermus aquaticus* (Taq) DNA polymerase and gDNA in a total volume of 12.5 µl for samples that were to be sequenced.

In the first stage of cycling, the samples were heated to 94°C for 10 minutes. Only denaturation of DNA takes place at this stage, as indicated in Table 3.1. In the second stage, the samples were incubated at 94°C for 30 seconds, at the annealing temperature (Table 3.2) for 30 seconds and at 72°C for 30 seconds. This cycle was repeated a total of 25 times during which amplification occurred. In the third stage, the samples were incubated at 72°C for 7 min. This stage ensures that all PCR products are synthesised to full length. In the last stage, the samples were cooled to 4°C and held at that temperature indefinitely until purification of the PCR products.

Table 3.1: PCR conditions for amplification of mitochondrial DNA

PCR step	# of cycles	Purpose	Temperature	Duration
1	1	Denaturation	94°C	10 min
2	25	Denaturation	94°C	30 s
		Annealing	x°C	30 s
		Synthesis	72°C	30 s
3	1	Synthesis of incomplete fragments	72°C	7 min
4	1	Hold	4°C	Indefinite

= number, PCR =polymerase chain reaction, x = the annealing temperature for each pair of primers as indicated in Table 3.2.

In general the annealing temperature for amplification is optimised starting from a temperature 2°C below the calculated mean melting temperature (T_m) of the primer set for the specific DNA region of interest. The $MgCl_2$ concentration is optimised by varying its concentration until good amplification has been achieved. The duration of the cycles is also varied one at a time until optimised.

The estimated annealing temperature (T_a) of the primer set serves as the starting point from where it can be increased or decreased to achieve optimal amplification. The Oligonucleotide Properties Calculator software (Oligonucleotide Properties Calculator, 2004) was used to determine the T_m for each primer pair through nearest neighbour and thermodynamic calculations by using the values published by Sugimoto *et al.* (1996).

Table 3.2: Primers used for identifying polymorphisms that characterise African haplogroups

np of SNP	Primer	Sequence (5'-3')	CRS reference	T_m	avg T_m	T_a	Size (bp)
3308 3594	L3073	F: aaagtcctacgtgatctgagttc	3051-3073	61.0	60.8	55	639
	H3670	R: ggcgtagtttgagtttgatgc	3690-3670	60.6			
3693	L3644	F: gccacctctagcctagccgt	3625-3644	66.6	62.6	58	623
	H4227	R: atgttggagattgtaatgggt	4247-4227	58.7			
4767 5096 5147	L4750	F: ccaatactaccaatcaatactc	4729-4750	57.1	60.8	52	599
	H5306	R: ggtgatggtggctatgatggtg	5327-5306	64.5			
6150 6221	L5781	F: agccccggcaggtttgaagc	5762-5781	66.6	64.6	58	626
	H6367	R: tggcccctaagatagaggaga	6387-6367	62.6			
7055	L6869	F: ccggcgtcaaagtatttagc	6850-6869	60.4	61.6	58	578
	H7406	R: gggttcttcgaatgtgtggtag	7427-7406	62.7			
9072	L8799	F: ctcggactcctgcctcactca	8779-8799	66.5	63.4	58	638
	H9397	R: gtggccttggtatgtgcttt	9416-9397	60.4			

Table 3.2 continued ...

np of SNP	Primer	Sequence (5'-3')	CRS reference	T_m	avg T_m	T_a	Size (bp)
9554 9755 9818	L9362	F: ggcctactaaccaacacacta	9342-9362	60.6	59.9	56	609
	H9928	R: aaccacatctacaaaatgccagt	9950-9928	59.2			
10400	L9886	F: tccgccaactaatatttcactt	9865-9886	57.1	56.7	55	617
	H10462	R: aatgaggggcatttggtaaa	10481-10462	56.3			
10810 10819 10873	L10403	F: aaaggattagactgaaccgaa	10383-10403	56.7	59.6	56	612
	H10975	R: ccatgattgtgaggggtagg	10994-10975	62.5			
11899 11914	L11486	F: aaaactaggcggctatggta	11467-11486	58.4	60.4	56	629
	H12076	R: ggagaatgggggataggtgt	12095-12076	62.5			
13708	L13612	F: aagcgcctatagcactcgaa	13593-13612	60.4	61.5	56	614
	H14186	R: tggttgaacattgtttgttgg	14206-14186	56.7			
14769 14905	L14650	F: ccccattactaaacccacactc	14629-14650	62.7	61.7	58	597
	H15211	R: ttgaactaggtctgtcccaatg	15232-15211	60.8			
15849	L15676	F: tccccatcctccatatatcc	15657-15676	60.4	59.1	56	524
	H16157	R: tgatgtggattgggtttttatgta	16180-16157	57.7			

Avg = average, bp = base pairs, CRS = Cambridge reference sequence, F = forward primer, np = nucleotide position, R = reverse primer, SNP = single nucleotide polymorphism, T_a = annealing temperature, T_m = melting temperature. Adapted from Maca-Meyer *et al.* (2001).

3.6 AGAROSE GEL ELECTROPHORESIS

Two percent (w/v) agarose gels in a 1 x TBE buffer (8.915 mM Tris® pH 8.1, 8.895 mM boric acid, 0.2498 mM Na_2EDTA) were used in electrophoresis at 100 volts (V), 10V.cm^{-1} for 30 min for verification of amplification of the mtDNA regions of interest prior to cycle sequencing. A volume of 2.5 µl of ethidium bromide (EtBr) of concentration 10 mg.ml^{-1} was added to 40.0 ml of gel. A volume of ca. 2.5 µl of each amplified DNA sample was mixed with the 2 X loading buffer (0.04% orange G and 50% glycerol) and loaded onto the gel.

The remaining DNA samples were stored at 4°C for purification and sequencing. A volume of 2.5 µl of the 100 bp molecular weight marker [Promega] was mixed with the loading buffer and loaded onto the gel. Visualisation of the amplified DNA fragments was performed with a ultra-violet (UV) lamp.

3.7 DETERMINATION OF HAPLOGROUPS

mtDNA of the patients was analysed for single nucleotide polymorphisms (SNPs) using primers indicated in Table 3.2 via automated cycle sequencing. The amplified DNA fragments were purified of excess primers, nucleotides, polymerases, salts and mineral oil,

as discussed in Section 3.7.1, sequenced and the DNA precipitated as discussed in Sections 3.7.2 and 3.7.3 respectively. The haplogroups of the patients were determined by using sets of SNPs presented in Figure 3.1.

3.7.1 Purification of PCR products

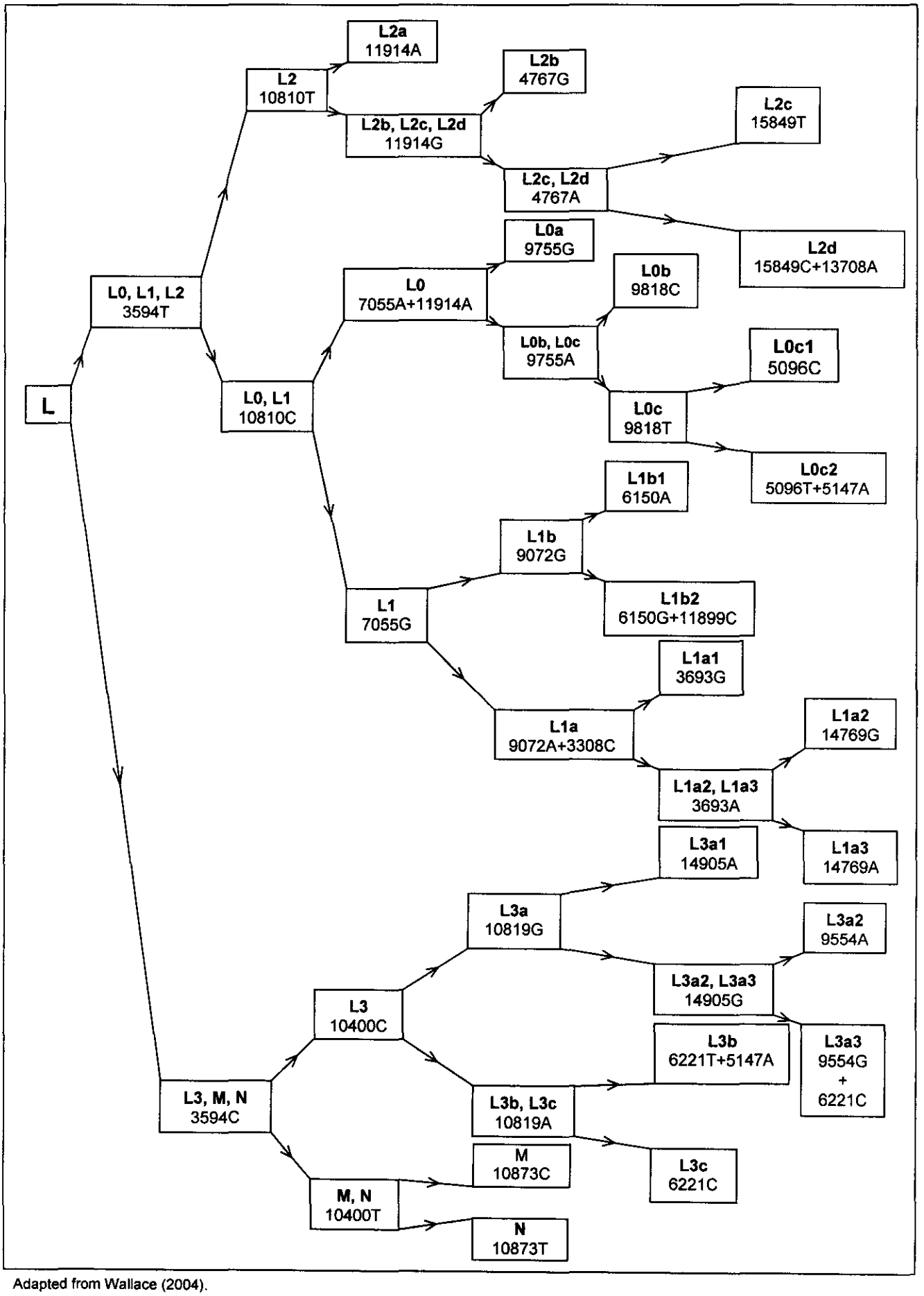
Purification of the PCR products was performed using the QIAquick[1] PCR purification kit as outlined below. Fragments ranging from 100 bp to 10 kb are purified from mineral oil, primers, nucleotides, polymerases and salts. All centrifugation steps are conducted at 17,900 x g in a conventional tabletop microcentrifuge. DNA selectively adsorbs to the uniquely designed silica membrane in the presence of high salt, while contaminants pass through the column. The pure DNA is finally eluted from the column with Tris buffer.

Five volumes of buffer PB excluding the mineral oil were added to every volume of the PCR samples. The QIAquick spin column was placed in a 2 ml collection tube. The sample was applied to the QIAquick column and centrifuged for 30 – 60 s. The flow-through was discarded and the QIAquick column placed back into the same tube. To wash the DNA of impurities, 0.75 ml buffer PE was added to the QIAquick column and the contents centrifuged for 30 – 60 s. This step serves to remove the impurities or unbound material. The flow-through was discarded again, and the QIAquick column placed back in the same tube. The column was centrifuged for an additional 1 min to remove any residual ethanol.

The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube and the DNA eluted in 50 µl EB buffer via centrifugation for 1 min. Alternatively, for increased DNA concentration (if the electropherogram had weak bands of patient DNA compared to molecular weight marker), 30 µl of elution buffer was added to the centre of the QIAquick membrane. The column was left to stand for 1 min and then centrifuged. The concentration of the purified DNA was determined via spectrophotometry.

[1] QIAquick® is a registered trademark of QIAGEN, Clifton Hill, Victoria, Australia.

Figure 3.1: Single nucleotide polymorphisms used to characterise African mtDNA haplogroups



Adapted from Wallace (2004).

3.7.2 Automated sequencing analysis

Automated sequencing analysis (Wilson *et al.* 1990) was used to identify all the SNPs presented in Figure 3.1. Automated sequencing was performed using the ABIPrism®[1] BigDye™[2] Terminator version 3.1 (v3.1) Ready Reaction Cycle Sequencing Kit and the primers in Table 3.2 in a Thermo Hybaid®[3] MBS®[4] thermocycler using a modification of the procedure described by Sanger *et al.* (1977). The amount of DNA used in each sequencing reaction varied from 10 – 20 ng.

The following were pipetted in the order indicated: ddH_2O, 2.0 µl of 5 x sequencing buffer, 2.0 µl of ready reaction mix, 1.0 µl of primer (3.2 µM) and DNA into tubes. The amount of DNA added was 10 - 20 ng and the total volume of DNA and water in each tube was 5.0 µl. All the other volumes in the protocol remained fixed.

The samples were cycled at 94°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 min for a total of 25 cycles. The samples were rapidly thermal-ramped to 4°C and held until ready to precipitate.

3.7.3 PCR product precipitation

The sequenced DNA samples were centrifuged, and to each sample was added 62.5 µl of 99.8% ethanol, 3.0 µl of 3.0 M sodium acetate (pH 4.6) and 14.5 µl of ddH_2O, in 1.5 ml centrifuge tubes. The DNA was left in the precipitation mixture for ca. 15 min followed by centrifugation at 10,600 x *g* for 20 min. The supernatant was discarded and the pellet centrifuged for 10 min at 10,600 x g after the addition of 250 µl of 70% ethanol in each tube. The supernatant was discarded and the DNA was allowed to air dry for 15 min.

