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MtDNA heteroplasmy in longevity: a puzzling story

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Strana è la nostra condizione qui sulla terra.

Ciascuno di noi viene per una breve visita,

senza sapere perchè,

eppure a volte presagendo uno scopo.

Dal punto di vista della vita quotidiana,

però, una cosa sappiamo per certo:

l' uomo è qui per il bene di altri uomini,

soprattutto di quelli dal cui sorriso e

dal cui benessere dipende la sua felicità.

Albert Einstein

Data included in this thesis were obtained as a part of the EU FP6 Integrated Project on Genetics of Healthy Ageing (GEHA). Permission to use these data in this thesis has been granted by the GEHA Consortium. It should be noted that future publications by the GEHA Consortium may include these results possibly with additional data and/or analyses. Should this occur, the results presented in the publications by the GEHA Consortium and not this thesis shall be regarded as definitive.

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Sommario

Studi sull' insorgenza e l' accumulo di mutazioni somatiche a carico della regione di controllo del DNA mitocondriale hanno rivelato la presenza di mutazioni tessuto-specifiche in siti importanti per la replicazione e la trascrizione del DNA mitocondriale stesso (Michikawa et al., 1999; Wang et al., 2001). In particolare, una di esse, la transizione C150T, rimodellando una delle due origini di replicazione del DNA mitocondriale, risulta essere correlata alla probabilità di raggiungere età molto avanzate (Zhang et al., 2003). Inoltre, nello stesso studio, dall' analisi di concordanza dei livelli della mutazione in gemelli monozigoti e dizigoti, è stato ipotizzato un controllo genetico nucleare sull' insorgenza e l'accumulo di tale mutazione. Uno studio analogo, condotto su campioni provenienti da una diversa popolazione, ha invece riportato una bassa frequenza della transizione C150T e, di contro, un' alta incidenza della transizione T152C, sebbene non associata ad un rimodellamento dell' origine di replicazione (Iwata et al., 2007).

Alla luce di queste considerazioni, l' attività da me condotta durante il corso del Dottorato di Ricerca è stata rivolta a chiarire se l' insorgenza e l' accumulo di mutazioni somatiche a carico della regione di controllo del DNA mitocondriale possano rappresentare un fenomeno geneticamente controllato, teso a favorire la sopravvivenza in età avanzate.

A tal fine è stato condotto un primo studio in famiglie di centenari reclutate nell' ambito del progetto europeo ECHA (European Challenge for Healthy Aging). I risultati di questo studio hanno rilevato che l' accumulo di mutazioni eteroplasmiche a carico della regione di controllo del DNA mitocondriale è un fenomeno più frequente in soggetti centenari e i loro discendenti (figli e nipoti) rispetto a soggetti di controllo appartenenti alla stessa popolazione. Inoltre, i livelli di eteroplasmia risultavano essere correlati nelle coppie centenari-figli e non associati alla variabilità ereditata del DNA mitocondriale. Quindi, considerando l' aggregazione familiare del fenotipo longevità, questi risultati suggeriscono che l' insorgenza e l' accumulo di mutazioni somatiche nella regione di controllo del DNA mitocondriale siano un fenomeno geneticamente controllato (verosimilmente dal genoma nucleare) che favoriscono la longevità.

Per investigare ulteriormente tale controllo genetico abbiamo condotto un secondo studio su coppie di fratelli ultranovantenni reclutati nell' ambito del progetto europeo

GEHA (GEnetics of Healthy Aging). L' utilizzo di tali campioni ci permetteva anche di analizzare i livelli di eteroplasmia in diversi tipi cellulari (granulociti e linfo-monociti) appartenenti allo stesso soggetto, oltre che di analizzare campioni reclutati in diverse aree geografiche (nord e sud Italia, Finlandia) al fine di rilevare possibili differenze popolazione-specifiche. Questo studio è stato condotto utilizzando 2 diversi approcci molecolari: uno rivolto a rilevare la presenza e l' accumulo di mutazioni somatiche nella regione studiata (DHPLC quantitativa), l' altro rivolto allo screening specifico della mutazione C150T (metodo PARFAH).

L'analisi tramite DHPLC quantitativa ha rivelato una correlazione significativa dei livelli di eteroplasmia tra fratelli appartenenti alla stessa coppia in tutte le popolazioni analizzate. Inoltre è stato evidenziato, applicando metodi di permutazione, come la correlazione osservata fosse dovuta alla relazione di parentela tra i fratelli, come atteso in base all' ipotesi di un controllo genetico sulla presenza e l' accumulo di mutazioni eteroplasmiche a carico di questa regione.

Lo screening della mutazione C150T ha dimostrato la presenza di eventi somatici associati all' insorgenza e l' accumulo della mutazione (analisi nei granulociti e linfo-monociti appartenenti allo stesso individuo) e una forte correlazione tra fratelli della stessa coppia. Questi risultati sono in accordo con quelli riportati precedentemente da Zhang (2003), confermando la presenza di un controllo genetico nucleare sui livelli di eteroplasmia della mutazione C150T, presente a livello di diversi tipi cellulari (granulociti e linfo-monociti) e in campioni appartenenti a diverse popolazioni (Italiana e Finlandese).

Combinando le informazioni ottenute dall' applicazione dei due diversi metodi di analisi molecolare (DHPLC e PARFAH), ci è stato possibile distinguere le mutazioni a carico della posizione 150 dalle mutazioni somatiche a carico di altre posizioni nella stessa regione. L' analisi di correlazione della presenza/accumulo di tali mutazioni ha rivelato una correlazione significativa nelle coppie finlandesi ed in quelle reclutate nel nord Italia, ma non in quelle reclutate nel sud Italia, suggerendo la possibile presenza di fattori popolazione-specifici coinvolti nell' accumulo di mutazioni somatiche a carico di questa regione del DNA mitocondriale. Tali mutazioni potrebbero far parte di un rimodellamento generale (quindi non a carico della sola mutazione C150T) della regione di controllo, controllato dal nucleo, teso a ripristinare la funzionalità mitocondriale in seguito, ad esempio, ad un deterioramento età-correlato.

Summary

Studies on heteroplasmy occurring in the mitochondrial DNA Control Region (mtDNA CR) region demonstrated an age-related accumulation up to homoplasmy of the C150T somatic transition in leukocytes from Italian centenarians in comparison to younger controls. Moreover, 5' end analysis of nascent heavy mtDNA strands consistently revealed a new replication origin at position 149, substituting for that at 151, only in molecules carrying the C150T mutation. Finally, the concordance in mutation levels observed in twins indicated a possible nuclear genetic control on the contribution of somatic events leading to the accumulation of the mutation (Zhang et al., 2003).

In order to further understand the mechanisms leading to the occurrence and the accumulation of the C150T mutation and its relationship with longevity, during my PhD appointment I studied the occurrence and the accumulation in two population samples collected in the frame of two European research projects aimed at studying the biological basis of longevity.

The first sample was collected in Calabria (southern Italy) in the frame of the project European Challenge for Healthy Aging (ECHA) (http://biologia.unical.it/ECHA/). The sample was composed by 100 centenarians, 100 of their children, 100 of their nephews/nieces and of a control group made of 100 geographically matched subjects, belonging to the same age cohort of the children and the nephews/nieces of the centenarians. Through means of an original protocol performed by Denaturing High Performance Liquid Chromatography (DHPLC), we were able to quantify heteroplasmy levels in a fragment of the CR (16531-261 nt, 300bp). We found that the levels of heteroplasmy were similar in centenarians and their relatives (despite the different ages) and significantly higher than those found in controls. Taking into account that longevity runs in families of centenarians, the above results supported the hypothesis that high levels of heteroplasmy in the CR may provide a survival advantage. In addition, we also found that heteroplasmy levels were significantly correlated in parent-offspring pairs, but independent of mtDNA inherited variability. Taken together these results suggest that the occurrence/accumulation of heteroplasmy in the mtDNA CR can be a nuclear genetically controlled phenomenon that provides a survival advantage (Rose et al., 2007).

The second sample was collected in the frame of the Project Genetics of Healthy Aging (GEHA). The sample was composed by 195 ultra-nonagenarians sib-pairs (130 from Italy; 65 from Finland).
DHPLC-based analyses showed that the heteroplasmy levels were significantly correlated in sib-pairs in both populations. What is more, the correlation was nullified when the familial relationship was destroyed by permutation procedures. This finding was in agreement with a genetic control on the heteroplasmy levels in the mtDNA CR. Afterward, we applied a PCR Amplicon Restriction Fragment Analysis by High performance liquid chromatography (PARFAH) (Procaccio et al., 2006) for assessing, in the same sample analyzed by DHPLC, the mutation levels of the C150T transition. This analysis revealed a strong correlation of mutation levels between sibs. Moreover, the analysis of the C150T mutation in different leukocytic cells (granulocytes and lympho-monocytes) of the same subject confirmed that somatic event(s) are involved in such a phenomenon, because in some cases, no concordance was observed between granulocytes and lympho-monocytes. In addition, focusing our attention on the mtDNA CR heteroplasmy not due to the C150T transition, we found a significant correlation between sibs both in Finnish and northern Italians. Intriguingly, such a correlation is not observed between sibs from southern Italy. This finding suggests that population-specific factors play a role in such a phenomenon, and that specific somatic mutations, additional to the C150T, may occur in different populations.

On the whole, the work presented in this PhD thesis indicates that: i) mtDNA CR heteroplasmy is genetically controlled; ii) it is beneficial for longevity, as it runs in families of long lived subjects; iii) it can be somatically acquired. In addition, as for other aspects of the correlation between mtDNA variability and longevity, population-specific factors are involved.

List of abbreviations

Acetyl-CoA	Acetyl-Coenzyme A
ADP	Adenosine DiPhosphate
AIF	Apoptosis Inducing Factor
ANT	Adenine Nucleotide Translocator
Apaf-1	Apoptotic protease activating factor-1
ATP	Adenosine TriPhosphate
BC	Buffy Coats
bp	base pair
COX	Cytochrome c OXidase
CR	Control Region
CSB	Conserved Sequence Block
CYB	CYtochrome B
Cytc	Cytochrome c
DHPLC	Denaturing High Performance Liquid Chromatography
D-loop	Displacement-loop
DNA	DeoxyriboNucleic Acid
DZ	DiZygotic
ECHA	European Challenge for Healthy Aging
F	Females
FAD^+	oxidized Flavin Adenine Dinucleotide
FADH	reduced Flavin Adenine Dinucleotide
FMN	Flavin MonoNucleotide
GEHA	GEnetics of Healthy Aging
GPx	Glutathione Peroxidase
GPx	Glutatione Peroxidase
GR	Granulocytes
GTP	Guanosine TriPhosphate
GTPase	Guanosine TriPhosphatase
H1and H2	Heavy strand transcription sites
HPLC	High Performance Liquid Chromatography
HSP	Heavy Strand Promoter