At Inqaba Biotechnical Industries (Pty) Ltd, the sequenced and precipitated DNA samples were resuspended in 6.0 µl Hi-DiTM deionised formamide and 3.0 µl was injected into a SpectruMedixTM (SCE2410) Genetic Analysis System sequencer for capillary gel electrophoresis and detection. The relationship between the BigDye™ terminator v3.1 colours and the bases eluted via the detector is as indicated in Table 3.3.

[1] ABI Prism® is a registered trademark of Applera Corporation, Forster City, CA, U.S.A.
[2] BigDye™ is a trademark of Applera Corporation, Forster City, CA, U.S.A.
[3] Thermo Hybaid® is a registered trademark of Hybaid Limited, Ashford, Middlesex, UK.
[4] MBS® is a registered trademark of Thermo Electron Corporation, Milford, MA, U.S.A.

Table 3.3: Colours of the bases on a SpectruMedix TM (SCE2410) Genetic Analysis System sequencer

DNA base	Terminator	Colour of peak on detector
A	V3 Dye 2	Green
C	V3 Dye 4	Blue
G	V3 Dye 1	Black
T	V3 Dye 3	Red

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

The sequences were analysed and aligned with the RCRS using the BioEdit sequence Alignment Editor (BIOEDIT, 2004). The relationship between the haplogroups and the SNPs that characterise them, as presented in Figure 3.1, was used to analyse results from automated sequencing for the purpose of haplogrouping patients from whom DNA was obtained. For example, the SNP at position 10810 distinguishes haplogroup L2 from haplogroups L0 and L1. Haplogroup L2 has a T while L0 and L1 have a C nucleotide at this position.

CHAPTER FOUR

RESULTS AND DISCUSSION

Twenty-seven South African paediatric patients, clinically diagnosed with suspected mtDNA disorders, were analysed for polymorphisms of phylogenetic importance via PCR and automated sequencing. DNA was isolated from blood for 21 and from muscle for six patients. The optimisation of experimental protocols followed by the results for patient DNA analysis are presented and discussed.

4.1 OPTIMISATION OF EXPERIMENTAL PROCEDURES

The experimental procedures used in this study included PCR, electrophoresis, purification of PCR products, DNA quantification via spectrophotometry, automated sequencing, precipitation of sequenced products and electrophoresis of the sequenced products. The extraction of DNA from the blood samples was performed by former students of the Centre for Genome Research as mentioned in chapter three using the Wizard® Genomic DNA purification kit and the QIAamp® DNA mini kit. Apart from PCR, which had many factors that required optimisation, the other experimental procedures involved the use of commercially available kits, whose outcome necessitated no optimisation.

4.1.1 Optimisation of polymerase chain reactions

Three samples of DNA with a concentration of 50 $ng.\mu l^{-1}$ (working DNA concentration, obtained by appropriately diluting stock DNA) from controls were first amplified using the set of conditions used by Maca-Meyer *et al.* (2001), Prosser (2001), Van Brummelen (2003) and Van der Merwe (2003). Amplification of the three DNA samples with each pair of the entire forward and reverse primers utilised (see Table 3.2) was achieved under the conditions specified in the table. A negative control having ddH_2O instead of DNA in the other three tubes was included in each amplification phase to rule out any contamination of the samples.

Any one of the three optimised individuals' DNA samples was used as a positive control in each reaction batch for amplification of patient DNA samples. The addition of dimethyl

sulphoxide or formamide was not required in any of the amplification reactions, as mtDNA generally does not form secondary structures that interfere with PCR amplification.

4.1.2 Electrophoresis and PCR product purification

Electrophoresis was used to determine whether there was successful amplification of DNA fragments via PCR before purification was performed. The PCR products were run on a 2% agarose gel in a 1 x TBE buffer at 10 $V.cm^{-1}$ for 30 min and visualisation of fragments under UV light reflected successful amplification. All 13 pairs of primers utilised for amplification of DNA in this study had photographs taken via a video documentation system for proof of amplification. Electrophoresis is an efficient and cost-effective method to confirm that the correct fragment length was amplified successfully before proceeding with PCR product purification and sequencing. All the fragments amplified with the 13 pairs of primers were ca. 600 bp in length, as indicated in Table 3.2.

The amplified fragments were purified using the QIAquick® PCR purification kit as outlined in Section 3.7.1. The concentration of the purified DNA was determined through spectrophotometry. The sample DNA concentrations varied between 8.0 and 32.3 $ng.\mu l^{-1}$ for the different regions amplified.

4.1.3 Cycle sequencing

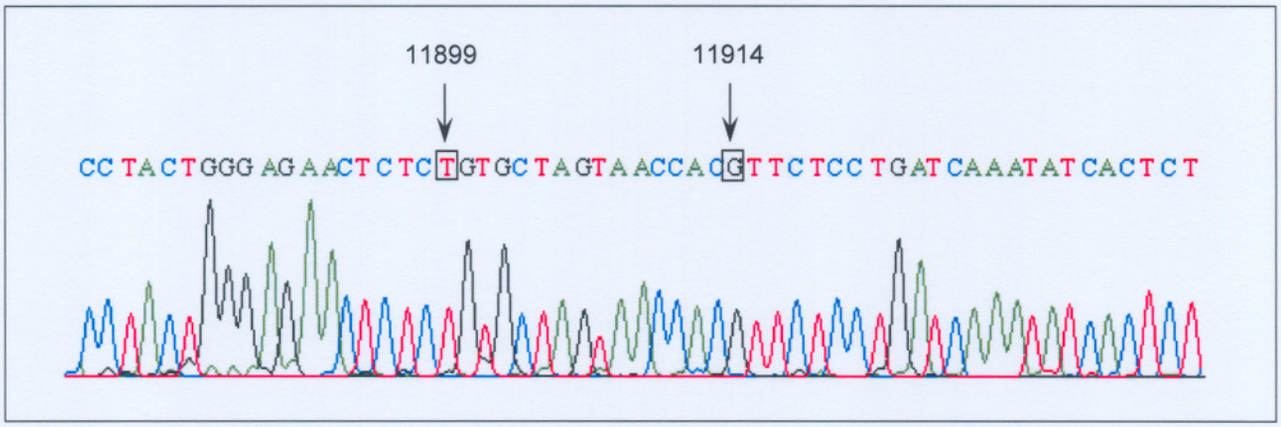
The success of cycle sequencing depends upon the concentration and quality of the PCR product, the quality of PCR purification and the quality of the precipitation. A range of 10 - 20 ng of purified DNA was used for cycle sequencing in all the reactions. Excess template leads to poor separation during electrophoresis and broad peaks of which individual bases are poorly resolved. Insufficient template leads to peaks with low amplitude that is masked by background noise. If the quality of the template is poor, for example when non-specific secondary amplification occurs, excess background noise is observed. Care needs to be taken when discarding or withdrawing the supernatant during precipitation of the sequenced products to avoid discarding of the precipitate (DNA pellet), since it is small and hardly visible. It was also ensured that the pellet was completely air-dried, as moisture could have destabilised it.

The cycle sequencing technique was easier to perform and optimisation was also easier to achieve than for PCR, since only short fragments from the PCR products are sequenced.

Only a single primer is used in each sequencing reaction, unlike in PCR where both the forward and reverse primers are used in each reaction. All 13 primers used in sequencing involved use of the same sequencing programme, unlike in PCR where annealing temperatures varied between different types of primers. Only forward primers were utilised for sequencing except for the primer H5306 utilised to amplify the region from np 4750 to 5306. This region involved analysis of SNPs at np 4767, 5096 and 5147. The SNP at np 4767 could not be detected when sequencing was performed by using the forward primer.

Figure 4.1 is an electropherogram obtained after automated sequencing using sequencing primer L11486 for the DNA sample of control individual C20. The nucleotide changes at the two nucleotide positions of phylogenetic value, 11899 and 11914, are alleles T and G respectively. Similar electropherograms were obtained using the other 12 sequencing primers, and information derived from them was used to haplogroup the individuals.

Figure 4.1: Representative electropherogram of mtDNA sequence of individual C20 encompassing SNPs 11899 and 11914



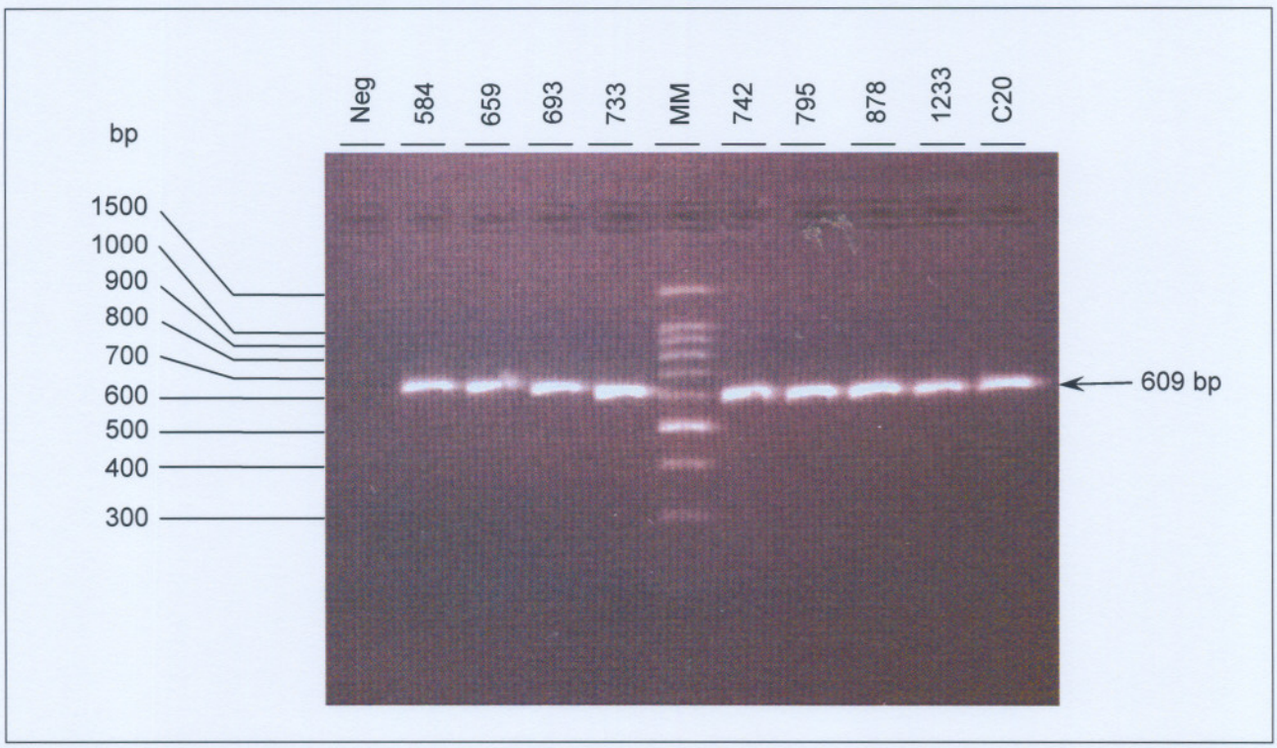
A = adenine; C = cytosine; G = guanine, T = thymine. Arrow indicates SNP(s) of phylogenetic interest. Nucleotide numbering of the sequence is according to MITOMAP (2004).

Automated sequencing detects bases over a large region of the amplified fragments. Much more information is obtained from a single sequence compared to RFLP analysis technique. However, low sensitivity and failure to detect low levels of heteroplasmy are its major drawbacks. Low levels of heteroplasmy are detectable to some extent by RFLP analysis. Bases very close to the beginning and end of the amplified region may also not be detected by automated sequencing since peaks in such regions usually have too much overlap due to poor resolution.

4.2 PATIENT DNA ANALYSIS

DNA was extracted from blood or muscle tissue of the patients as described in Section 3.3. Amplification via PCR, purification and automated cycle sequencing was carried out on the DNA products as described in Section 3.5. A representative agarose gel from electrophoresis of the indicated patients from DNA amplified using primers L9362 and H9928 is presented in Figure 4.2.

Figure 4.2: Representative photograph of agarose gel electrophoresis of the amplified mtDNA for haplogroup analysis



bp = base pairs, C = positive control DNA from patient number 20, MM = 100 base pair molecular weight marker, Neg = Negative control sample. Sample numbers are indicated at the top of the picture.