H-strand	guanine-rich Heavy-strand of mtDNA
HVRI and HVRII	HyperVariable Region I and II
IAPs	Inhibitors of Apoptosis
L	Light strand transcription sites
L- strand	cytosine-rich Light-strand of mtDNA
LDH	Lactate DeHydrogenase
LSP	Light-Strand Promoter for transcription of the mtDNA
LY	LYmpho-monocytes
M	Males
MnSOD	Manganese SuperOxide Dismutase
mRNA	messenger RNA
mtDNA	mitochondrial DNA
mtPTP	mitochondrial Permeability Transition Pore
mtTFA	mitochondrial Transcription Factor A
MZ	MonoZygotic
NAD^+	oxidized Nicotinamide Adenine Dinucleotide
NADH	reduced Nicotinamide Adenine Dinucleotide
OAA	OxalAcetic Acid
O_{H1} and O_{H2}	primary and secondary Origins of replication of the Heavy strand of mtDNA
O_L	Origin of replication of the Light strand of mtDNA
Opa1	Optic atrophy 1
OXPHOS	OXidative PHOSforilation
PARFAH	Pcr Amplicon Restriction Fragment Analysis by HPLC
PCR	Polymerase Chain Reaction
r	correlation coefficient
RFLP	Restriction Fragment Length Polymorphism
RNA	RiboNucleic Acid
ROS	Reactive Oxygen Species
rRNA	ribosomal RNA
SAM	Sorting and Assembly Machinery
sTIMs	small TIM proteins
TCA	Tricarboxylic Acid Cycle

TIM	Translocase of the mitochondrial Inner Membrane
TOM	Translocase of the mitochondrial Outer Membrane
tRNA	transfer RNA
UCP	UnCoupling Protein
VDAC	Voltage-Dependent Anion Channel

1. Introduction

Aging is a natural phenomenon which is characterized by a progressive decline of the normal physiological functions, an increase of morbidity and an increasing risk of death. Although aging is a generalized phenomenon, there is no doubt that a noticeable inter-individual variability exists with respect to the rate and the quality of aging. The continuous increase of life expectancy and of autonomous life expectancy which has occurred in the last decades in western societies clearly suggests that environmental conditions are essential to slow down human aging and to attain longevity. On the other hand, different studies have shown that the rate and the quality of aging are also influenced by genetic factors. Indeed, Perls and co-workers reported examples of familial clustering of longevity (Perls et al., 2000). For instance, Perls et al. (2002) investigated the familial predisposition for longevity in 444 centenarian pedigrees (age major or equal to 100 years) by comparing death rates and survival probabilities of siblings of centenarians with data from the same birth cohort. Interestingly, relative survival probability for these siblings increased markedly at older age, and was significantly higher than that people belonging to the same birth cohort. Moreover, siblings of centenarians maintained a life-long reduction in risk of death of approximately one-half, even up through very old age. The same result was confirmed in relatives (siblings and parents) of super-centenarians (age major or equal to 110 years) where a substantial survival advantage was reported in particular for siblings and mothers of super-centenarians. The familial clustering of extended survival has been confirmed also in the Dutch population by the Leiden Longevity Study, where a significantly low Standardized Mortality Ratios (SMR= observed N° of deaths/expected N° of deaths in the general population, adjusted for sex and calendar period) was found in parents and offspring of long-living subjects, compared to their spouses (Schoenmaker et al., 2006). However, although these studies supported a familial component for extreme longevity they do not further differentiate how much such a component is due to genetic *versus* environmental factors family members may share. On the other hand, studies on twins allowed to separate the genetic component from the environmental one in the variation of life span. These studies reported that the percentage of the variation in human life span which can be attributed to genetic

differences among individuals ranges from 22% to 33% (McGue et al., 1993; Herskind et al., 1996; Ljungquist et al., 1998). In addition, the studies on twins allowed the heritability of longevity to be estimated as 0.26 for males and 0.23 for females (Herskind et al., 1996). The remaining variation can be divided among conditioning factors that rise in the first part of life (month of birth, education, socio-economic state of parents) and the life style (Vaupel et al., 1998). Moreover, another study on twins demonstrated that having a co-twin surviving to old ages substantially and significantly increases the chance of reaching the same old age, and this chance is higher for MonoZygotic (MZ) than for DiZygotic (DZ) twins (Hjelmborg et al., 2006). These evidences support the existence of a genetic component affecting longevity in humans, especially at advanced ages.

Many studies have been undertaken to find out which genes affect by their variability the genetic component of longevity. These studies have been mainly carried out through case-control approach. However, more recently great efforts have been performed on linkage analysis in long lived sib pairs, which requests larger datasets. These studies have shown different genetic loci affecting longevity: 'cardiovascular genes' (APOE, APOC3, MTTP, ACE), 'immune system genes' (IL6), 'metabolism-related genes' (IGF1, GH1, HFE) and mitochondrial polymorphisms (Christensen et al., 2006 and references therein). The results obtained so far need to be confirmed by longitudinal studies which are currently ongoing. However, their soundness is suggested by the consistency between these results and those observed in model organisms. On the whole, these studies indicate that aging is mainly correlated to the decline of homeostatic capacity of the organism which declines with age, and makes less efficient not only the metabolic pathways for the conservation, the mobilization and the use of nutrients but also the response to external and internal stress.

1.1 Aging and mitochondria

It is generally assumed that damages accumulated after a variety of cellular stresses are the underlying cause of aging. The living organisms have developed different systems in order to protect the cells by curbing and repairing damages to the cellular systems. When the repair or replacement systems are unable to maintain the positive balance, the aging phenotype slowly becomes manifest.

Harman (1956) was the first to propose the *free radical theory of aging*: aging, as well as associated degenerative diseases, could be attributed to the deleterious effects of Reactive Oxygen Species (ROS) on various cell components. The mitochondrial respiratory chain is a major site of ROS production in the cell and it has been suggested that mitochondria are prime targets of oxidative damage; consequently, the accumulation of defective mitochondria can be one of the major contributors to aging (Kirkwood, 2005). ROS damage to the mitochondria, mitochondrial DNA (mtDNA) and host cell is supposed to be one of the most important factors in determining age-related cellular decline. As shown in Fig. 1, an increased oxidative stress can activate mitochondrial transition pores (mtPTP) leading to apoptosis; this process generates, during time, the loss of cells up to exceed the tissue functional threshold. Harman (1972) proposed a refined version of the free radical theory, the "mitochondrial theory of aging": mtDNA is one of the first targets of ROS, and accumulation of mtDNA damages can be one of the phenomena which lead cells to their functional age-related decline (Fig.1).

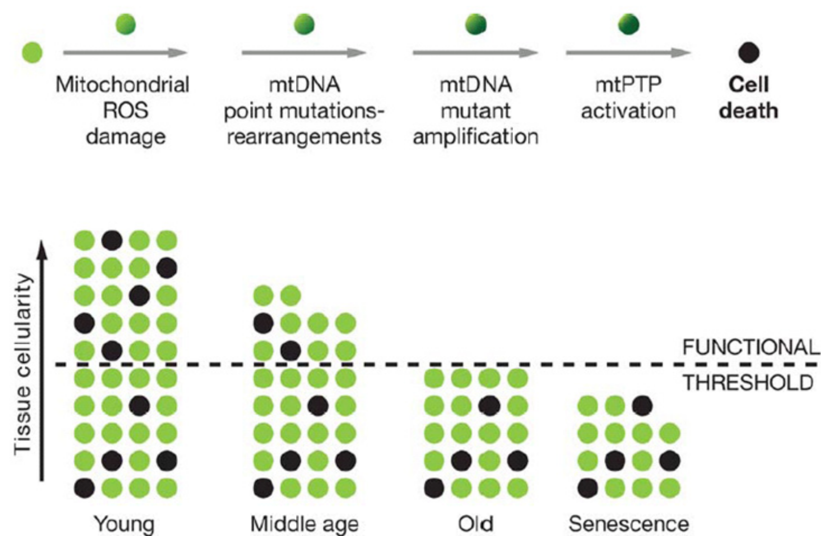


Figure 1. Mitochondrial and cellular model of aging
Representation of the mitochondrial role in the energetic life and death of a cell. The bottom diagram represents the loss of cells in a tissue over the life. The minimum number of cells for the tissue to function normally is indicated by the dashed line (modified from Wallace, 2005).

1.2 The human mitochondrial genome

The mitochondrion

Structurally, the mitochondrion is a membrane-enclosed organelle ranging from 1–10 micrometers in size (Henze and Martin, 2003). The number of mitochondria in a cell varies widely by organism and by tissue type. Many cells have only a single mitochondrion, whereas others can contain several thousand of mitochondria. As shown in Fig.2, mitochondria contain both an inner and an outer membrane, leading to the formation of two aqueous compartments, the matrix and the inter-membrane space, where several specialized functions take place.

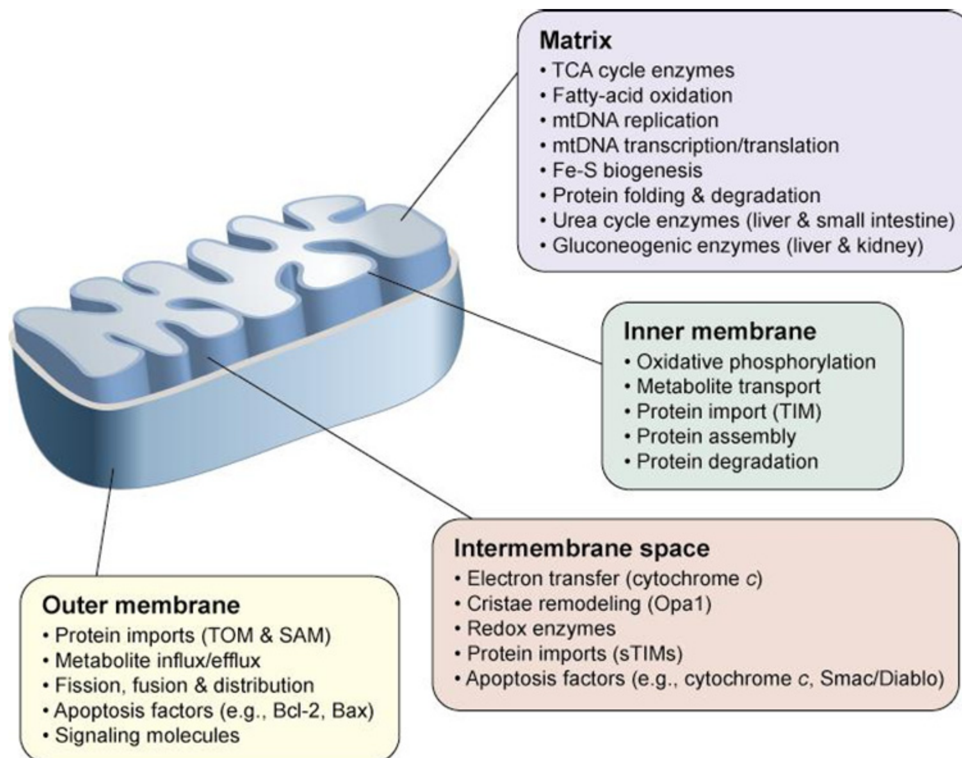


Figure 2. The mitochondrial sub-compartments and specialized functions
Examples of compartment-specific processes and proteins are depicted. The main function of the mitochondria is the energy conversion (fatty acid metabolism, citric acid cycle, ATP or heat production); nevertheless it is involved in a series of processes that are crucial for the cell activity (amino acid metabolism, regulation of apoptosis, regulation of cellular calcium, production of oxygen free radicals). Abbreviations: Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma protein 2; Opa1, Optic atrophy 1; SAM, Sorting and Assembly Machinery; sTIMs, small TIM proteins; TIM, Translocase of the mitochondrial Inner Membrane; TOM, Translocase of the mitochondrial Outer Membrane (modified from Ryan and Hoogenraad , 2007).

The main mitochondrial function is energy production. The process by which the mitochondrion generates energy is called OXidative PHOSphorylation (OXPHOS). The OXPHOS process, consisting in the oxidation of organic acids and fats to create a capacitor across the mitochondrial inner membrane, generates energy stored as adenosine triphosphate (ATP) molecules (Fig. 3). The OXPHOS process is efficient, but a small percentage of electrons may prematurely reduce oxygen, forming Reactive Oxygen Species (ROS), such as superoxide, as a toxic by-product of OXPHOS. This can cause oxidative stress within mitochondria and may contribute to the decline in mitochondrial function. As shown in Fig. 3, the mitochondria are also the major regulators of apoptosis, accomplished via the mitochondrial permeability transition pore (mtPTP) (Chipuk et al., 2006). The opening of mtPTP can be stimulated by: a) the mitochondrial uptake of excessive Ca2+, b) an increase of the oxidative stress, c) a decrease of the mitochondrial membrane potential, d) a decrease of the intra-mitochondrial ATP concentration (Wallace DC, 2005).

It is important to note that mitochondria should not be considered as individual organelles independent from each other, but as a dynamic network undergoing directed movements as well as fission and fusion (Ono et al., 2001). In fact, the state of the mitochondrial network morphology appears to influence a variety of other cellular functions. Mitochondrial fusion has been suggested to play an important role in mitochondrial proliferation (Chen et al., 2004) and in the propagation of signals through the organellar population (Chan et al., 2006). The mitochondrial fission and fusion machineries as well as proteins involved in mitochondrial distribution have also been shown to act in cell differentiation (Park et al., 2001), in regulating apoptotic events (Suen et al., 2008) as well as in aging (Lopez.Lluch et al., 2008).

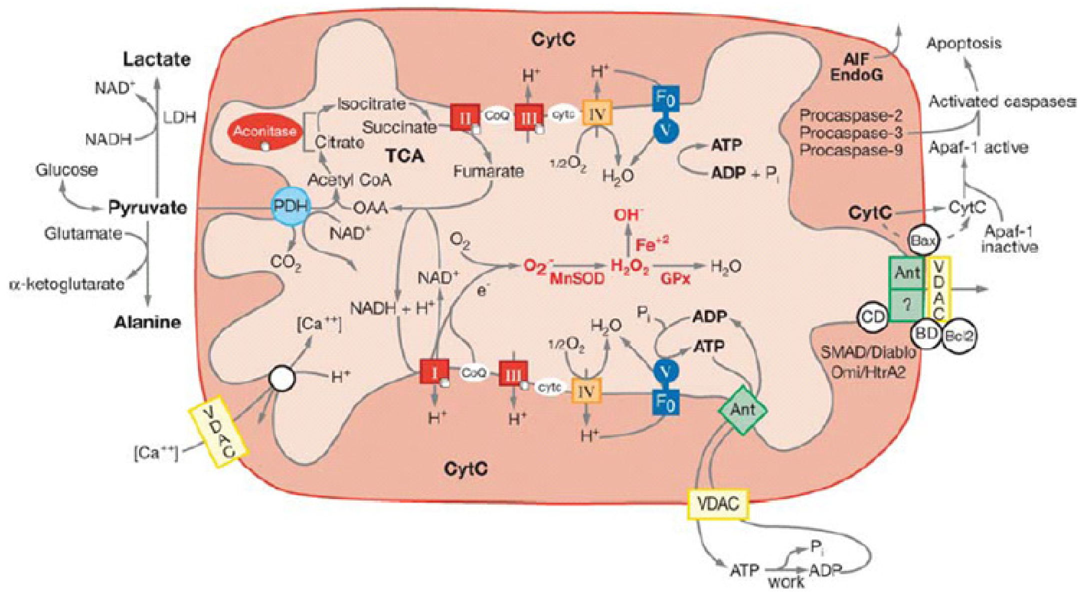


Figure 3. Mitochondrial functions

The diagram shows the relationships of mitochondrial OXPHOS to (a) energy (ATP) production, (b) reactive oxygen species (ROS) production, and (c) initiation of apoptosis through the mitochondrial permeability transition pore (mtPTP). Abbreviations: Acetyl-CoA, acetyl-coenzyme A; ADP or ATP, adenosine di- or triphosphate; ANT, adenine nucleotide translocator; Apaf-1, apoptotic protease activating factor-1; cytc, cytochrome c; GPx, glutathione peroxidase-1; LDH, lactate dehydrogenase; MnSOD, manganese superoxide dismutase; NADH, reduced nicotinamide adenine dinucleotide; SMAD/Diablo, antagonizes inhibitors of apoptosis (IAPs); Omi/Htr A2, serine protease 24; TCA, tricarboxylic acid cycle; VDAC, voltage-dependent anion channel; I, II, III, IV, and V, OXPHOS complexes I to V (modified from Wallace, 2005).

The human mitochondrial genome, located in proximity of the inner mitochondrial membrane and organized into nucleoprotein complexes (nucleoids), is a circular DNA molecule of 16,569 base pairs which corresponds to a molecular weight of about 10 million Daltons. The normal mtDNA state is thought to be as a super coiled structure (Richter et al., 1988). In most cells it represents only the 0.5–1 % of the total DNA content (Anderson et al., 1981). The two strands of the mtDNA can be distinguished because of their different G+T content and can be separated by density in denaturing gradients, giving a Heavy or H-strand and a light or L-strand.

One of the main characteristics of animal mtDNA is the compact gene organization, with all the coding sequences contiguous to each other or separated by a few bases (Anderson et al., 1981). The human mtDNA contains only 37 genes (coding for 13 polypeptides, 22 tRNAs, and 2 rRNAs) and two non-coding regions (the control region

for mtDNA transcription and replication and a short segment containing the L-strand origin of replication) (Fig.4).

Figure 4. Map of the human mtDNA
Human mitochondrial genome has two strands, a guanine-rich heavy strand (H) and a cytosine-rich light (L) strand. Twenty-four encode for the translational machinery of the mtDNA (22 tRNAs, 2 rRNAs). The remaining 13 genes encode subunits of the OXPHOS system: ND1-6 and NDL4 for complex I (NADH dehydrogenase); CYB for complex III (cytochrome b); CO1-3 for complex IV (cytochrome c oxidase); ATPase 6 and 8 for complex V (ATP synthetase). MtDNA is replicated from two origins, O_H for the H-strand,and O_L for the L-strand (modified from Tom Strachan and Andrew Read, Human Molecular Genetics 3rd Edition, 2003).

All the 13 polypeptides are subunits of the OXPHOS machinery of the inner membrane. The remaining 76 subunits of the respiratory chain, and proteins involved in the mitochondrial activity (about 1500 genes) are encoded by the nuclear genome and must be imported into the mitochondria (Fig.5). From this perspective, to understand human cell biology and its relationship with health and diseases, it is necessary to pay attention to the intricacy of relationships among mitochondria and between mitochondrial and nuclear genomes.

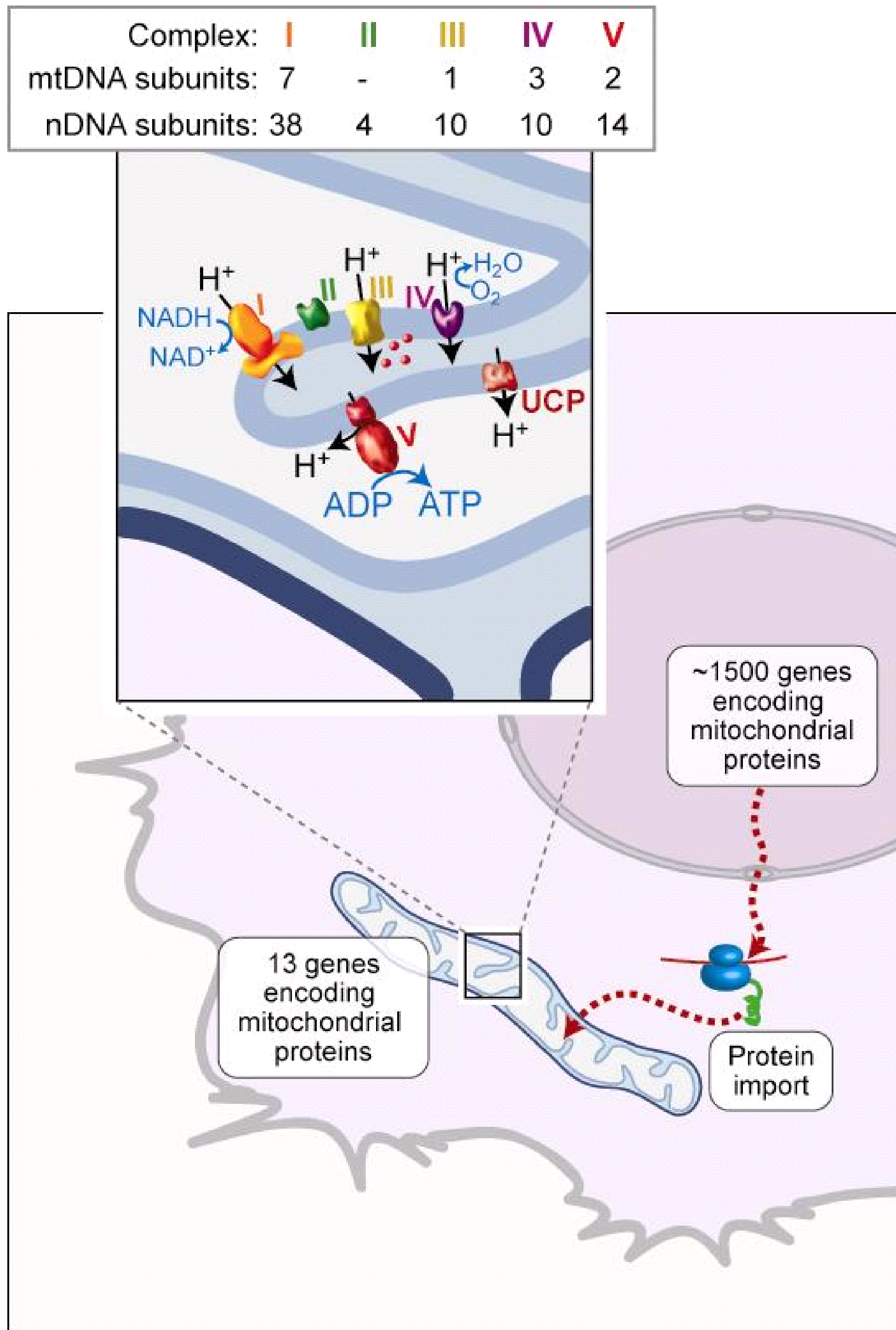


Figure 5. Mitochondrial biogenesis and the OXPHOS machinery
The protein complement of mitochondria consists of 13 subunits, encoded by mtDNA, which are synthesized in the organelle. The remaining proteins are encoded by nuclear genes and are made in the cytosol and imported into mitochondria (Calvo et al., 2006). Inset: the OXPHOS machinery of the inner membrane consists of complexes I–IV, involved in electron transfer and proton export to the intermembrane space, whereas complex V uses the proton gradient to generate ATP. Uncoupling protein (UCP) uses proton flow to generate heat. Cytochrome c (red) is found in the intermembrane space. The total number of subunits encoded by nuclear and mtDNA are shown for each of the OXPHOS complexes (modified from Ryan and Hoogenraad , 2007).

Mitochondrial genetics

The studies in the field of mitochondrial genetics have shown a series of peculiarities and differences compared to the nuclear genome.