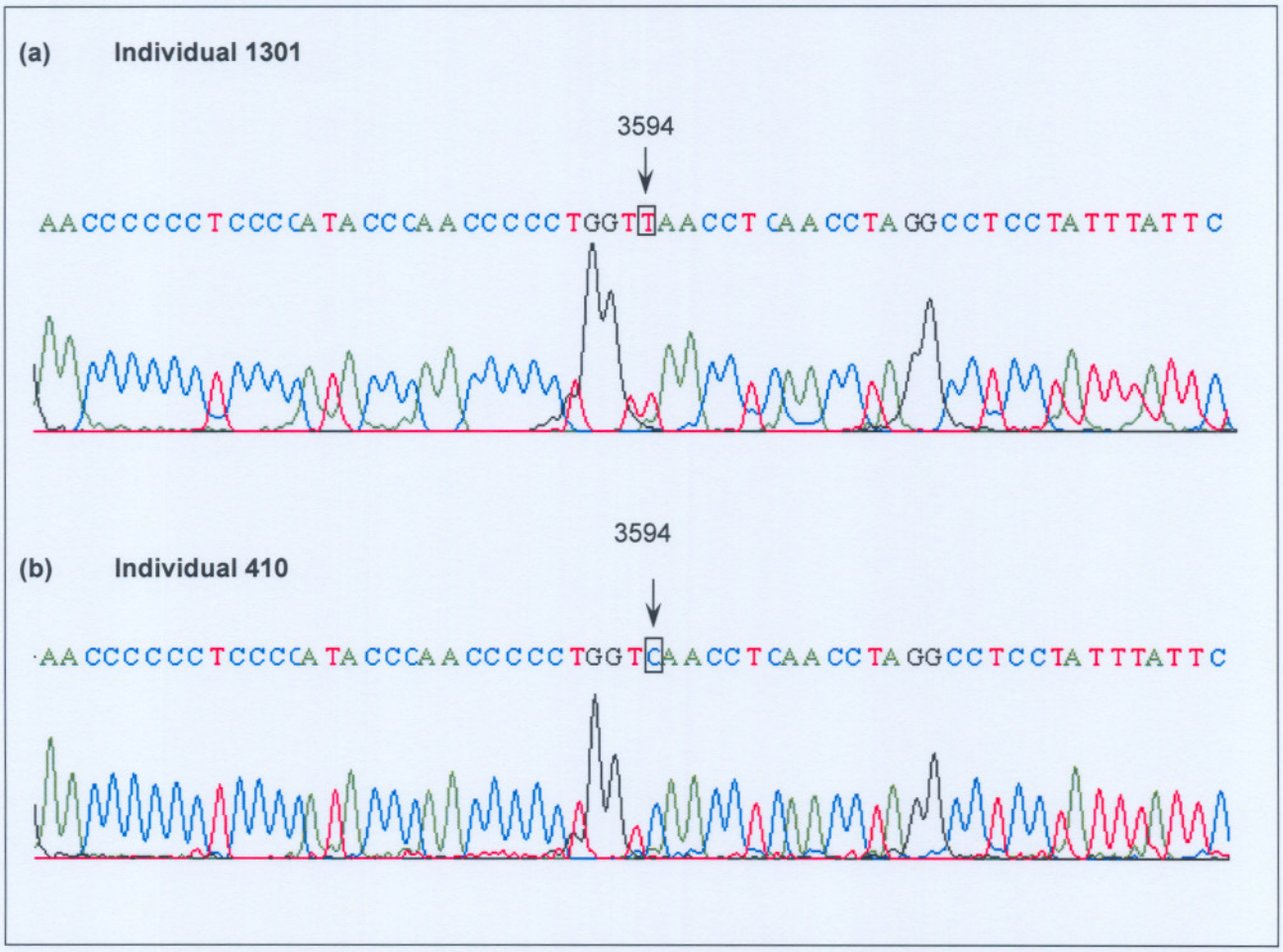
The African specific macrohaplogroup L is divided into two groups of sub-lineages L0, L1 and L2, and L3, M and N. L0, L1, L2 and L3 are African-specific haplogroups whereas M and N are Eurasian haplogroups. Figure 3.1 illustrates the SNPs that differentiate and characterise the various haplogroups. In this section, the results from DNA sequencing are presented, the patients haplogrouped and any polymorphisms or variations in mtDNA sequence from the RCRS noted and discussed.

4.2.1 Single nucleotide polymorphism at position 3594

Polymorphisms at np 3594 are used to assign an individual to macrohaplogroup L with individuals harbouring a T nucleotide belonging to L0, L1 or L2 haplogroup. Those with a C

nucleotide at this position belong to haplogroup L3, M or N (see Table 4.3). Figure 4.3 illustrates the electropherograms of two individuals, one with a T and the other with a C nucleotide at position 3594.

Figure 4.3: Representative electropherogram of the mtDNA sequence encompassing SNP 3594



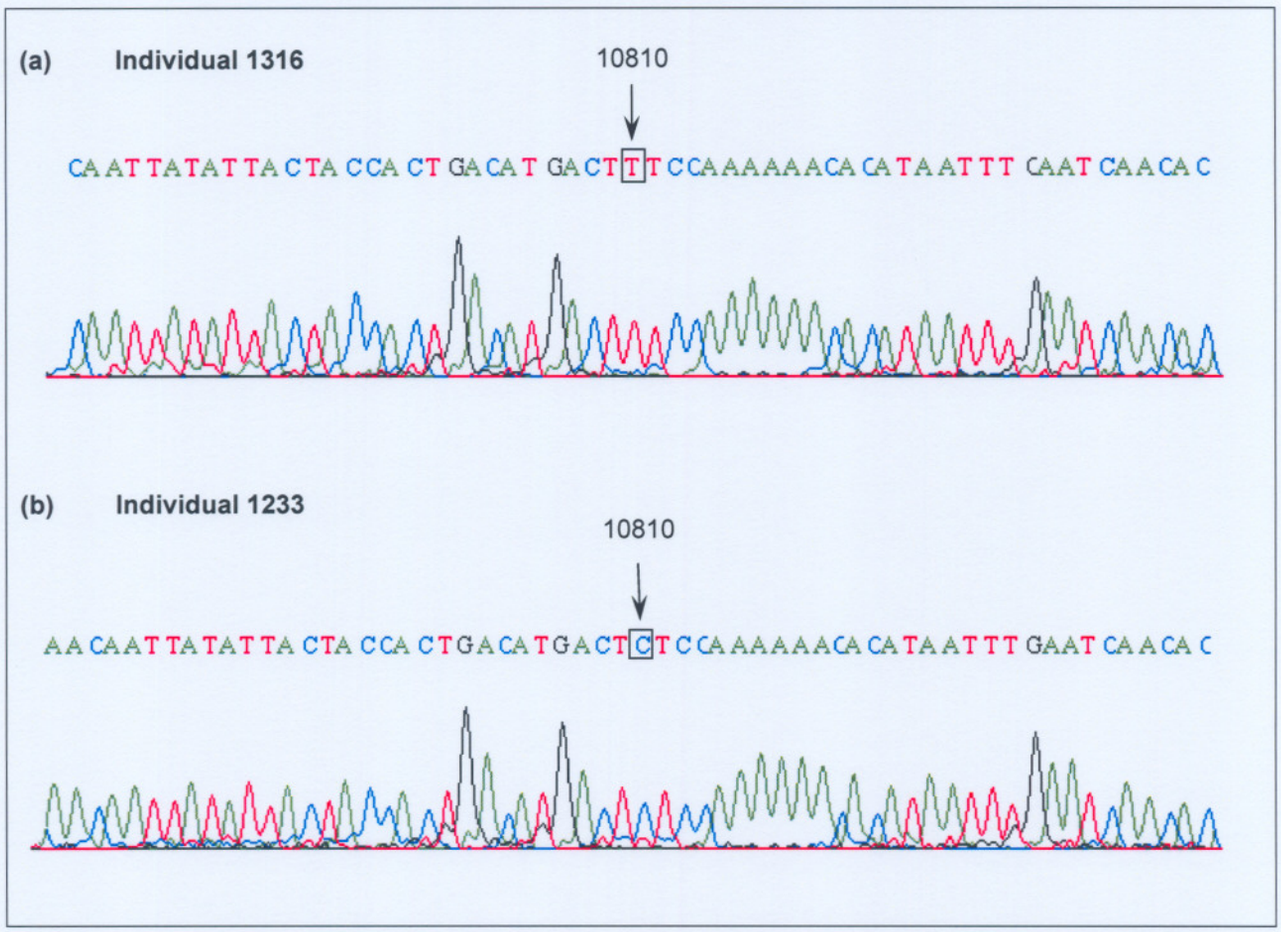
The arrows indicate the SNP used for phylogenetic analysis. The nucleotide numbering of the sequence is according to MITOMAP (2004).

Patients 584, 659, 693, 733, 795, 1233, 1301, 1316 and 1336 had a T nucleotide at this position and were assigned to haplogroup L0, L1 or L2. In contrast, patients 387, 388, 389, 401, 410, 412, 426, 441, 451, 460, 475, 655, 676, 742, 764, 800, 878 and 1289 all harboured a C nucleotide at this position and were assigned to haplogroups L3, M or N. The electropherogram in Figure 4.3(a) had multiple overlapping peaks. This could be due to too much DNA used in the sequencing reaction or the quality of precipitation of the sequenced products being poor. This, however, did not interfere with the final result, as the sequence could still be used in the current form.

4.2.2 Single nucleotide polymorphism at position 10810

All the patients that had a T nucleotide at position 3594 had their DNA analysed for polymorphisms at np 10810. An individual with a C nucleotide at this position belongs to haplogroup L0 or L1, while one with a T nucleotide at the same position belongs to haplogroup L2 (see Table 4.3). Figure 4.4 is a representation of electropherograms of two individuals, one with a T and the other with a C nucleotide at position 10810.

Figure 4.4: Representative electropherogram of the mtDNA sequence encompassing SNP 10810



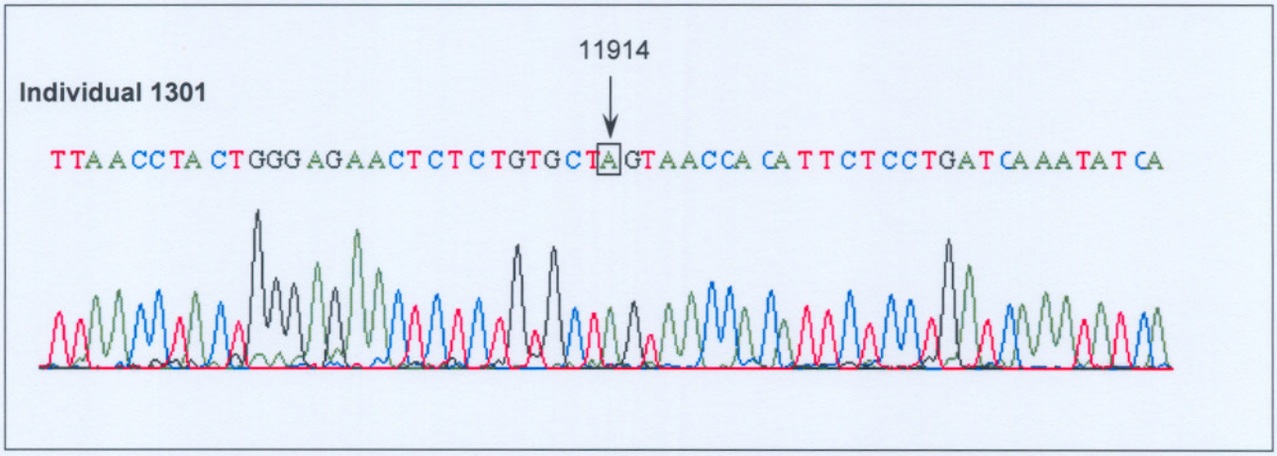
The arrows indicate the SNPs used for phylogenetic analysis. Nucleotide numbering of the sequence is according to MITOMAP (2004).

Patients 584, 659, 693, 733, 795 and 1233 had a C nucleotide at position 10810 and hence belong to haplogroup L0 or L1. However patients 1301, 1316 and 1336 harbour a T nucleotide at the same position and therefore belong to haplogroup L2.

4.2.3 Single nucleotide polymorphism at position 11914

Polymorphisms at position 11914 can be used to delineate haplogroup L2 to L2a and L2b, L2c or L2d. Individuals with a G11914A transition were assigned to haplogroup L2a, while those with a G allele at np 11914 were designated to belong to the L2b, L2c or L2d haplogroups (see Table 4.3). All the individuals analysed in L2 harboured an A nucleotide at this position (i.e. 1301, 1316 and 1336) and were assigned to haplogroup L2a. Figure 4.5 is a representative electropherogram of an individual with an A nucleotide at this particular nucleotide position.

Figure 4.5: Representative electropherogram of the mtDNA sequence encompassing SNP 11914

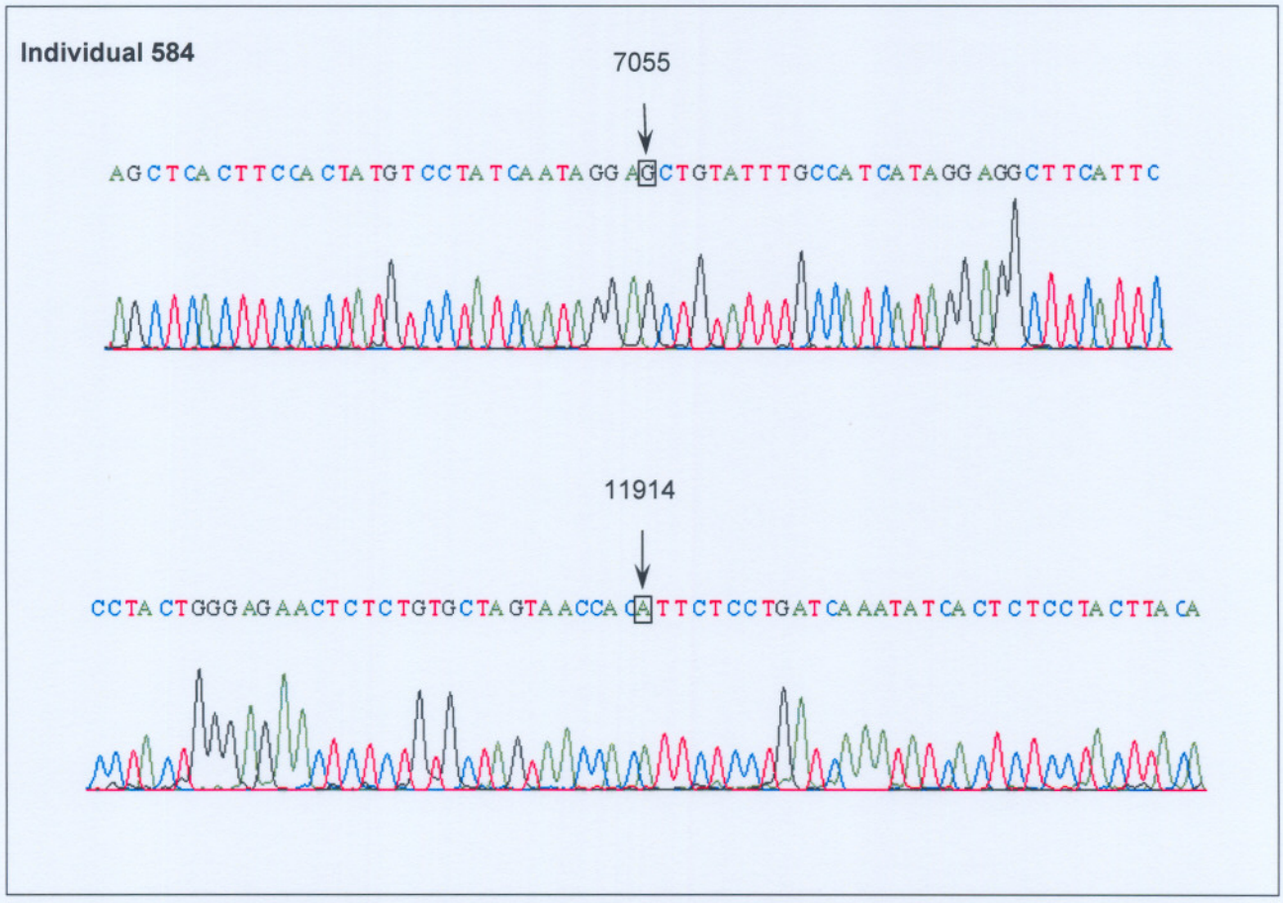


The arrow indicates the SNP used for phylogenetic analysis. Nucleotide numbering of the sequence is according to MITOMAP (2004).

4.2.4 Single nucleotide polymorphism at position 7055 and 11914

An individual harbouring both the A allele at np 7055 and the G11914A polymorphism is designated as haplogroup L0. Individuals with only the A7055G nucleotide change are of haplogroup L1 (see Table 4.3). All the individuals analysed for these two SNPs belonged to haplogroup L0 and included patients 584, 659, 693, 733, 795 and 1233. Figure 4.6 is a representative electropherogram of an individual with the A allele at np 7055 and the G11914A mtDNA polymorphism.

Figure 4.6: Representative electropherograms of the mtDNA sequence encompassing SNPs at nucleotide positions 7055 and 11914

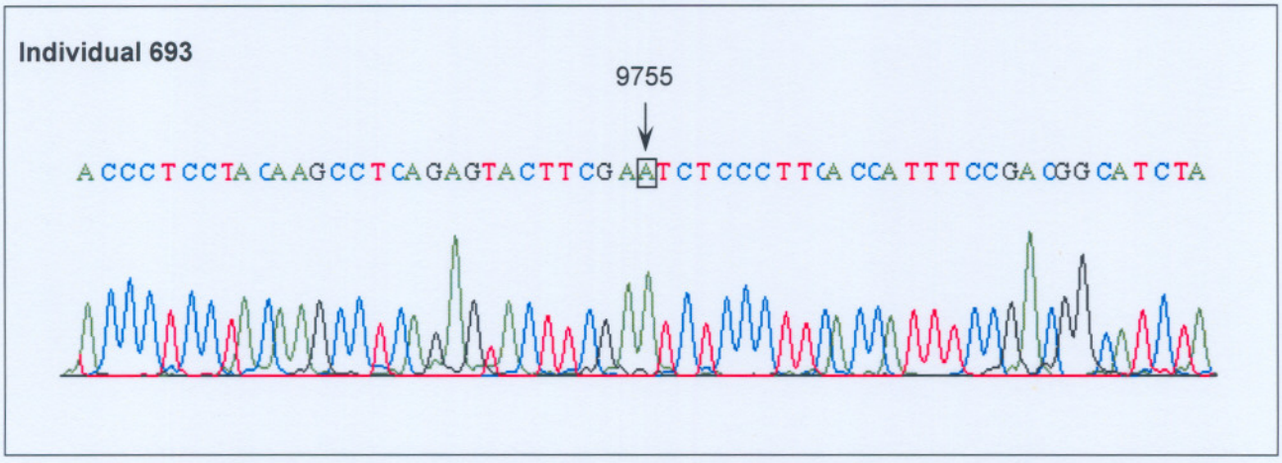


The arrows indicate the SNPs used for phylogenetic analysis. Nucleotide numbering of the sequence is according to MITOMAP (2004).

4.2.5 Single nucleotide polymorphism at position 9755

All the individuals in the L0 haplogroup, as discussed in Section 4.2.4, had a G9755A transition at this position and hence belonged to either the L0b or L0c haplogroups (see Section 4.2.4 above). An individual with a G nucleotide at this position would have been assigned to haplogroup L0a. Figure 4.7 is a representative electropherogram of an individual with the G9755A mtDNA polymorphism.

Figure 4.7: Representative electropherogram of the mtDNA sequence encompassing SNP at nucleotide position 9755

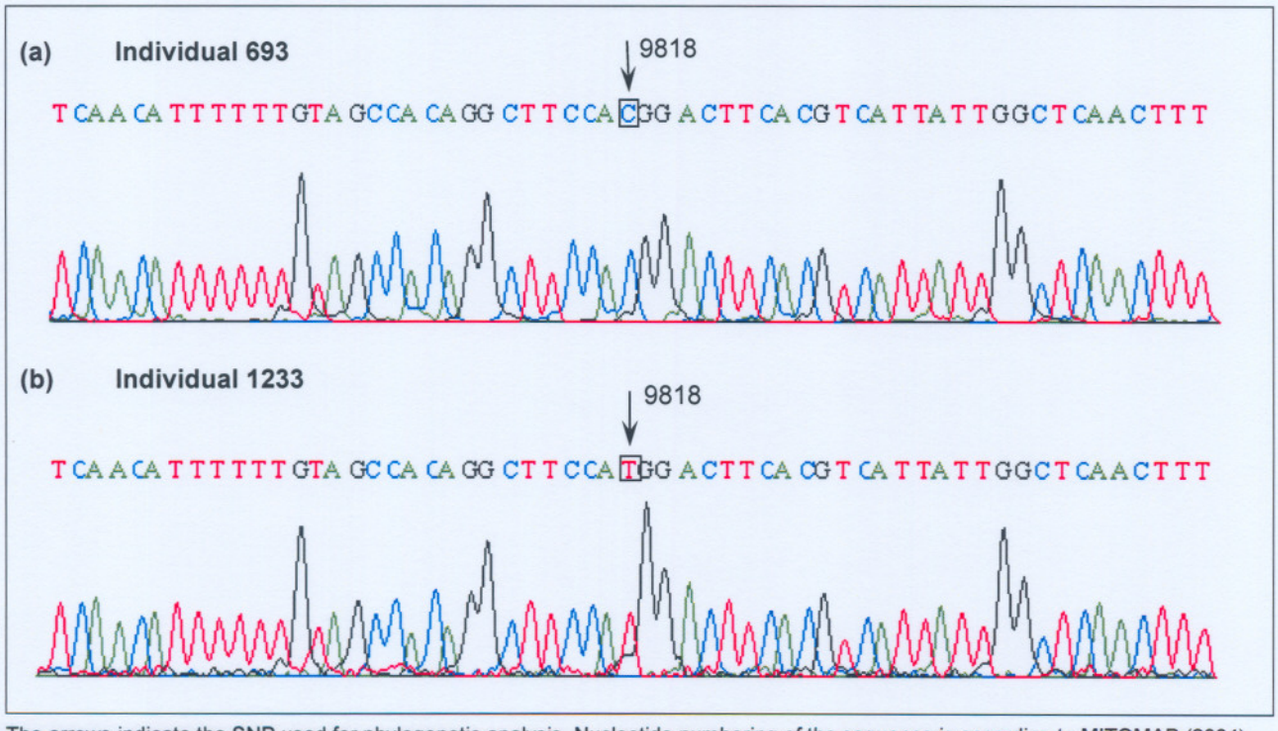


The arrow indicates the SNP used for phylogenetic analysis. Nucleotide numbering of the sequence is according to MITOMAP (2004).

4.2.6 Single nucleotide polymorphism at position 9818

An individual with allele C at np 9818 belongs to haplogroup L0b and one with C9818T polymorphism to haplogroup L0c (see Figure 3.1). Individuals 584, 659, 693, and 795 belonged to haplogroup L0b. In contrast, patients 733 and 1233 belonged to haplogroup L0c. Representative electropherograms of mtDNA polymorphisms at np 9818 are presented in Figure 4.8.

Figure 4.8: Representative electropherograms of the mtDNA sequence encompassing SNPs at nucleotide position 9818

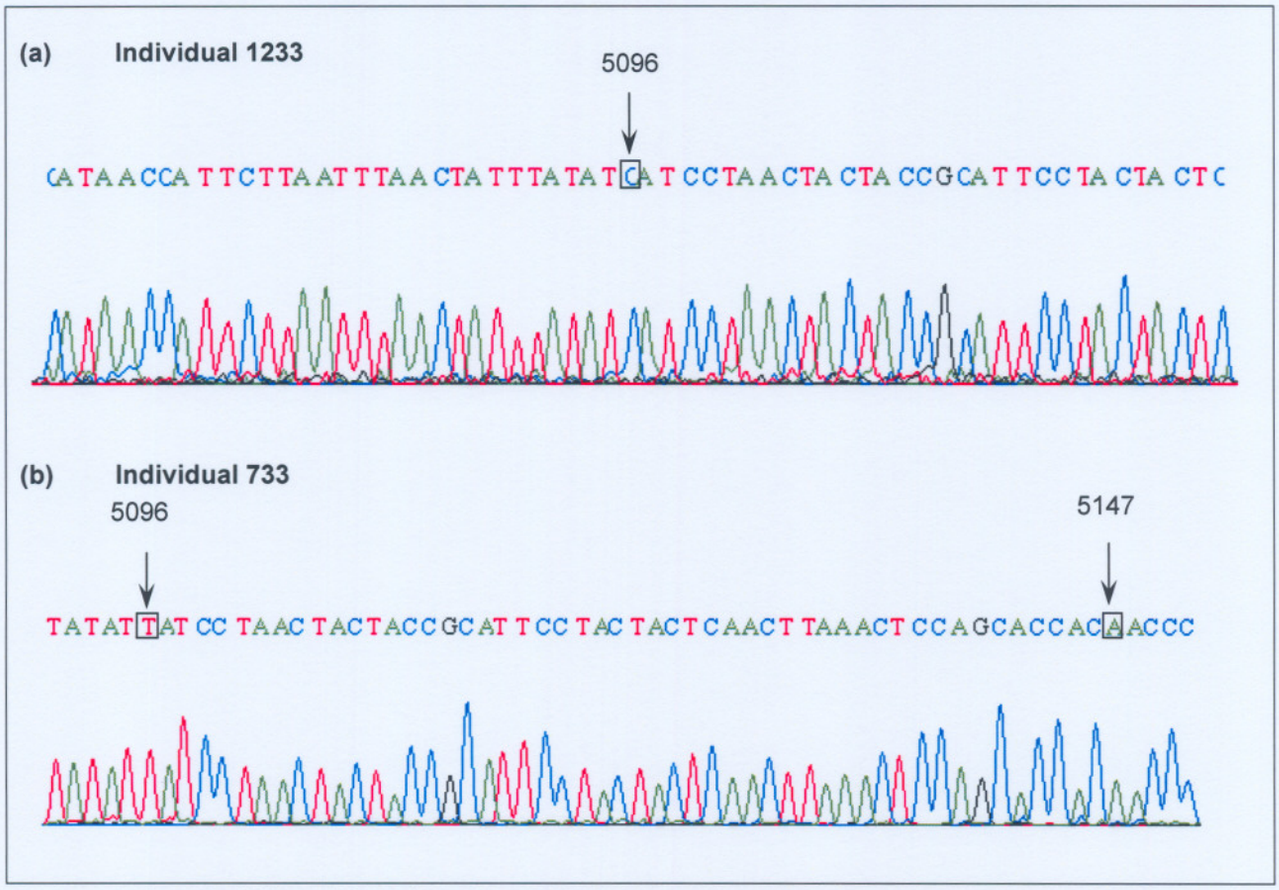


The arrows indicate the SNP used for phylogenetic analysis. Nucleotide numbering of the sequence is according to MITOMAP (2004).