1) The mitochondrial genome is maternally inherited; the few mitochondria from the sperm cell that could enter the oocyte during fertilization are actively eliminated by a ubiquitin-dependent mechanism (Sutovsky et al., 2000). During oogenesis, mtDNA suffers a bottleneck phenomenon by which only a small subset of mtDNA molecules are amplified and transmitted to the offspring (Marchington et al., 1998). On this topic recent data are emerging: by studying the fate of random mtDNA mutations in the mammalian germ-line Stewart and co-workers (Stewart et al., 2008) demonstrate the importance of a purifying (negative) selection in shaping mitochondrial sequence diversity. Moreover, in the same paper, the authors propose that mtDNA in germ cells is under a different selective regime than mtDNA in somatic cells. The massive proliferation of mtDNA during oogenesis (up to about 100,000 mtDNA molecules) and the subsequent reduction of mtDNA copies (approximately 950-1,550) provide ample opportunities for functional testing of mtDNA during female germ-line development. Future research is required to unravel molecular nature and mechanism for this selection.

2) The evolution rate of mtDNA is much faster than that of the nuclear genome (Brown et al., 1979). Several reasons are invoked to explain this fact: mtDNA is less protected by proteins, it is physically associated with the mitochondrial inner membrane where damaging reactive oxygen species are generated, and it appears to have less-efficient repair mechanisms than the nucleus (Pinz & Bogenhagen, 1998).

3) Cells are polyploid with respect to mtDNA: most mammalian cells contain hundreds of mitochondria and, in turn, each mitochondrion contains several (2–10) copies of mtDNA (Wiesner et al. 1992). If all mtDNA copies are identical, we have a condition known as homoplasmy, but if the mtDNA copies are not identical, we have a condition known as heteroplasmy (Fig. 6). At cell division mitochondria and their genomes are randomly distributed to daughter cells and hence, starting from a given heteroplasmic situation, different levels of heteroplasmy up to homoplasmy can arise in different cell lineages (Fig. 6).

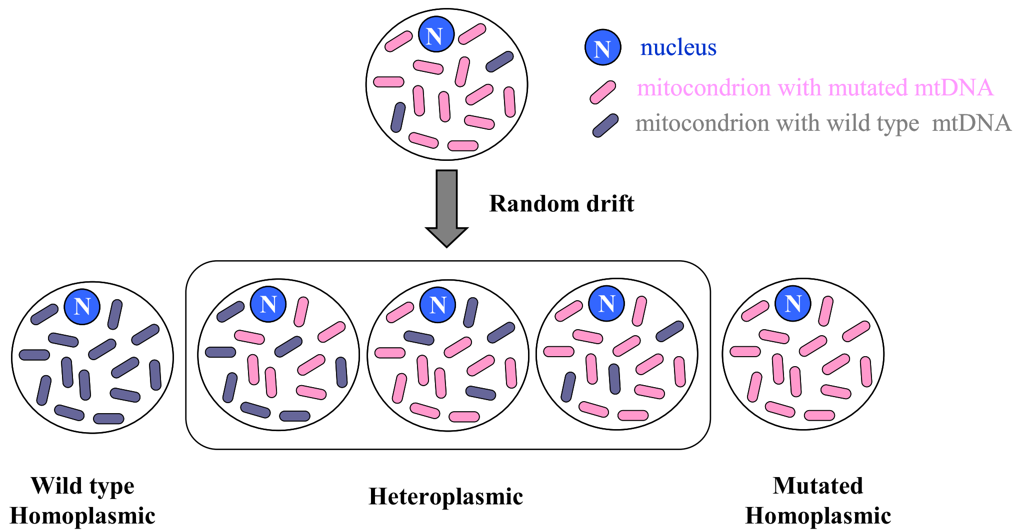


Figure 6. Schematic representation of homoplasmy and heteroplasmy

Therefore, in a tissue, heteroplasmy can be observed at three different levels:

- intercellular, in which mutant and wild-type mtDNA occur in different cells
- intracellular and inter-mitochondrial, in which mutant and wild-type mtDNAs occur in different mitochondria in the same cell
- intracellular and intra-mitochondrial, in which mutant and wild-type mtDNAs occur in the same organelle.

1.3 MtDNA variability

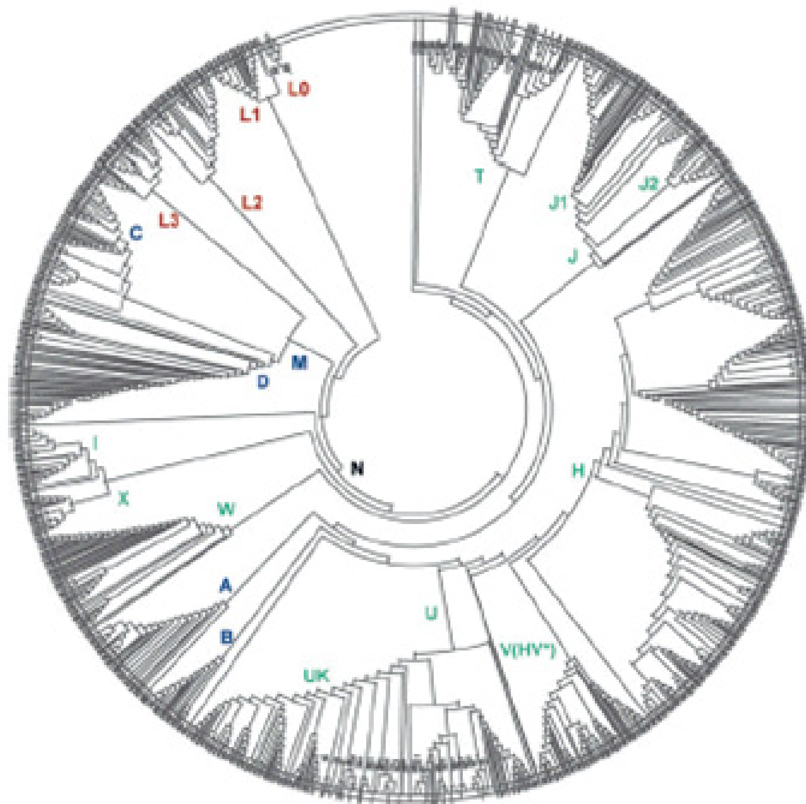
Features and function of mtDNA have attracted the attention of many researchers in the last decades. In fact, human mtDNA is largely studied for both inherited and somatic variability.

Inherited mtDNA variability

The inherited mtDNA variability implies both rare (mutations) and common (polymorphisms) variants. It goes through to replicative segregation (random drift of mtDNA molecules) and selection (negative or positive selection) during oogenesis.

The inherited mtDNA mutations in humans often cause severe pathologies. These pathogenic mtDNA mutations fall into three categories: rearrangement mutations (insertions and deletions), polypeptide gene missense mutations, and protein synthesis (rRNA and tRNA) gene mutations. These mutations, escaping purifying selection

during the oocyte development, are inherited and, when they exceed their functional threshold, the pathologic phenotype becomes manifest. To date, about a hundred of different mtDNA inherited mutations associated with mitochondrial diseases are known (Di Mauro, Hirano, 2005). On the other hand, during human evolution, a number of non-pathogenic variants reached polymorphic frequencies. MtDNA uniparental inheritance and high mutation rate make the study of mtDNA polymorphisms a powerful tool to reconstruct genetic relationships among human populations and human evolution (Ingman et al., 2000; Finnila et al., 2001; Maca-Meyer et al., 2001; Herrnstad et al., 2002; Tanaka et al., 2004; Coble et al., 2004; Kivisild et al., 2006). In fact, clusters of haplotypes (haplogroups) have been recognised and utilized to display human radiations across the continents (Torroni et al., 1996; Finnila et al., 2001; Herrnstad et al., 2002; Silva et al., 2002; Kong et al., 2003). As a result, the mutation history of human mtDNA sequence variation can be reconstructed as a single sequential mutational tree radiating from the last common mtDNA ancestor, that existed between 150,000 and 200,000 years before present (YBP) (Fig.7). Although originally attributed to genetic drift, considerable evidences suggest that the branches of the mtDNA tree correlate with the geographic origin of indigenous human populations (Wallace et al., 2003). In fact, the major transitions in mtDNA types strongly correlate with latitude, suggesting that climatic selection played a role in shaping the present worldwide distribution of mtDNA variation. In particular, the variants affecting ATP and heat production are more frequent in cold areas where they confer an advantage (Wallace, 2007). Evidence that climatic adaptation has influenced the geographic distribution of mtDNA diversity was obtained by analyzing regional gene-specific variation (*cob*, *cox1*, *atp6* genes, various *nad* subunit genes) (Mishmar et al., 2003). As an example, the atp6 gene was highly variable in the Arctic but was strongly conserved in tropical and temperate zone: this suggests that climatic selection may have favoured mtDNA variants that lowered the coupling efficiency of ATP production in order to increase heat production, thus permitting humans to survive in the cold northern climates (Mishmar et al., 2003).



Africa Europe Asia

Figure 7. Phylogenic tree of human mtDNA coding sequence variants
Haplogroups, groups of related haplotypes harbouring characteristic mtDNA sequence polymorphisms, are derived from a founding haplotype. Each haplogroup is designated by a letter with or without a subdividing letter. In this tree, the ticks around the perimeter of the circle represent the individual mtDNA sequences analyzed (1,125 sequences) to reconstruct the phylogenic tree (Ruiz-Pesini et al., 2004). The mtDNA haplogroups have proven to be geographically associated (Wallace, 2005) (modified from Wallace, 2007).

Evidences suggesting that mtDNA common variability is not neutral supported the idea of a *mitochondrial paradigm* for metabolic and degenerative diseases in which mtDNA common variability would play a role (Wallace, 2005). This is also in agreement with the observation that some mtDNA polymorphisms correlate with mitochondrial diseases (Torroni et al., 1997; Reynier et al., 1999) and complex traits (Wallace, 2005). Interestingly, mtDNA polymorphic variants were shown to affect also longevity phenotypes (Tanaka et al., 1998; De Benedictis et al., 1999; Ross et al., 2001; Niemi et al., 2003, Bilal et al., 2008). Multiple studies have demonstrated that in Europeans the haplogroup J is enriched in extremely old people and thus it seems to slow the aging process (De Benedictis et al., 1999; Ross et al., 2001; Niemi et al., 2003). A similar observation has been made for haplogroup D in the Japanese

population (Tanaka et al., 1998; Bilal et al., 2008). In addition, the European haplogroups J and U have been found to be protective for Parkinson's disease (Ghezzi et al., 2005). The association of the European haplogroups J and U with longevity and neuroprotection may be explained by assuming that polymorphisms occurring in haplogroups J and U partially uncouple OXPHOS, generating heat and leaving fewer excess of electrons to generate ROS: this would be protective of neurodegenerative diseases and aging which are thought to be caused by a elevated mitochondrial ROS damage (Wallace, 2007). Of course, experimental studies are needed to verify the above hypothesis.

Somatic variability

MtDNA somatic variability is due to stochastic mutations and their replicative segregation in post-mitotic tissues. Several studies suggest a link between mtDNA mutations and aging phenotypes (Trifunovic et al., 2004 and references therein). Indeed, many studies have reported an age related accumulation of mtDNA somatic mutations. However, it is still debated whether mtDNA mutations are just markers of biological age, or they are among the causes of the age related decline. To clarify this point several studies on murine models have been carried out in the recent years. In these studies, to have direct insights on the effects of mtDNA mutations in aging, mouse models were engineered to have defects in the proofreading function of mitochondrial DNA polymerase (Pol γ). This led to the progressive, random accumulation of mtDNA mutations during the course of mitochondrial biogenesis. In these experiments the mtDNA mutator mouse displayed a completely normal phenotype at birth and in early adolescence, but subsequently it acquired many features of premature aging (Trifunivic et al., 2004). Moreover, studies in human muscle cells with atrophy have highlighted that mtDNA deletions are largely clonal and absent from phenotypically normal regions within individual muscle fibers (Fayet et al., 2002). Similar results have also been observed in buccal, epithelial and neuronal cells where age-related dysfunctions had been diagnosed (Cottrell et al., 2001; Cottrell et al., 2002; Kraytsberg et al., 2006). Although these studies do not prove causality, they provide strong evidence in support of the hypothesis that mtDNA deletions contribute to aging in mammals. Probably, senescence leads to mtDNA deletions which, on turn, exacerbate the effects of the age related decline (Wallace 2007).