4.2.7 Single nucleotide polymorphism at positions 5096 and 5147

The presence of a T5096C polymorphism designates an individual as belonging to the L0c1 haplogroup, whereas the presence of a T allele at nucleotide position 5096 together with a G5147A polymorphism designates the L0c2 haplogroup (see Table 4.3). Patient 733 in the L0c profile was eventually classified into the L0c2 haplogroup, while patient 1233 belonged to L0c1. The electropherograms representing the polymorphisms at these nucleotide positions, 5096 and 5147, are presented in Figure 4.9.

Figure 4.9: Representative electropherograms of the mtDNA sequence encompassing SNPs at nucleotide positions 5096 and 5147



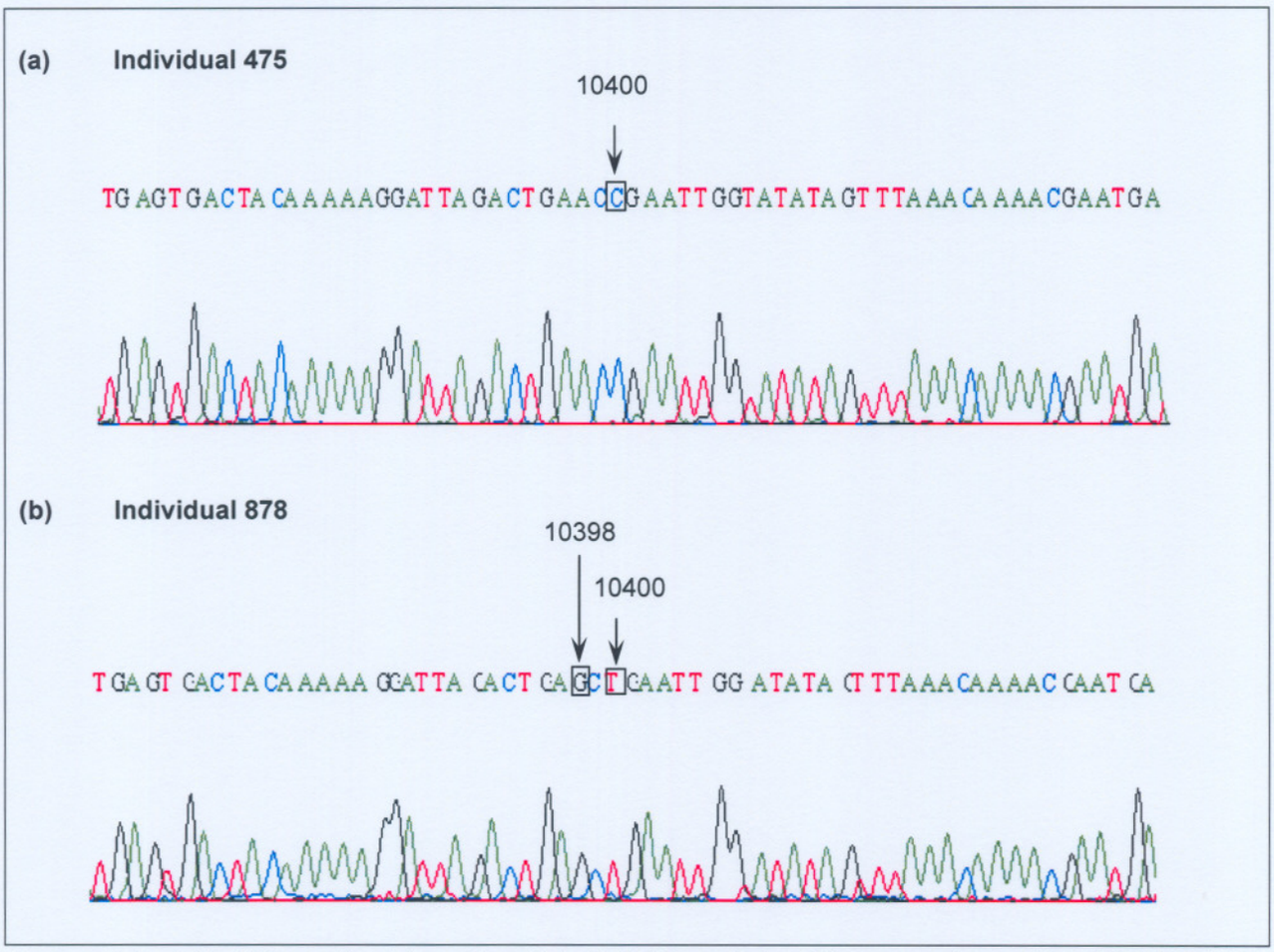
The arrows indicate the SNPs used for phylogenetic analysis. Nucleotide numbering of the sequence is according to MITOMAP (2004).

4.2.8 Single nucleotide polymorphism at position 10400

Polymorphisms at np 10400 are used to differentiate haplogroup L3 from M and N. An individual belongs to haplogroup L3 if he or she has a C nucleotide at position 10400. An individual with a T nucleotide at position 10400 belongs to haplogroup M or N (see Table 4.3).

Patients 387, 388, 389, 401, 410, 412, 426, 441, 451, 460, 475, 655, 676, 742, 764, 800 and 1289 had a C nucleotide at position 10400 and where assigned to haplogroup L3. Only patient 878 had a T nucleotide at this position and hence belonged to haplogroup M or N. Figure 4.10 represents two electropherograms of this SNP, where both the C and T alleles of the SNP are indicated. Figure 4.10 also represents another polymorphism, A10398G, in patient 878, reported as common in African macrohaplogroup L (Wallace *et al.*, 1999; Herrnstadt *et al.*, 2002).

Figure 4.10: Representative electropherogram of the mtDNA sequence encompassing SNP 10400



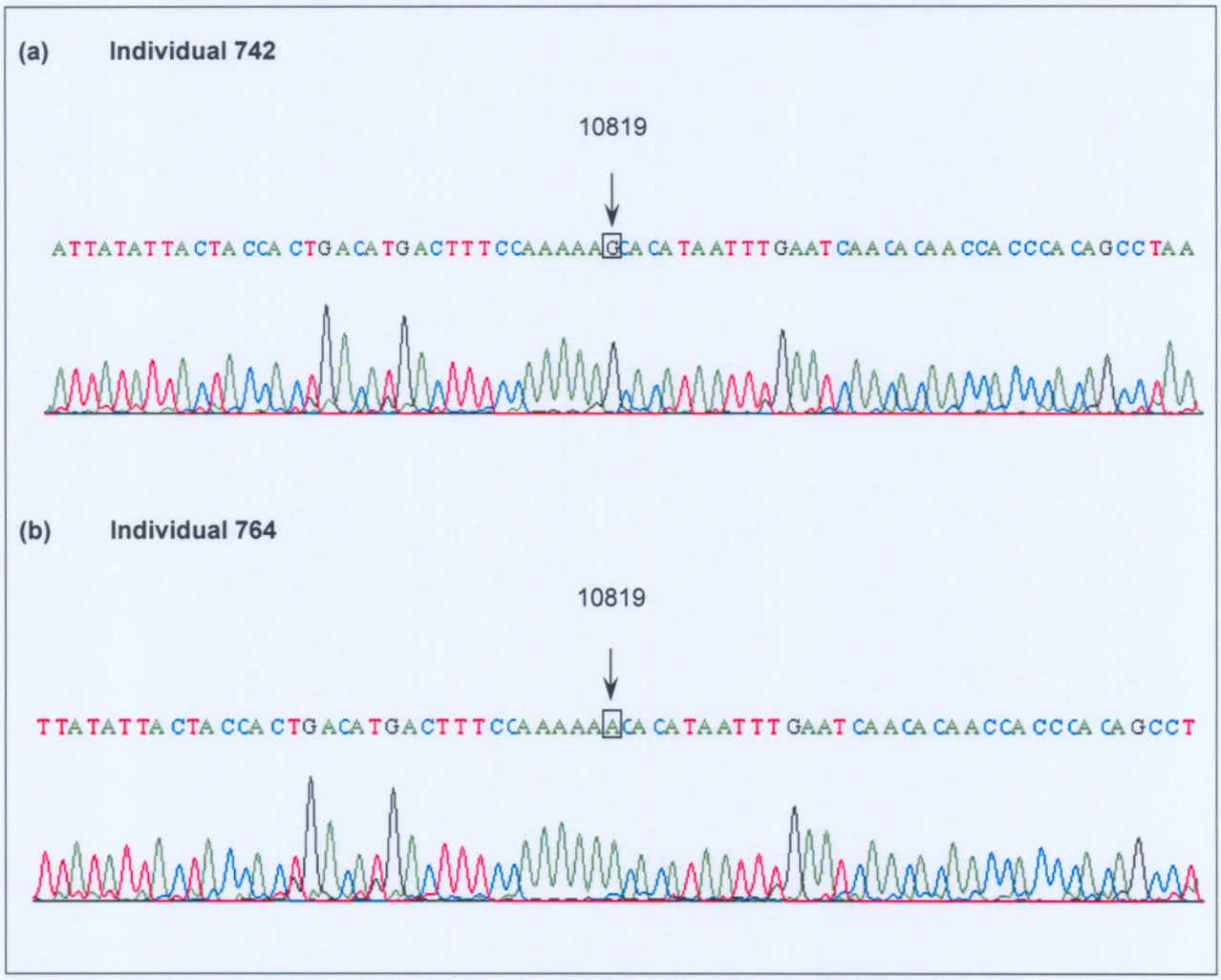
The arrows indicate the SNPs used for phylogenetic analysis. Nucleotide numbering of the sequence is according to MITOMAP (2004). Individual 878 also has an A10398G polymorphism.

4.2.9 Single nucleotide polymorphism at position 10819

Polymorphisms at np 10819 are used to distinguish individuals in haplogroup L3a from those in haplogroup L3b and L3c (see Table 4.3). Possession of an A nucleotide at this position places individuals in either the L3b or L3c haplogroup, whereas a G nucleotide at this position tags the individual to haplogroup L3a (see Figure 3.1). Figure 4.11 is a

representative electropherogram of two individuals, one with an A and the other with a G nucleotide at position 10819.

Figure 4.11: Representative electropherogram of the mtDNA sequence encompassing SNP 10819



The arrows indicate the SNPs used for phylogenetic analysis. Nucleotide numbering of the sequence is according to MITOMAP (2004).

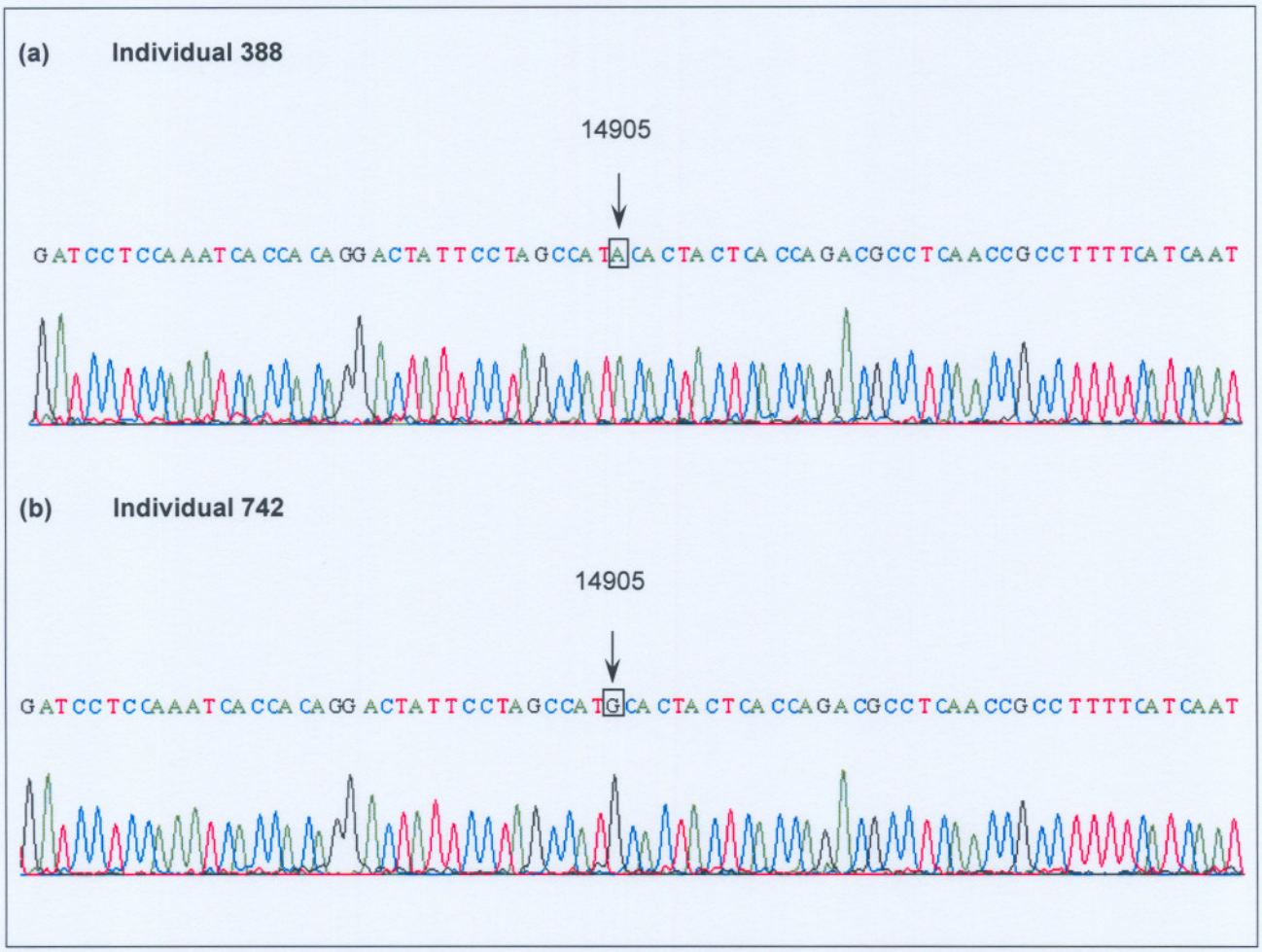
Patients 387, 389, 401, 410, 412, 426, 441, 451, 460, 475, 655, 764 and 1289 had an A allele at np 10819 and are hence designated as belonging to haplogroup L3b or L3c. Patients 388, 676 and 742 harboured an A10819G polymorphism and belonged to haplogroup L3a.

4.2.10 Single nucleotide polymorphism at position 14905

An individual with an A nucleotide at position 14905 belongs to haplogroup L3a1, whereas a G nucleotide would classify the individual as belonging to haplogroup L3a2 or L3a3 (see Table 4.3). Patients 388 and 676 harboured an A nucleotide at position 14905 with respect to the RCRS and hence are of haplogroup L3a1 origin. In contrast, patient 742 had a G

nucleotide at this position and therefore belongs to haplogroup L3a2 or L3a3. Two electropherograms are presented in Figure 4.12, with both the A and G alleles of the SNP at position 14905 indicated.

Figure 4.12: Representative electropherogram of the mtDNA sequence encompassing SNP 14905

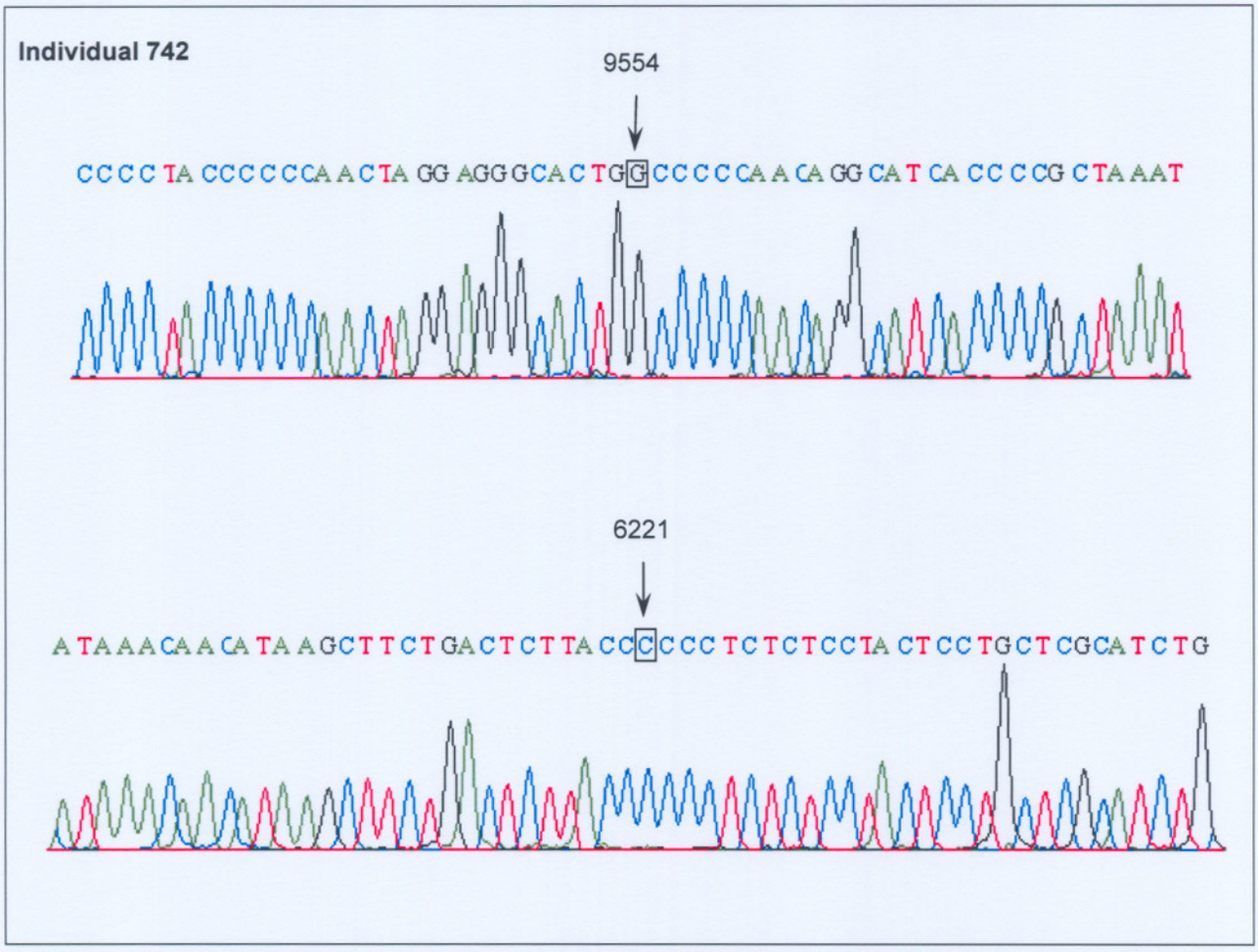


The arrows indicate the SNPs used for phylogenetic analysis. Nucleotide numbering of the sequence is according to MITOMAP (2004).

4.2.11 Single nucleotide polymorphism at position 9554 and 6221

The G9554A polymorphism designates haplogroup L3a2 whereas the L3a3 haplogroup is characterised by the G allele at position 9554 together with the T6221C polymorphism (see Table 4.3). Individual 742 harboured a G nucleotide at position 9554 and a C nucleotide at 6221 and was designated as belonging to haplogroup L3a3. Figure 4.13 represents electropherograms of this individual at the two nucleotide positions in question.

Figure 4.13: Representative electropherogram of the mtDNA sequence encompassing SNPs 9554 and 6221

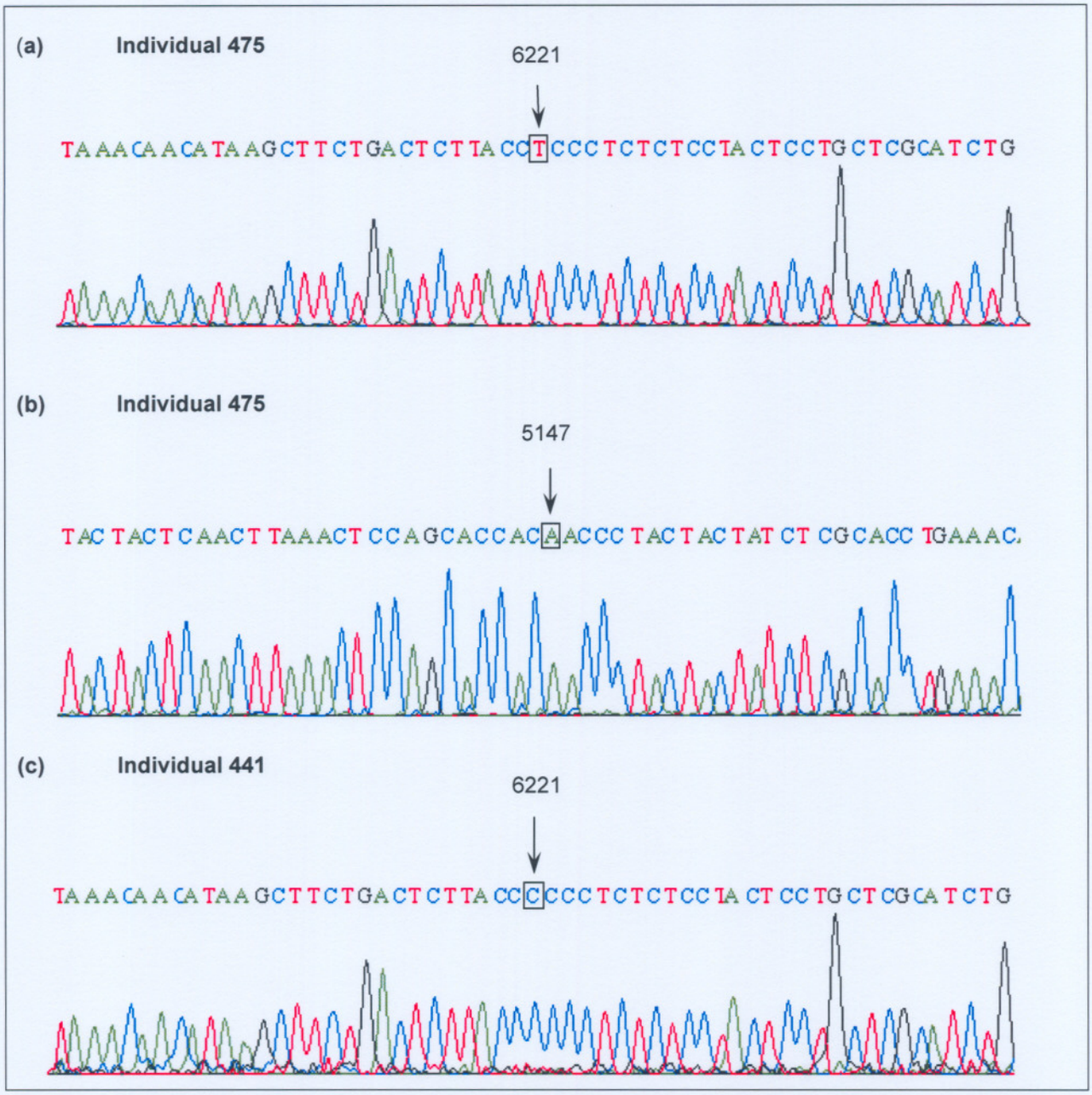


The arrows indicate the SNPs used for phylogenetic analysis. Nucleotide numbering of the sequence is according to MITOMAP (2004).

4.2.12 Single nucleotide polymorphism at positions 6221 and 5147

An individual with a T6221C polymorphism is classified as belonging to haplogroup L3c, whereas individuals with a T allele at np 6221 and G5147A polymorphism belong to L3b (Wallace, 2004). In this profile, patients 475, 800 and 1289 harboured the alleles for haplogroup L3b, as indicated in Figure 4.14 (a and b), while patients 387, 389, 401, 410, 412, 426, 451, 460, 655 and 764 harboured the T allele at np 6221 but had G instead of an A allele at np 5147 (see Figure 3.1). This information suggests that haplogroup L3b may need to be subdivided into L3b1 and L3b2, for which further investigations will be required. Patient 441 had the T6221C polymorphism of haplogroup L3c as illustrated in Figure 4.14 c.

Figure 4.14: Representative electropherograms of the mtDNA sequence encompassing SNPs 6221 and 5147

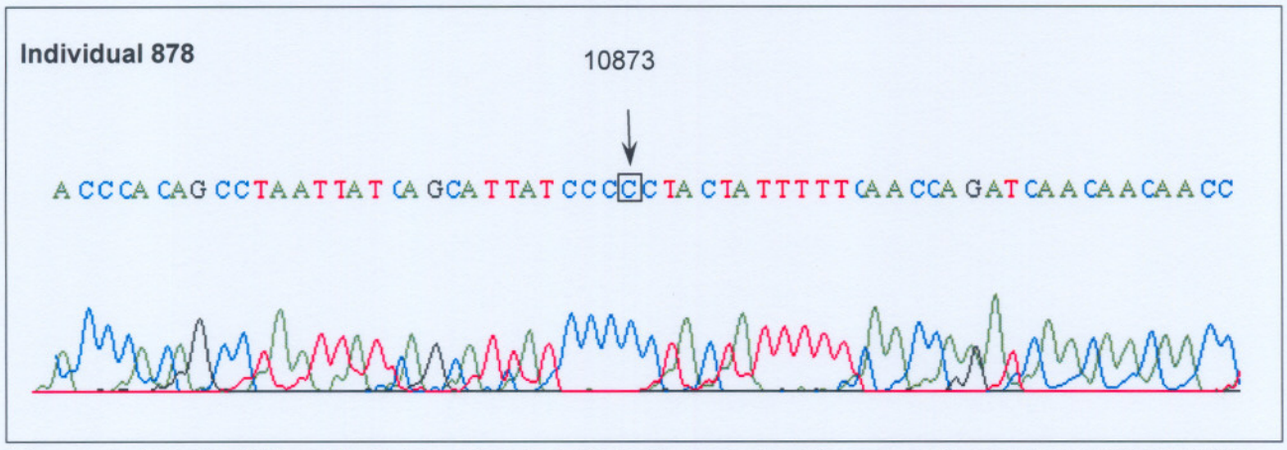


The arrows indicate the SNPs used for phylogenetic analysis. Nucleotide numbering of the sequence is according to MITOMAP (2004).