Although the presence of deletions in the mtDNA of aged people has been known for a long time, the presence of point mutations has been recognised (Michikawa et al., 1999; Wang et al., 2001). In particular, Michikawa et al. (1999) found that long living subjects accumulate a number of tissue specific point mutations at critical positions of the mtDNA Control Region (CR).

1.4 Point mutations of the mtDNA control region in human aging

The human mtDNA CR is the main regulatory region for mtDNA transcription and replication(Fig. 8). The mtDNA CR spans about 1.1 kb between tRNAPhe and tRNAPro and contains:

1. the two initiation sites for the transcription of the H strand (H1 and H2 sites),
2. the promoter for the transcription of the L strand (L), which is also the promoter for the RNA primer of the H-strand synthesis,
3. three conserved sequence blocks (CSB),
4. the Displacement-loop (D-loop), a triple-stranded structure of 500–700 nucleotides that remains annealed to the parental L-strand, in which the primary (OH1) and secondary (OH2) initiation sites of H-strand synthesis are localized.

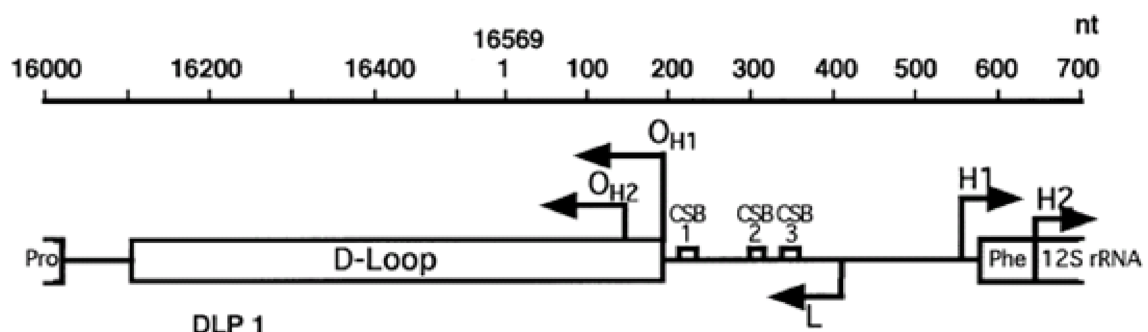


Figure 8. Schematic representation of the mtDNA control region
O_{H1} and O_{H1}= primary and secondary origin of H-strand Synthesis. CSB1-3= conserved sequence blocks 1-3 (Walberg and Clayton, 1981). H1, H2, L: start sites for transcription of rDNA, whole H-strand and light (L)-strand, respectively (Ghivizzani et al., 1994). Pro, Phe: tRNAPro and tRNAPhe genes (modified from Attardi, 2002).

The mtDNA D-loop region is the most variable non coding portion of the human mtDNA, containing two hypervariable regions (HVR), HVRI and II (Hasegawa and Horai, 1991; Stoneking et al., 1991). Although the function for most of the CR is not well understood yet, the observation that the central domain of this region is highly evolutionarily conserved suggests that the CR is selectively constrained (Sbisà et al., 1997).
Researchers have recently focused their attention on the accumulation of mutations in the mtDNA CR. This choice is chiefly based on the consideration that this region has a greater susceptibility to mutations than other mtDNA regions (Hasegawara and Horai, 1991). If the same mechanisms operating to create variation in evolution also operate during aging, this region could represent a good model for getting insights into the mitochondrial theory of aging (Attardi, 2002). In addition, this region also contains functional elements important for mtDNA transcription and replication: mutations on this region can affect the entire mitochondrial functions influencing mtDNA replication and transcription.

Studies on the control region have established that some specific base-substitution mutations can reach high levels in fibroblast cells derived from aged individuals (Michikawa et al., 1999) and also in skeletal muscle (Wang et al., 2001) (Fig.9). The reason why these specific mutations accumulate in mtDNA is unclear, but they are tissue-specific and occur in mtDNA control sites for replication and transcription. The localization of these aging-dependent mutations at critical control sites provides strong evidence that the mutations themselves may confer a segregation advantage to the mutant molecules. Moreover, the tissue-specificity of these point mutations and their localization at critical sites for H-strand mtDNA synthesis, suggest the involvement of cell type-specific proteins or cofactors that could provide a segregation advantage to the mutant molecules or, alternatively, could affect the susceptibility of these mtDNA sequences to oxidative damage or to replicative errors.

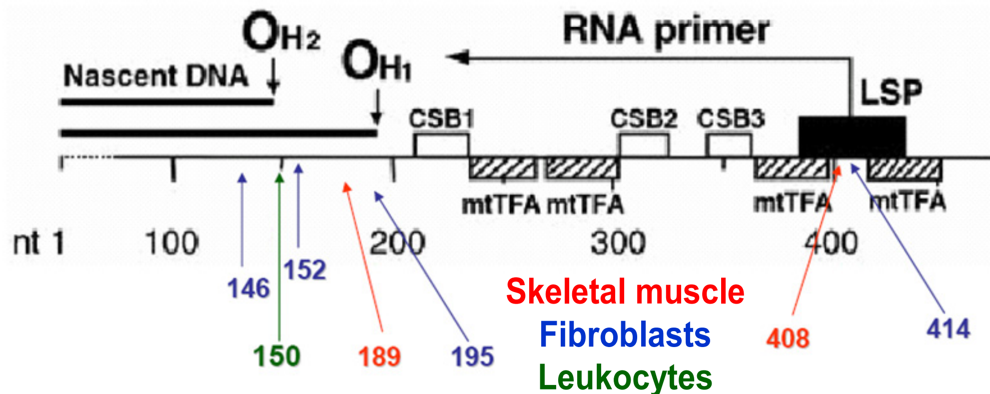


Figure 9. Tissue-specific aging-dependent somatic mutations identified in human mtDNA main control region

O_{H1} and O_{H2}, primary and secondary origin of H-strand synthesis; LSP, promoter for transcription of L-strand and synthesis of primer for H-strand synthesis; CSB1, CSB2, and CSB3, conserved sequence blocks 1, 2, and 3. The positions of binding of mitochondrial transcription factor A (mtTFA; the densely hatched rectangle indicates a position of high-affinity binding) are shown . Blue arrows and numbers indicate the fibroblast-specific point mutations, red arrows and numbers indicate the skeletal muscle-specific point mutations, green arrow and numbers indicate the C150T transition found in leukocytes (modified from Zhang et al., 2003).

The special case of C150T mutation

One of the most important findings on the correlation between CR somatic mutations and aging came from the study of Zhang and co-workers (Zhang et al., 2003).

By a large-scale screening of the mtDNA CR in leukocytes from an Italian population, the authors observed that the homoplasmic C150T transition was more frequent in centenarians than in younger individuals. The analysis of the C150T mutation in Lympho-monocytes (LY) and Granulocytes (GR) from the same individual suggested that the mutation can be both inherited and/or somatically acquired. In addition, by the analysis of concordance in mutation levels between leukocytes of Monozygotic and Dizygotic twins, a higher concordance between MZ than DZ twins was observed. In the same study, by using fibroblast longitudinal studies, the authors showed an age-related somatic expansion of the C150T mutation. Finally, the authors found that 5' end analysis of nascent heavy mtDNA strands in fibroblasts or immortalized lymphocytes consistently revealed a new replication origin at position 149, substituting for that at 151, only in samples carrying the C150T mutation.

On the whole, the above findings led the authors to conclude that somatic events, probably under nuclear genome control, contribute to the selective accumulation of the C150T mutation in centenarians and twins (Zhang et al., 2003).
In a subsequent study, a significant greater frequency of the inherited C150T transition was found in leukocytes of very old Finnish and Japanese subjects than in leukocytes from younger individuals (Niemi et al., 2005). On the other hand, the analysis of the C150T transition in leukocytes from three groups of an Ashkenazi Jew population (female centenarians, their mixed gender offspring, and mixed gender control subjects) (Iwata et al., 2007) revealed a different situation with respect to the report of Zhang et al. (2003). In particular, the frequency of the C150T mutation was very low and, by contrast, the T152C and T195C transitions showed a fairly high frequency in all three groups of subjects. Furthermore, it was found that the heteroplasmic form of the T152C transition (presumably originated from somatic events) increases with age, but it is not associated with changes in the replication origin. This finding suggests that, if a nuclear genetic control on the accumulation of the C150T mutation (or other type of mutations in the mtDNA CR) exists, different polymorphic forms of nuclear genes and/or environmental changes may have a different effectiveness on the occurrence/accumulation of mtDNA somatic mutations. In line with these considerations, population-specificity has been already reported in relation to mtDNA inherited polymorphisms and human longevity (Dato et al., 2004). So it is arguable that the environmental changes and/or the specificity of the population gene pools can affect strongly the phenomena linked to the occurrence/accumulation of mtDNA CR somatic mutations, leading to different situations.
Taken together, these findings add intriguing clues to the relationship among mtDNA somatic mutations, aging and longevity phenotype. In addition, they underlie the relevance of the cross-talk between nucleus and mitochondria in complex traits and offer an interesting background to further investigations.
In order to study the somatic variability of mtDNA in aging and its correlation with longevity, a great advantage is provided by population samples including relatives of centenarians of different origin. This allows to verify if somatic mutations are shared by relatives of centenarians, if a genetic control of their occurrence exists, and, finally, if population-specific patterns can highlighted. For this purpose we take advantage by large population samples collected in the frame of the ECHA and GEHA projects.

1.5 ECHA and GEHA designs

The European Challenge for Healthy Aging (ECHA) and the GEnetics of Healthy Aging (GEHA) are two study designs aimed at identifying the key factors (genetic and environmental factors) involved in modulating the quality of human aging across Europe.

The ECHA project (http://biologia.unical.it/ECHA/) used for the first time an original study design based on the concordance/discordance between siblings of the longevity phenotype. The ECHA project started from the consideration that sib-pairs in which both sibs attained longevity (concordant pairs) are expected to share genetic and intra-familiar environmental factors favouring longevity, while sib-pairs in which a sole sib shows the longevity trait (discordant sib-pairs) are expected to share intra-familiar environmental factors but not the longevity genetic factors (De Rango et al., 2008). According to such a consideration, in the frame of the ECHA project centenarian families that were composed by a centenarian (and his/her brother/sister for the concordant families), his/her child, his/her nephew (the child of the centenarian's sibling) were recruited (Fig.10).

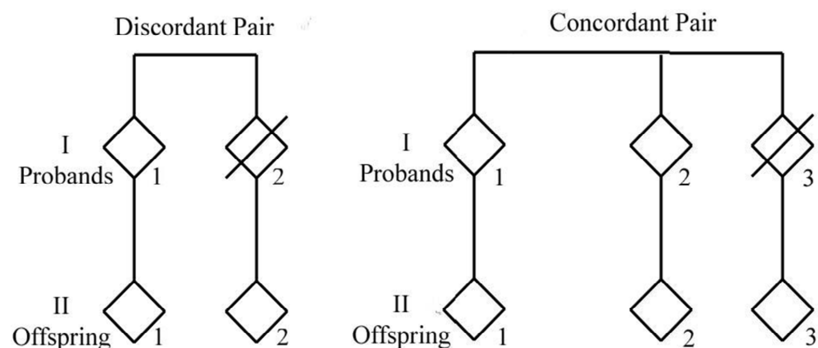


Figure 10. The structure of the ECHA families

The recruited subjects came from three different European areas: southern Denmark (Denmark), Languedoc-Roussillon (France) and Calabria (Italy).