4.2.13 Single nucleotide polymorphism at position 10873

This SNP is used to differentiate individuals in haplogroup M from those in N. An individual with a C nucleotide at this position belongs to haplogroup M whereas individuals designated as belonging to haplogroup N have a T nucleotide at this position (see Table 4.3). Only patient 878 met all the requirements to be assigned to the M or N haplogroups. This patient had a C nucleotide at position 10873, and is hence of haplogroup M origin. Figure 4.15 is a representative electropherogram of the mtDNA sequence of SNP at np 10873 for this patient.

Figure 4.15: Representative electropherogram of the mtDNA sequence encompassing SNP 10873



The arrow indicates the SNP used for phylogenetic analysis. Nucleotide numbering of the sequence is according to MITOMAP (2004).

4.2.14 Summary of patient haplogroup analysis

The individuals' haplogroups as delineated in Sections 4.2.1 to 4.2.13 are presented in Table 4.1. The ethnicity of the individuals involved is also indicated. The haplogroups that were eventually assigned are not as initially expected. For instance, patients 426 and 659, who are of South African Indian ancestry, have been designated to haplogroups L3b and L0b respectively. However, they where expected to belong to haplogroup M or N, which are common in Asia (Maca-Meyer *et al.*, 2001).

Table 4.1: Haplogroups of South African paediatric patients clinically diagnosed with suspected mitochondrial disorders

Patient #	Ethnicity	Haplogroup	Patient #	Ethnicity	Haplogroup
387	C	L3b	676	A	L3a1
388	C	L3a1	693	A	L0b
389	C	L3b	733	A	L0c2
401	C	L3b	742	A	L3a2
410	C	L3b	764	C	L3b
412	C	L3b	795	Co	L0b
426	I	L3b	800	A	L3b
441	C	L3c	878	C	M
451	C	L3b	1233	A	L0c1
460	C	L3b	1289	C	L3b
475	C	L3b	1301	A	L2a
584	C	L0b	1316	A	L2a
655	C	L3b	1336	A	L2a
659	I	L0b			

= number, A = Black South African, C = Caucasian South African, Co = Coloured South African, I = Indian South African.

A summary of the patients' haplogroups is presented in Table 4.2. The most common haplogroup was L3b (48%), with no patients belonging to L1. In a study conducted on a different group of control individuals for specific facioscapulohumeral muscular dystrophy rearrangements (Van der Merwe, 2003) but within the same region in South Africa, no L1 individuals were detected. Of the 41 individuals Van der Merwe (2003) analysed, 61% belonged to haplogroup L0, 0% to L1, 27% L2 and 12% to L3.

Table 4.2: Prevalence of haplogroups in the mitochondrial patient population

R	Haplogroup	# of patients	% of patients	R	Haplogroup	# of patients	% of patients
a	**M**	**1**	**4**	**k**	**L2**	**3**	**11**
b	**N**	**0**	**0**	l	L2a	3	11
c	**L0**	**6**	**22**	m	L2b	0	0
d	L0a	0	0	n	L2c	0	0
e	L0b	4	15	o	L2d	0	0
f	L0c	2	7	**p**	**L3**	**17**	**63**
g	**L1**	**0**	**0**	q	L3a	3	11
h	L1a	0	0	r	L3b	13	48
i	L1b	0	0	s	L3c	1	4
j	L1c	0	0	**a-s**	**Total**	**27**	**100**

R = letter of row, # = number; Rows c, g, k and p reflect totals of their respective subhaplogroups.

4.3 ANALYSIS OF PATIENT DNA SEQUENCES FOR MUTATIONS

The variations in sequence of patient DNA from that of the RCRS are indicated in Table 4.3. Most of the variations were reported polymorphisms as discussed in Section 4.3.1. Thirty-seven different polymorphisms were detected, of which seven were unreported (see Section 4.3.2) according to a search of MITOMAP (2004) mtDNA polymorphism database and other literature cited. Twenty three of the polymorphisms were synonymous substitutions in various coding regions of the mtDNA investigated. Noteworthy is the previously unreported deletion at np 4820 in the ND2 gene. All the individuals investigated at the nucleotide position harboured this deletion and therefore warrant further screening in a bigger patient population with control individuals.

Table 4.3: mtDNA polymorphisms in South African paediatric patients with suspected mitochondrial disorders

Alteration	Individuals	AA change	Gene	Reference
T3197C	389, 412	~	16S rRNA	Sternberg *et al.*, 1998
T3398C	389	Met-Thr	ND1	Sternberg *et al.*, 1998
G3438A	451, 584, 795	Syn	ND1	Thomas *et al.*, 1996
C3450T	441	Syn	ND1	Pulkes *et al.*, 2003
A3480G	460	Syn	ND1	Howell *et al.*, 1993
C3516A	584, 659, 693, 733, 795, 1233	Syn	ND1	Herrnstadt *et al.*, 2002; Van Brummelen, 2003
C3594T	584, 659, 693, 733, 795, 1233, 1301, 1316, 1336	Syn	ND1	Chen *et al.*, 1995; Bandelt *et al.*, 2001
G4820DEL	387, 389, 410, 412, 426, 460, 584, 655, 659, 800, 1233		ND2	Not yet reported
T5096C	1233	Leu-Ser	ND2	Herrnstadt *et al.*, 2002; Wallace, 2004
G5147A	475, 733, 800, 1289	Syn	ND2	Kobayashi *et al.*, 1991; Noer *et al.*, 1991
T6221C	441, 742	Syn	COI	Macaulay *et al.*, 1999
A7154G	659	Ile-Val	COI	Not yet reported
T7389C	584	Syn	COI	Not yet reported
T9540C	584, 659, 693, 733, 742, 795, 1233	Syn	COIII	Lertrit *et al.*, 1994; Nishino *et al.*, 1996
A9545G	733	Syn	COIII	Herrnstadt *et al.*, 2002; Silva *et al.*, 2002
G9554A	733	Syn	COIII	Herrnstadt *et al.*, 2002; Wallace, 2004
G9755A	584, 659, 693, 733, 795, 1233	Syn	COIII	Pulkes *et al.*, 2003
C9818T	733, 1233	Thr-Met	COIII	Wallace, 2004
A10086G	441, 451	Syn	ND3	Chen *et al.*, 2000
C10142T	426	Thr-Met	ND3	Not yet reported
A10398G	878	Thr-Ala	ND3	Obayashi *et al.*, 1992
C10400T	878	Syn	ND3	Sternberg *et al.*, 1998; Moraes *et al.*, 1993
T10410C	412	~	$tRNA^{Arg}$	Hayashi *et al.*, 1994; Sternberg *et al.*, 2001
T10643G	1289, 1316	Leu-Trp	ND4L	Not yet reported
T10645G	389	Cys-Gly	ND4L	Not yet reported
G10688A	659, 733, 1233	Syn	ND4L	Herrnstadt *et al.*, 2002
A10810C	584, 693, 733, 795, 878, 1233	Phe-Leu	ND4	Torroni *et al.*, 1993
A10819G	388, 676, 742	Syn	ND4	Wallace *et al.*, 1988a; Marzuki *et al.*, 1991
C10864G	451, 584, 655, 795	His-Asp	ND4	Not yet reported
T10873C	388, 584, 659, 693, 733, 742, 795, 800, 878, 1233, 1301, 1316, 1336	Syn	ND4	Ozawa *et al.*, 1995; Sudoyo *et al.*, 2002
T10915C	659, 733, 800, 1233	Syn	ND4	Yoneda *et al.*, 1990
A11641G	1233	Ser-Gly	ND4	Herrnstadt *et al.*, 2002
G11719A	584, 659, 693, 795, 1301, 1316, 1336	Syn	ND4	Johns, 1991; Brown *et al.*, 2002
G11914A	584, 659, 693, 795, 1233, 1301, 1316, 1336	Syn	ND4	Herrnstadt *et al.*, 2002
G12007A	584, 659, 693, 795, 1233	Syn	ND4	Herrnstadt *et al.*, 2002
G14905A	388, 676	Syn	Cyt b	Finnilä *et al.*, 2001; Herrnstadt *et al.*, 2002
C14766T	388, 676	Syn	Cyt b	Herrnstadt *et al.*, 2002

AA = amino acid, COI = cytochrome oxidase unit 1, COIII = cytochrome oxidase unit 3, Cyt b = cytochrome b, ND 1 to 4L = NADH dehydrogenase subunits 1 to 4L, RNA = ribonucleic acid, $tRNA^{Arg}$ = gene encoding transfer RNA for arginine, 16S rRNA = gene encoding 16S ribosomal RNA, Syn = synonymous. ~ = mutation not found in protein coding region.

4.3.1 Reported polymorphisms

The T3197C polymorphism in the mitochondrial 16S rRNA gene was reported by Sternberg *et al.* (1998) in patients lacking the A3243G and A8344G mutations and was a frequent Caucasian polymorphism in control patients. The polymorphism was suggested to be of phylogenetic importance (Sternberg *et al.* 1998; Herrnstadt *et al.*, 2002). The T3398C polymorphism in the ND1 region is a homoplasmic missense variation (Sternberg *et al.*, 1998), which is associated with the heteroplasmic G4450A mutation in $tRNA^{Met}$ and was observed in only one patient in this study.

The T10410C polymorphism is a homoplasmic variation in the $tRNA^{Arg}$ gene (Hayashi *et al.*, 1994) with a frequency of 1% in the population (Sternberg *et al.*, 1998). The polymorphism was found in only one patient in the current study. The A3480G mutation in the ND1 gene (Howell *et al.*, 1993) and the G11719A in the ND4 gene (Johns *et al.*, 1991; Brown *et al.*, 2002) were found to be in association with the G15257A polymorphism associated with LHON.

The polymorphisms at position 3438 (Thomas *et al.*, 1996) and 3450 (Pulkes *et al.*, 2003) were described as being haplogroup-associated in African populations. The polymorphisms at position 3594 (Chen *et al.*, 1995; Bandelt *et al.*, 2001) and 10086 (Chen *et al.*, 2000) are commonly used polymorphisms for phylogenetic analysis of African macrohaplogroup L. The alleles at np 5147 (Kobayashi *et al.*, 1991; Noer *et al.*, 1991), 5096 (Herrnstadt *et al.*, 2002; Wallace, 2004), 6221 (Macaulay *et al.*, 1999), 9554 (Wallace, 2004), 9755 (Pulkes *et al.*, 2003), 9818 (Herrnstadt *et al.*, 2002; Wallace, 2004), A10398G (Obayashi *et al.*, 1992), 10400 (Ozawa *et al.*, 1991; Moraes *et al.*, 1993; Sternberg *et al.*, 1998), G10688A (Herrnstadt *et al.*, 2002), 10810 (Torroni *et al.* 1993), 10819 (Wallace *et al.*, 1988a; Marzuki *et al.*, 1991), 10873 (Ozawa *et al.*, 1991; Ozawa *et al.*, 1995, Sudoyo *et al.*, 2002), 10915 (Yoneda *et al.*, 1990), 11914 (Herrnstadt *et al.*, 2002) and 14905 (Finnilä *et al.*, 2001; Herrnstadt *et al.*, 2002) are also phylogenetically informative polymorphisms that segregate in human mtDNA.

The A9545G polymorphism in the COIII gene was used in addition to the A13263G to characterise Native American haplogroup C individuals (Herrnstadt *et al.*, 2002; Silva *et al.*, 2002). The C14766T polymorphism was defined as being common to all African haplotypes as well as to all but one Asian haplotype (Herrnstadt *et al.*, 2002) and was also found in a similar group of patients to those used in this study by Van Brummelen (2003).

The results obtained from this study restrict the polymorphism to only the L3a1 sub-lineage whereas individuals investigated by Van Brummelen (2003) also had this SNP but belonged to L0 and L2 lineages. It still remains to be investigated whether this polymorphism is haplogroup-specific or haplogroup-associated in a study with a much larger sample size. The C14766T polymorphism in the cyt b gene of the cytochrome reductase complex of the RC was synonymous and was therefore likely to have no phenotypic or functional effect on the enzyme complex.

The C3516A synonymous polymorphism in the ND1 gene was observed in six of the 27 patients investigated in this region. This polymorphism was observed in only one haplogroup L0, however all the L0 haplogroup patients this polymorphism. The polymorphism was also reported in patient 504 and 525 by Van Brummelen (2003) on the same cohort but different patients, whose data designate the patients to haplogroup L0b. The polymorphism may therefore be haplogroup-specific (Herrnstadt *et al.*, 2002). This polymorphism was absent in all L0 normal individuals (four) in a study conducted on 10 individuals (data not shown). In addition, the six non-L0 haplogroup individuals also did not have this polymorphism. The significance of this SNP in rendering haplogroup L0 individuals susceptible to mitochondrial disorders remains to be proven. The G12007A polymorphism occurred in all except one individual in the L0 sub-cluster and was also described by Van Brummelen (2003) in patients 504 and 525, who belonged to the same haplogroup. However, Herrnstadt *et al.* (2002) described the polymorphism as being associated with more than one haplogroup.

4.3.2 Unreported polymorphisms

Of the seven unreported alterations, the deletion at np 4820 occurred in the ND2 gene in eleven patients. Five of the seven unreported alterations (see Table 4.3) occurred in the first enzyme complex of the RC. These entire complex I polymorphisms were non-synonymous. The C10142T in ND3 replaces threonine, a hydrophilic amino acid, with methionine, which is also hydrophilic and hence may have no effect on electron transfer in this complex. The T10643G and the C10864G polymorphisms replace an amino acid with a more hydrophilic one in the ND4L and ND4 gene loci respectively, whereas the T10645G and the A11641G polymorphisms result in substitution of a hydrophilic with a less hydrophilic amino acid in their respective loci. Replacements involving amino acids of dissimilar properties may be responsible for mitochondrial dysfunction and this, coupled

with the high proportion of unreported alterations, make the first enzyme complex a hot spot for investigation of the aetiology of mitochondrial disorders in these patients.

Two of the seven unreported mutations occurred in the COI gene region. The A7154G polymorphism resulted in the substitution of isoleucine with valine, both hydrophobic amino acids, while the T7389C polymorphism was synonymous.

4.4 CORRELATION BETWEEN HAPLOGROUPS AND CLINICAL PHENOTYPES

The most energetically dependent tissues, such as skeletal and heart muscles, are affected most (Ozawa *et al.*, 1995; Budd and Nicholls, 1998; Holt, 2003) when the function of the mitochondrial genome is compromised. The relationship between the haplogroups and clinical phenotypes of patients included in the study is presented in Table 4.4. Lactic acidosis was a major symptom observed in almost all the patients, except for patients in haplogroup L0b or L0c1 or L0c2. Only one patient out of six (17%) in the L0 haplogroup had lactic acidosis compared to 67% in haplogroup L2a and 54% in haplogroup L3b, suggesting that haplogroup L0 may offer protection against lactic acidosis. In contrast, haplogroups L2a and L3b may increase susceptibility to lactic acidosis. Seizures were present in 33% of L3a, and 31% in L3b haplogroups, but absent in L0c and L2a haplogroups. There was a high prevalence of ophthalmological symptoms (100%) in haplogroup L0c and L2a, as compared to 25%, 33% and 39% in haplogroups L0b, L3a and L3b respectively. There was also a high prevalence of hypotonia in haplogroups L0b (75%) and L0c (100%) but 0% hypertonia in these haplogroups.

Table 4.4: Relationship between haplogroups and clinical phenotypes

Haplogroup	# of patients	% of patients with clinical phenotype										
		Seizures	Ophthalmological symptoms	Hypertonia	Hypotonia	Lactic acidosis	Hypermobile joints	Mucopoly-saccharides	Failure to thrive	Short stature	Oligo-saccharides	Delayed development
L0b	4	25	25	0	75	25	0	25	50	75	50	100
L0c	2	0	100	0	100	0	50	100	100	50	100	50
L2a	3	0	100	0	67	67	33	0	67	33	33	67
L3a	3	33	33	33	67	33	0	67	33	67	33	0
L3b	13	31	39	31	46	54	8	23	23	8	15	72
L3c	1	100	100	0	100	100	0	0	0	0	0	0
M	1	0	100	100	0	100	0	0	0	0	0	100

= number.

Patients in the L0c and L2a haplogroups had no defects affecting their central nervous system as presented in Table 4.5, but all (100%) had eye defects. The skeletal muscle turned out to be the most affected organ with 78% of all the patients having muscular defects. The kidney was the least affected organ with only 4% (1/27) affected patients, namely a patient in haplogroup L0b.

Table 4.5: Relationship between haplogroups and affected organs

Haplogroup	# of patients	% of patients with organ/system affected								
		CNS	Eye	Muscle	Liver	Endocrine	Skeleton	Kidney	CNS/Eye/ Muscle	Muscle/ Endocrine
L0b	4	25	25	75	25	75	25	25	0	50
L0c	2	0	100	100	0	50	50	0	0	50
L2a	3	0	100	67	33	33	33	0	0	33
L3a	3	33	33	67	0	33	0	0	0	33
L3b	13	54	54	69	23	8	15	0	31	8
L3c	1	100	100	100	100	0	0	0	100	0
M	1	100	100	100	0	0	0	0	100	0

= number, CNS = central nervous system.

In the L0 haplogroup, 67% of the individuals had abnormalities in their endocrine systems. This was in contrast to 33% in L2a, 33% in L3a and 8% in the L3b haplogroups. These findings may demonstrate how haplogroups can affect the involvement of different tissues and organs in patients with possible mitochondrial disorders.

CHAPTER FIVE

CONCLUSIONS

In this investigation, 27 DNA samples, 21 extracted from blood and six from muscle, of South African paediatric patients that were clinically diagnosed with mitochondrial disorders were haplogrouped. In patients from the same cohort that were previously screened for MERRF, LS, and MELAS (Prosser, 2001; Van Brummelen, 2003), no reported causative mutations were observed. Haplogroup analyses were performed in order to evaluate this data in the context of disease expression (Torroni, 2000; Wallace *et al.*, 1999). The samples were obtained from individuals who had MDC scores of 6, 7 or 8, and from patients with classical clinical manifestations of mitochondrial disorders.

The investigation presented here forms part of an extended research programme in which the mtDNA of 104 patients has been screened to date. Most were screened for alterations in the mitochondrial $tRNA^{Leu(UUR)}$, $tRNA^{Lys}$ and ATPase 6 genes involving DNA extracted from muscle and blood, and in some instances from both muscle and blood for particular patients (Prosser, 2001; Van Brummelen, 2003). Few muscle samples were used, in view of the highly invasive nature of this sampling method. Whole mtDNA genome sequencing was performed in previous investigations for 10 patients (Prosser, 2001; Van Brummelen, 2003). In this entire group a single patient harboured the A3243G mutation in the $tRNA^{Leu(UUR)}$ gene (Prosser, 2001) associated with MELAS (Goto *et al.*, 1990). To date, no other reported pathogenic mutations have been detected in this cohort of South African patients, including the investigation presented here.

In the patients of whom only the $tRNA^{Leu(UUR)}$, $tRNA^{Lys}$ and ATPase 6 mitochondrial genes were screened, and in the patients screened in this study over a wider region of mtDNA, it is possible that the pathogenic mutations could be located in areas of the mtDNA genome that, as yet, have not been screened. Whole mtDNA genome screening will have to be performed on these patients before reported causative alterations in the mtDNA can be excluded from the aetiology of their disorders.

The fact that no reported mutations were detected could also be due to heteroplasmy and heterogeneity in the distribution of mutated mtDNAs (Larson and Clayton, 1995). It is possible that the expected mutations could be present in tissues other than blood and muscle. Sampling of tissues such as the heart, liver, kidney, brain and eyes is difficult in humans and yet these are some of the tissues in which the mutations are expressed. The ubiquitous nature of the mitochondria and their unique genetic features contribute to the clinical, biochemical and genetic heterogeneity of mitochondrial diseases (Bauer *et al.*, 1999). Further studies including the development of animal models are necessary to advance understanding of the pathogenesis of mitochondrial disorders to enable, in turn, the development of novel therapies and genetic rescue strategies for the treatment of human disease (Larson and Clayton, 1995).

Another possibility for the perceived absence of reported mutations in the mitochondrial patient population could be that mutations causing the observed mitochondrial dysfunction are nuclear-encoded, since some of the proteins in the mitochondrion are encoded by the nDNA. mtDNA is dependent on nuclear-encoded proteins for maintenance and faithful propagation (Spelbrink, 2003). However, the nDNA genome is more complex than the mtDNA genome, and screening for pathogenic mutations in the nuclear genome in this group of mitochondrial patients would not involve only those regions coding for respiratory chain subunits. Many proteins involved in mammalian mtDNA replication have not been fully characterised to date. Because of the complex nature of the nuclear genome, alterations in the mtDNA are generally first excluded prior to screening nDNA for pathogenic mutations, but both types of DNA damage may be relevant for an understanding of the molecular mechanisms underlying certain diseases that affect the mitochondria (Richter *et al.*, 1988).

It is also possible that after screening all the nuclear genes involved in the mitochondria, the pathogenic mutations would still not be detected. The complex network of interaction and communication between the nucleus and mitochondria is not fully understood yet. Disruption of these networks can still result in pathology and may not necessarily be evident from DNA sequencing analysis. The observed phenotype may also be due to a second-order mitochondrial defect such as malnutrition that causes the depletion of components that are essential for mitochondrial function (Gillis and Kaye, 2002). Defects at the level of hormones and neurotransmitters may cause abnormalities in mitochondrial metabolism or its regulation (Scholte, 1988). Extramitochondrial or extracellular acquired or inborn errors of metabolism can also cause depletion of essential components for

mitochondrial functioning, similar to the role played by phosphate in, for example, fructose intolerance (Scholte, 1988).

Most of the reported mutations were originally detected in patients belonging to non-African haplogroups. Of the 37 polymorphisms observed in the current study, 23 were synonymous (62%). African macrohaplogroup L mtDNAs have significantly lower frequencies of non-synonymous versus synonymous alterations compared to temperate and arctic haplogroups (Ruiz-Pesini *et al.*, 2004), due to the reduced impact of purifying and adaptive selection. A novel genetic aetiology may account for the majority of mitochondrial phenotypes observed in the South African population (Olckers *et al.*, 2001). As people migrated out of Africa into Europe and Siberia, some haplogroups were exposed to severe cold, which led to adaptive selection of mtDNA haplotypes with partially uncoupled oxidative phosphorylation. Uncoupling mutations decrease the production of ATP and reactive oxygen species, but increase the chances of energetic failure. Such haplogroups will have an increased susceptibility to disease of energy deficiency, but in turn serve as a protective factor from neurodegenerative diseases and aging (Ruiz-Pesini *et al.*, 2004). It is possible that alterations regarded as polymorphisms in other populations may become pathogenic when expressed against an African, or as in this particular patient cohort, the Southern African genetic background.

Analysis of mtDNA variation may ultimately elucidate a part of the pathophysiology of mtDNA disease (Wallace *et al.*, 1999). As none of the reported pathogenic mutations were detected, the molecular aberration responsible for the observed mitochondrial phenotypes in these patients could not be identified. The results generated in this study support the hypothesis that the aetiology of mitochondrial disorders is unique in this South African population (Olckers *et al.*, 2001; Prosser, 2001; Van Brummelen, 2003). This uniqueness is attributed to haplogroup-specific polymorphisms, which influence the expression of the clinical mitochondrial disorder phenotypes that are observed in these paediatric patients.

The results from phylogenetic analysis (Table 4.2) depicted distinct clustering of the affected patients in specific haplogroups. The majority of the patients belonged to haplogroup L3b (48%) and haplogroup L0 (22%), suggesting susceptibility of these haplogroups (total of 70%) to mitochondrial disease. The proportion of haplogroup L2a (11%) and L3c (4%) suggests that these haplogroups may offer protection to mitochondrial dysfunction. Further studies of a larger population may be warranted to prove this notion.

None of the 27 patients investigated harboured the alleles that characterise the L1 haplogroup. Such a strict association not only supports the hypothesis that a haplogroup itself can contribute to a disease as a predisposing factor or a protective factor, but also demonstrates that mitochondrial disease can result from complex genetic interactions (Brown *et al.*, 2002). However, future studies including a larger patient cohort from a wider geographical region are necessary to ensure that no L1 haplogroup individuals are indeed present in this population.

None of the patients in haplogroups L0c, L2a had seizures, while only one patient in the L0b haplogroup displayed this specific symptom, indicating that the phenotype is restricted to L3a and L3b haplogroups in which the incidence was ca. 33%. The L0c and L2a haplogroups harboured 100% incidence of phenotypes associated with ophthalmologic symptoms, while there was considerable resistance to such malfunction among individuals of L0b, L3a and L3b haplogroups. Haplogroups L3c (one patient) and M (one patient) had a small sample size and no significant conclusions could be drawn from these data. The skeletal muscle was the most affected organ (in 78% of the patients) but none of the patients with hypertonia (in 33% of the non-L0b, L0c or L2a haplogroup patients) belonged to any of the L0b, L0c or L2a sub-clusters. Lactic acidosis, with an incidence of 48% in the patient cohort investigated in this study, and a classical phenotype in mitochondrial disorders, was least in the L0 haplogroup, where only one patient out of six exhibited this symptom. The L3a and L3b haplogroups had the widest spectrum of clinical phenotypes and their susceptibility to mitochondrial disorders should be investigated further in the future. Information from such investigations may be very useful in the search for therapeutic strategies. Furthermore, it may help to define the precise relationship between the observed clinical symptoms and the absence of currently reported mutations in the research population. This information can also facilitate informed counselling and diagnosis of individuals suspected of having mitochondrial disorders (Wolf and Smeitink, 2002). Eventually delineation of the mechanism behind these phenotypes will also be useful from a nutrigenomic and pharmacogenomic point of view to derive optimal diets and drugs for these individuals.

Heteroplasmy, heterogeneity, the greater number of mitochondrial disorders, the high mtDNA mutation rate and the dual genetic control of the mitochondrial enzyme complexes make the search for the aetiology of mitochondrial disorders a formidable challenge and may necessitate many strategies for redress. The absence of the L1 haplogroup among the patients, the appreciable incidence of haplogroup L0 and the high prevalence of the

L3b haplogroup among the patients included in this study should serve as a guide to direct further studies into the susceptibility and protective roles haplogroups bestow upon populations, and the aetiology of mtDNA disorders in Africa.

CHAPTER SIX

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6.1 GENERAL REFERENCES

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