The GEHA project (http://www.geha.unibo.it/) is one of the largest and most ambitious projects ever financed in Europe: it gathers 24 European partners (coming from 11 different countries), and one Asian partner (from China). The idea is to overcome the fragmentation of the research of the genetics of aging in Europe by setting up a coherent and integrated program of research using a multidisciplinary approach (medical, genetic, demographical, bio-informatic and so on) on the study of human aging and longevity. According to many data indicating a strong familial component of longevity (Schoenmaker et al., 2006; Hjelmborg et al., 2006; Christensen et al., 2006), and according to studies demonstrating that the first degree of relatives (parents, siblings and offspring) of long lived subjects have a significant survival advantage (Perls et al., 2002; Perls et al., 2007) the GEHA consortium chose to collect an unprecedented number (2650) of trios, composed by two (or more) long living sibs (90+ years) and geographically matched younger controls (50-75 years), coming from 15 different European areas (Franceschi et al., 2007) (Fig.11).

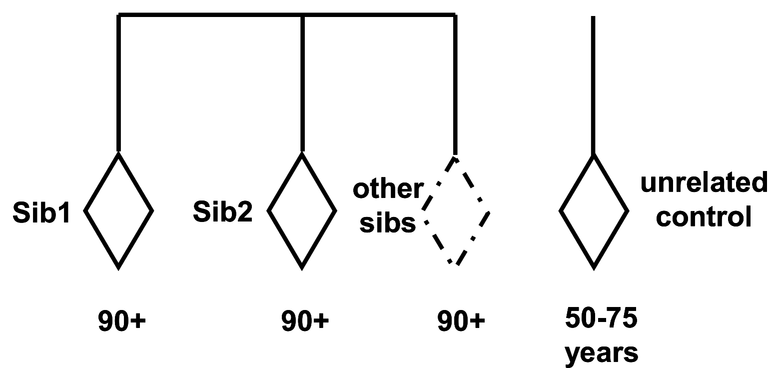


Figure 11. The structure of the GEHA families

2. Aim of the work

The human mtDNA Control Region (CR) is the main regulatory region for mtDNA transcription and replication. It contains functional elements important for mtDNA transcription and replication: mutation on this region can affect the entire mitochondrial functions influencing the mtDNA replication and transcription. In addition, the mtDNA D-loop region is the most variable non coding portion of the human mtDNA. Recent studies on this region reported that there are tissue-specific age-related point mutations altering critical control sites for mtDNA transcription and replication (Michikawa et al., 1999; Wang et al., 2001). In particular, one of them, the C150T mutation, is more frequent in leukocytes of centenarians than in younger controls. The occurrence and the age-related accumulation of this mutation seems to be genetically controlled, and it seems to favour longevity (Zhang et al., 2003). Taking into account these considerations, the aim of the work carried out during my PhD appointment was:

1. to clarify whether the occurrence/accumulation of heteroplasmy in the mtDNA control region is a genetically controlled event that may favour longevity;
2. to investigate whether different blood cell types are concordant in the occurrence/accumulation of heteroplasmy;
3. to investigate the occurrence/accumulation of heteroplasmy in the mtDNA control region in different populations.

To investigate the first point, we analyzed population sample collected in the frame of the ECHA project (http://biologia.unical.it/ECHA/). The results of this study have been already published (Rose et al., 2007). In summary, the results suggest that the occurrence/accumulation of heteroplasmy in the mtDNA CR can be a genetically controlled phenomenon, likely due to the nuclear genome, that provides a survival advantage.

To further investigate such a genetic control we performed a second study on sib-pairs selected for longevity collected in the frame of the GEHA project (http://www.geha.unibo.it/). The hypothesis of our work was that if the mtDNA CR heteroplasmy is genetically determined, so that genetic factors encoded by the nuclear genome are important in its occurrence/accumulation, we expect that sib-pairs selected

for longevity will correlate for mtDNA CR heteroplasmy levels. In addition, GEHA samples allowed to analyze heteroplasmy in different blood cells (granulocytes and lympho-monocytes) and in different populations. This study is described in Chapter 4 of this thesis.

3. Analysis of mitochondrial DNA control region heteroplasmy in families of centenarians (ECHA study)

(see END SECTION)

Abstract

Background: Studies on heteroplasmy occurring in the mitochondrial DNA (mtDNA) control region (CR) in leukocytes of centenarians and younger subjects have shown that the C150T somatic transition is over-represented in centenarians. However, whether the occurrence/accumulation of heteroplasmy is a *phenotypic consequence* of extreme ageing or a *genetically controlled event* that may favor longevity is a question that deserves further attention. To clarify this point, we set up a Denaturing High Performance Liquid Chromatography (DHPLC) protocol to quantify mtDNA CR heteroplasmy. We then analyzed heteroplasmy in leukocytes of centenarians (100 subjects), their offspring and nieces/nephews (200 subjects, age-range 65–80 years, median age 70 years), and in leukocytes of 114 control subjects sex- and age-matched with the relatives of centenarians.

Results: The centenarians and their descendants, despite the different ages, showed similar levels of heteroplasmy which were significantly higher than levels in controls. In addition we found that heteroplasmy levels were significantly correlated in parent-offspring pairs ($r = 0.263$; $p = 0.009$), but were independent of mtDNA inherited variability (haplogroup and sequence analyses).

Conclusion: Our findings suggest that the high degree of heteroplasmy observed in centenarians is genetically controlled, and that such genetic control is independent of mtDNA variability and likely due to the nuclear genome.

4. Analysis of mitochondrial DNA control region heteroplasmy in ultra-nonagenarian sib pairs (GEHA study)

4.1 Introduction

Studies on heteroplasmy occurring in the mtDNA CR of leukocytes from centenarians and younger subjects have shown that the C150T somatic transition is over-represented in centenarians (Zhang et al., 2003). In the same paper, the concordance in mutation levels observed in twins indicated a possible nuclear genetic control on the contribution of somatic events leading to the accumulation of the mutation. A confirmation of such a genetic control came from studies in families of centenarians, showing that the heteroplasmy levels are correlated in parent-offspring pairs (Rose et al., 2007). Consequently, heteroplasmy levels showed to be significantly higher in the descendants of centenarians than in controls. Taking into account that longevity runs in families of centenarians, the above results supported the hypothesis that high levels of heteroplasmy in the CR may provide a survival advantage. On the other hand, the analysis of the C150T transition in leukocytes from an Ashkenazi Jew population revealed a low incidence of the C150T transition, but a fairly high frequency of the T152C transition (Iwata et al., 2007). This evidence suggests a population-specificity on the occurrence/accumulation of mutations in the mtDNA CR.

To further investigate the genetic control on the occurrence/accumulation of heteroplasmy in mtDNA CR, we analysed the concordance of heteroplasmy levels in GEHA sib pairs. Moreover, for testing a possible population-specificity, the correlation was analysed in samples collected in both Italy and Finland.

4.2 Materials and Methods

Population samples

A total of 195 sib-pairs (390 subjects) were analysed: 63 sib-pairs (126 subjects) were recruited in the south of Italy (Calabria), 67 sib-pairs (134 subjects) in the north of Italy (Bologna) and 65 sib-pairs (130 subjects) in Finland (Tampere). For the Italian sib-pairs we analyzed DNAs coming from two different cell-types, granulocytes (GR) and lympho-monocytes (LY), for a total of 520 DNA samples (252 from the south of Italy, 268 from the north of Italy); for the Finnish sib-pairs we analyzed 130 DNAs coming from Buffy Coats (BC) (Table 1). The sampling was carried out in the frame of the GEHA project (Franceschi et al., 2007). It is important to notice that, taking into account the large heterogeneity of ethical rules in the different countries, the GEHA Consortium established criteria about recruitment and informed consent for privacy and confidentiality of data (Franceschi et al., 2007).

Recruitment Center	**Number of sib-pairs**	**Number of subjects**	**number of DNAs (from BC, LY and GR)**
Calabria (southern Italy)	63	126 (74F, 52M)	252 (126 from GR, 126 from LY)
Bologna (northern Italy)	67	134 (104F, 30M)	268 (134 from GR, 134 from LY)
Tampere (Finland)	65	130 (96F, 34M)	130 (from BC)
Total	195	390	650

Table 1. **Samples analyzed**
Abbreviations: F, females; M, males; BC, buffy coats; GR, granulocytes; LY, lympho-monocytes.

Biological samples

According to the standardization of procedure for the collection and processing of the biological material in the frame of the GEHA project, the biological samples (buffy coats, lympho-monocyte and granulocytes) were stored and shipped to the National Public Health Institute, KTL (Finland). KTL performed the centralized and automatic DNA extraction, quality control procedures and storage of the extracted DNAs. An aliquot of these DNAs was sent to us for the genetic analysis.

PCR DNA amplification

A 300 bp region of mtDNA encompassing the C150T site (region 16531-261) was amplified by 5'-AATAGCCCACACGTTCCCCTTA-3' forward primer and 5'-GCTGTGCAGACATTCAATTG-3' reverse primer (0.3 μM each) in a final volume of 50 μl, containing 80 ng DNA, 1.5 mM MgC12, 0.2 mM of each dNTP, and 2,5U of EuroTaq DNA polymerase (EuroClone). Amplification was performed in a Perkin Elmer Cetus 9600 PCR system. The amplification conditions were as follows: initial denaturation at 93°C for 30s, followed by 35 cycles at 93°C for 15s, 64°C for 20s, 72°C for 1 min. PCR products were checked by 2% agarose gel electrophoresis in TBE buffer with ethidium bromide. As a negative control we applied the same PCR protocol to DNA extracted from rho-zero cells (cells depleted of mitochondria).

Molecular cloning and DNA sequencing

The PCR products containing common (C150) and mutant (150T) sequences were cloned by using the pGEM-T Easy Vector Systems (Proniega), a system for the TA cloning of PCR products. The high-copy-number pGEM-T Easy Vector is already cut with EcoR V and provides a 3' terminal thymidine to both ends. These single 3'-T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization.

The PCR products with common (C150) and mutant (150T) sequences were ligated to pGEM-T Easy Vector in two reactions each containing. 5 μl of 2X Ligation Buffer, T4 DNA Ligase

• 1 μl of pGEM-T Easy Vector (50 ng)

• l μl of PCR product (100 ng)

• 1 μl T4 DNA Ligase (3 units)

• deionized water to a final volume of l0μl

The two reactions were mixed and incubated for 1 hour at room temperature. For each reaction, a positive control, that did not contain the PCR product, was carried out. Then, Top 10 E. coli cells were transformed with the ligation mixture by electroporation. First, we prepared electrocompetent cells. To this purpose, 300 ml of L-Broth (1% Bacto-Tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl) containing streptomycin (50 µg/ml) were inoculated with 6 ml of a overnight Top 10 E. coli cell culture and incubated on a rotary shaker at 37°C. The cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) every 45min-60min. When the OD_{600} value was equal to about 0.6 (log phase growth), the cells were removed from the shaker and placed on ice. Then the cells were centrifuged twice at 4000 rpm for 15 min at 4°C and the pellets were gently resuspended in ice-cold sterile water. Again the cells were centrifuged at 4000 rpm for 15 min at 4°C. The supernatants were removed and the pellets were resuspended in 6 ml of ice-cold 10% sterile glycerol. Then, the cells were centrifuged at 4000 rpm for 15 min at 4°C. Then pellets were suspended in 600 µl of ice-cold 10% glycerol. 20 µl aliquots of cells were prepared in pre-chilled 1.5ml eppendorf tubes.

20 µl of the electrocompetent cells and 2 µl of each ligation mixture were transferred in the 0,2 cm cuvette BIORAD and immediately electroporated at 250 kV, 25 µF, 200 ohms (Dower W.J. et al., 1988). 480 µl of liquid Luria-Bertani medium (LB) (1% Bacto-Tryptone, 0.5% Bacto-yeast extract, 1% NaCl) were added. Then the electropored cells were placed for 45 min on a rotary shaker at 37°C.100 µl of electropored cells were plated in a selective solid LB medium (1.6% agar) containing:

• ampicillin (50µg/ml),

• streptomycin (50µg/ml),

• Isopropil-Thio-b-D-gal actopiranoside (IPTG) (20mM)

• 5-Bromo, 4-Chloro, 3-Indolyl β-D-galactopiranoside (XGaI) (50 mg/ml).

Then, the cells were incubated overnight at 37°C. The following day, single white colonies were picked from the solid medium, transferred in a liquid LB medium and incubated at 37°C. Finally, the plasmids containing the common (C150) and mutant (150T) sequences were prepared by using Wizard Plus SV Minipreps DNA purification system (Promega). The correct insertion of the PCR product was verified by sequence analysis.

PCR-amplified fragments were purified by QIAquick PCR purification Kit (Qiagen), and sequenced by fluorescence-based automated direct sequencing with BigDye Terminator Cycle Sequencing Ready Reaction Kit in 310 DNA sequencer (PE Applied

Biosystems). Sequencing reaction mixtures contained 4 µl of Terminator Ready Reaction Mix, 200 ng of template, 3.2 pmol of each primer in a total volume of 20 µl. Cycle sequencing was carried out for 25 cycles at 96°C for l0s, 50°C for 5s, 60'C for 4min in GeneAmp PCR system 9600. The extension products were purified by using the Centri-SepTM spin columns (Princeton Separations).

Quantitative DHPLC

Accurate heteroplasmy level determinations of the mtDNA region encompassing the C150T site were carried out by using Denaturing High Performance Liquid Chromatography (DHPLC) and direct sequencing. After PCR fragments had been denatured for 3 min at 95°C, and gradually re-annealed from 95°C to 65°C in 30 min, about 500-600ng (10-20µl) of each sample were injected onto a DNASepTM column of a Transgenomic Wave Nucleic Acid Fragment Analysis System (Transgenomic, San Jose, CA). The amplicons were eluted in 0.1 M triethylammonium acetate, pH= 7, with a linear acetonitrile gradient at a flow rate of 0.9 ml/min. Mismatches were recognized by the appearance of two or more peaks in the elution profiles. Temperature conditions, which were chosen by computer simulation (available at http://insertion.stanford.edu/melt.html) were optimized to analyse the 16531-261 region, surrounding the position 150 and other mtDNA alternative replication origins. The DHPLC peak heights were measured by using WAVEMAKER 4.0 software (Transgenomic San Jose).

In order to build a reference curve for measuring the levels of heteroplasmy in the biological samples, plasmids containing the common (C150) and the mutant (150T) sequences were mixed in different proportions (0% T with 100% C; 5% T with 95% C; 10% T with 90% C; 20% T with 80% C; 30% T with 70% C; 40% T with 60% C; 50% T with 50% C) and again submitted to PCR amplification. By this approach, artificial samples having controlled conditions of C150T heteroplasmy were created. These samples were then submitted to DHPLC and a reference curve was assembled where the ratio between the height of the heteroduplex peak (Het) and that of the total peak (both heteroduplex and homoduplex peaks in the chromatogram, see Fig.12) was reported as a function of heteroplasmy, which varied according to the proportion between the two categories of cloned plasmids (Fig.13). Peak heights for both heteroduplex and homoduplex were determined by using WAVEMAKER 4.0 software (Transgenomic

San Jose). The levels of heteroplasmy in the biological samples were then estimated on the reference curve.

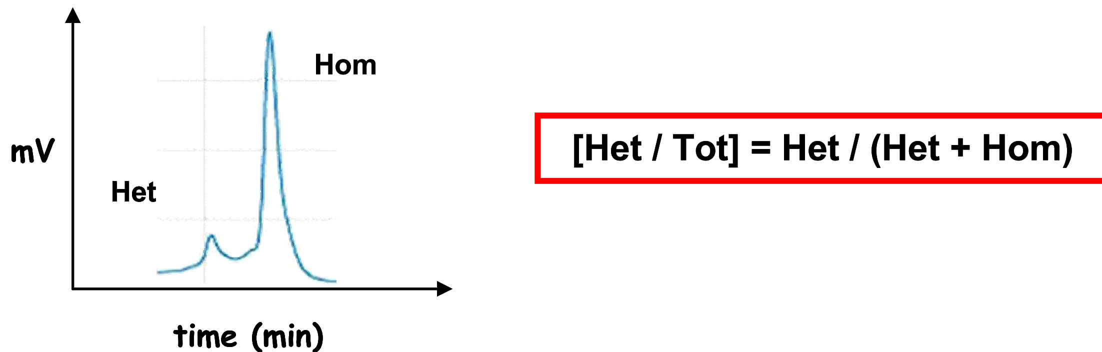


Figure 12. An example of DHPLC chromatogram of "artificial sample": 5% heteroplasmy level
Het = peak height of the heteroduplex peak; Hom = peak height of the homoduplex peak; Tot = Het + Hom; Het/Tot: relative height of the heteroduplex peak; x-axis: eluition time; y-axis DNA concentration measured in millivolts by optical density at 260nm.

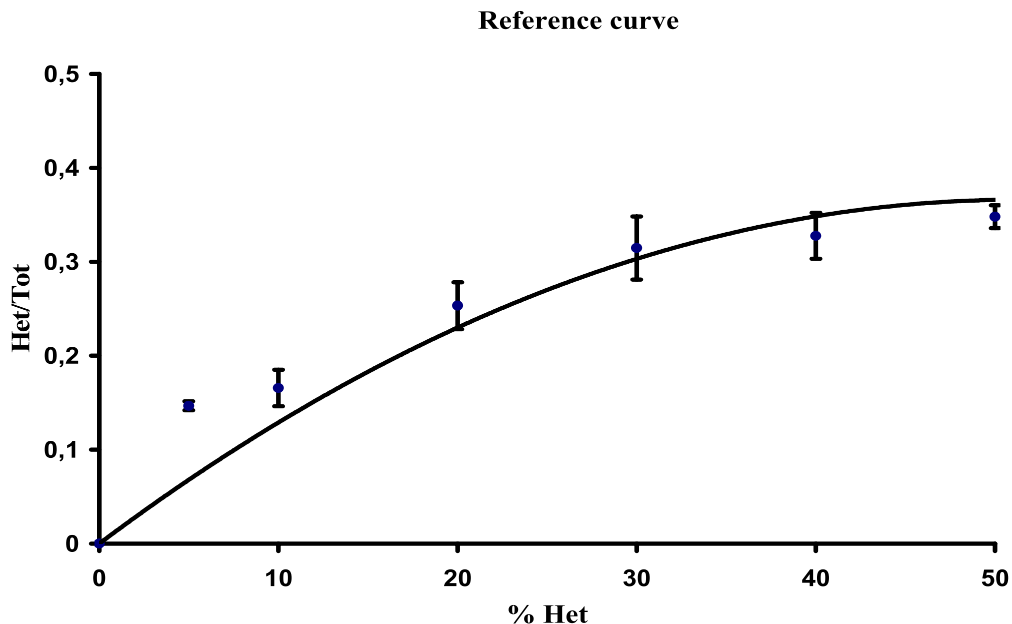


Figure 13. Reference curve
Reference curve based on the values obtained by measuring, for each "artificial" sample, Hp/Htot. Bars indicate the standard deviations in triplicate independent experiments. A 2nd degree of polynomial function $y= \beta_0 + \beta_1 x + \beta_2 x^2$ was used to fit DHPLC data of the heteroplasmic samples. y = -0,0001x2 + 0,0143x; R^2 = 0,9229.

Quantification of the C150T mutation levels by PARFAH

Occurrence and quantification of the C150T mutation was carried out by a technique based on the use of the WAVE system for high-performance liquid chromatography (HPLC)-mediated analysis of mutation-specific restriction fragments derived from mutant PCR amplicons (Procaccio et al., 2006). Briefly, the PCR Amplicon Restriction Fragment Analysis by HPLC (PARFAH) consists of:

1. PCR amplification of the fragment under study (region 16531-261);
2. Digestion with 10U of FokI endonuclease (Biolabs) at 37°C for 3 hours of about 600-800ng (30-40µl) of PCR product by adding directly the enzyme into the PCR mix;
3. Injection of digested products (20-25µl) into the WAVE system;
4. Separation of digested fragments using the buffer gradient condition summarized in table 2.

Gradient name	Time (minutes)	Buffer A (%)	Buffer B (%)
Loading	0	55	45
100bp (3.6 min)	0.5	50.2	49.8
225bp (3.6 min)	3.6	41.8	58.2
350bp (3.6 min)	6.8	38.2	61.8
475bp (3.6 min)	9.9	36.3	63.7
600bp (3.6 min)	13	35	65
Start clean	13.1	55	45
Stop clean	13.2	55	45
Start equilibrate	13.3	55	45
Stop equilibrate	13.4	55	45

Table 2. HPLC buffer gradient conditions used for the PARFAH method run on the Transgenomic WAVE system.

Buffer A is composed by triethyammonium acetate (TEAA) 0.1M in water and Buffer B is composed by TEAA 0.1M and 25% of acetonitrile (ACN) in water. The concentration of ACN increases over time based on this method. As the ACN concentration increases bridging capabilities of the triethyammonium (+) ions in the cartridge decrease, and the DNA fragments are released from the cartridge depending on their molecular weight.

FokI cleaves the 300bp wild-type (150C) fragment into two fragments (128bp and 172bp), but does not cleave the mutant (150T) fragment (300bp). WAVE system is equipped with an UV detector that can quantify the relative abundance of each fragment (128, 172 or 300bp) by the WAVEMAKER 4.0 software. In heteroplasmic samples the relative proportion of mutant and wild-type mtDNA were calculated by measuring the peak area percentage of the mutant fragments (Procaccio et al., 2006).

In order to check the sensitivity of the PARFAH method, we screened samples with known heteroplasmy levels obtained by mixing cloned sequences. By analyzing the samples with known heteroplasmy levels (0% of the 150T, 1% of the 150T, 2.5% of the 150T, 5% of the 150T, 95% of the 150T, 97.5% of the 150T, 99% of the 150T, 100% of the 150T) in three independent experiments, we found that the method can reliably detect up to the 2.5% of heteroplasmy.

Statistical analyses

The two-tailed Pearson test was used to perform correlation analyses (SPSS 14 software, SPSS Inc., Chicago, IL, USA). The linear correlation coefficient (r) measures the strength and the direction of a linear relationship between two variables; while the p-value measures the probability of getting a correlation as large as the observed value by random chance, when the true correlation is zero. The p-value is computed by transforming the correlation to create a t statistic having n-2 degrees of freedom, where n is the number of data pairs.

Permutation procedures were used to verify if the observed correlation in mtDNA CR heteroplasmy in sib pairs was due to the kinship between siblings or to their concordant age. Permutations were performed by implementing algorithms in MATLAB 6.1 software (MathWorks Inc.) as described in the following.

Let us consider the datasets where we applied the correlation analyses (Table 3).

Heteroplasmy in Sib1	**Heteroplasmy in Sib2**
a	a'
b	b'
c	c'
d	d'
.	.
.	.
.	.
k	j

Table 3. Dataset format used to perform correlation analyses and permutation procedures.
The letters indicate the levels of heteroplasmy in sib-pairs.

On these datasets we performed three numerical experiments.

Procedure 1. The first experiment consisted in computing the correlation coefficient r for 10,000 synthetic datasets obtained in the following way: the first column (heteroplasmy level in the sib1) is kept fixed, and the second column (heteroplasmy level in the sib2) is randomly permuted.

Procedure 2. The second experiment consisted in considering the n-1 data-sets obtained by shifting of one step, two steps, …, n-1 steps the second column of Table 3. For example the dataset corresponding to one shift was obtained by pairing a with b'; b with c'… k with a'. For each dataset the correlation coefficient and the respective p-value were computed.

Procedure 3. A third numerical experiment is based on permutations consisted in computing r for 10,000 data-sets obtained in the following way: the first and the second columns are pooled, the 2n dimensional array is randomly permuted, the resulting array is splitted into two n-dimensional arrays that are paired.

4.3 Results and discussions

MtDNA CR heteroplasmy in GEHA sib pairs

We analyzed the levels of heteroplasmy in 63 sib-pairs from southern Italy (Calabria) and 67 sib-pairs from northern Italy (Bologna). For each subject DNA was extracted from two fractions of leukocytic cells, lympho-monocytes (LY) and granulocytes (GR), previously separated. Thus, a total of 520 DNA samples was analyzed.

The mtDNA region under study (nt 16531-261, 300bp) was PCR amplified from each DNA sample, checked by electrophoresis and submitted to DHPLC. Then, the reference curve assembled by using cloned sequences with known levels of heteroplasmy (Fig.13, Materials and Methods) was used to quantify heteroplasmy. The results obtained are summarized in Fig.14, that shows the distribution of heteroplasmy levels in GR and LY from all the subjects collected in Bologna and Calabria.

We observe that the heteroplasmy levels in all the sample groups are not normally distributed as the majority of samples presents low levels of heteroplasmy (Fig.14).

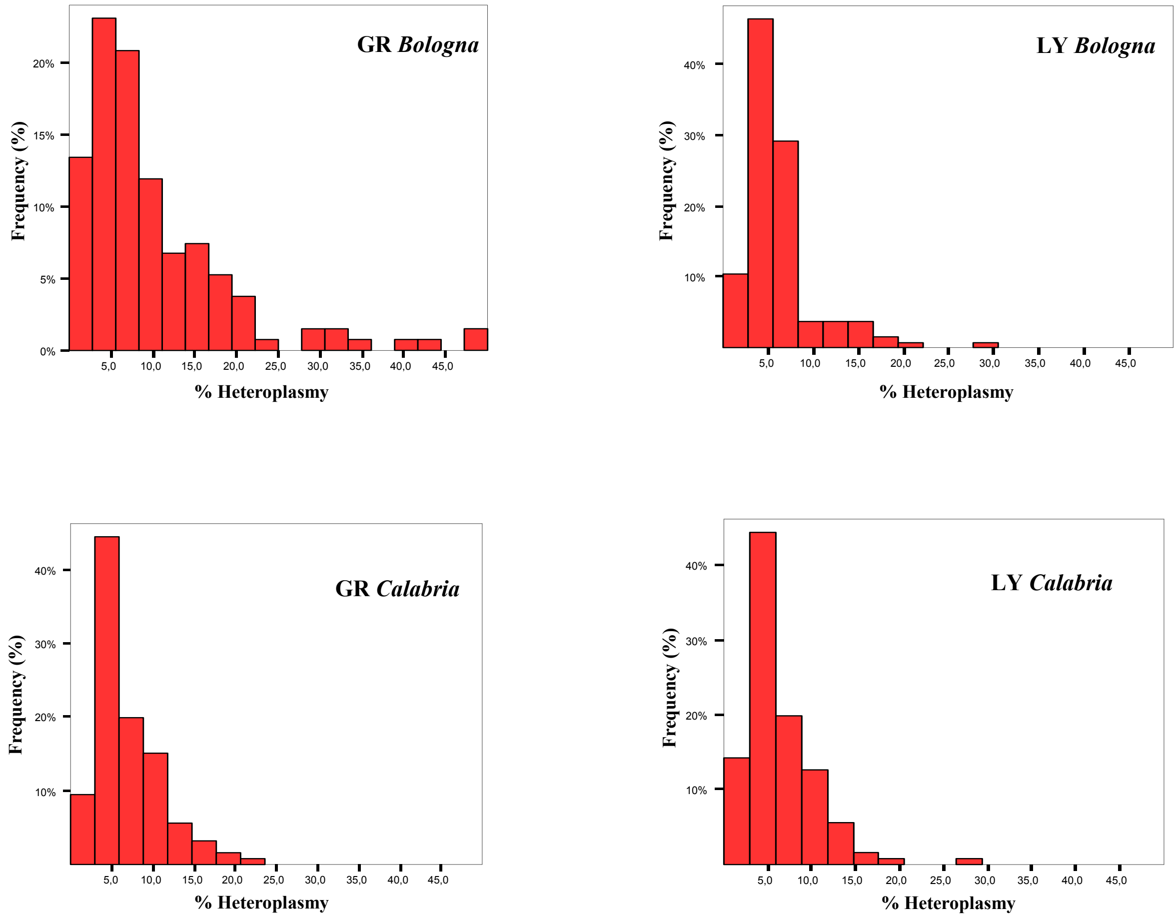


Figure 14. Heteroplasmy distribution in sib-pairs
The histograms show the levels of heteroplasmy in granulocytes (GR) and lympho-monocytes (LY) of sib-pairs from *Bologna* and *Calabria*. The percentages of heteroplasmy are estimated on the DHPLC reference curve of Fig.13.

In order to verify if a relation in heteroplasmy levels does exist between siblings, correlation analyses were performed. Fig.15 summarizes the obtained results. It can be seen that heteroplasmy levels are significantly correlated between siblings, both in the granulocytes and in the lympho-monocytes ($p<0.05$ in all the cases).

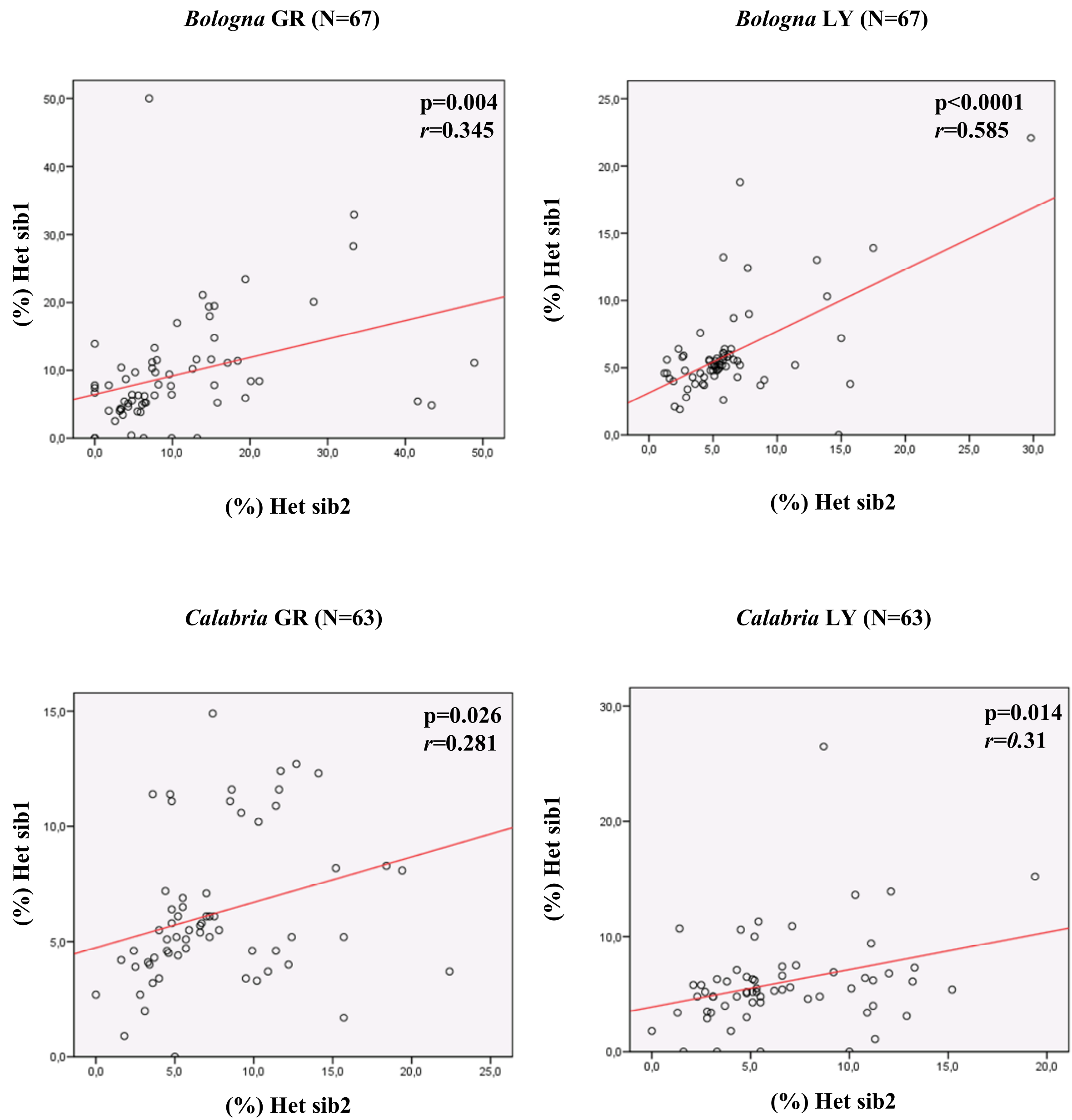


Figure 15. Correlation analyses in GEHA sib pairs
For each sample group regression lines and correlation coefficients (r) are shown. The p-values are obtained by 2-tailed Pearson test.

We wondered if the heteroplasmy correlation between siblings was due to the kinship (as expected for a genetically controlled trait), or, alternatively, to their concordant age (as expected for an age-related phenomenon). In order to answer this question we applied permutation procedures (as described in Materials and Methods; pagg. 30-31) to shuffle the coupling between siblings.

By this procedure we expected that, if the observed correlation was due to kinship, the correlation would be nullified; on the contrary, if the observed correlation was due to the concordant age between sibs, the correlation would be conserved.

We applied the three procedures reported in Materials and Methods. The results relevant to procedure 1 are shown in Fig. 16, which shows the empirical distribution of the r-values in the four sample groups.

We can observe that, in all the sample groups analyzed, the fraction of simulated r values exceeding the value observed in sib pairs are always lower than 0.05, as expected in the case that the kinship between siblings (and not their concordant age) is relevant in determining the value of the variable r. We obtained the same results (data not shown) by applying procedures 2 and 3 (see Materials and Methods).

On the whole, results indicate that heteroplasmy levels in mtDNA CR have a genetic component.

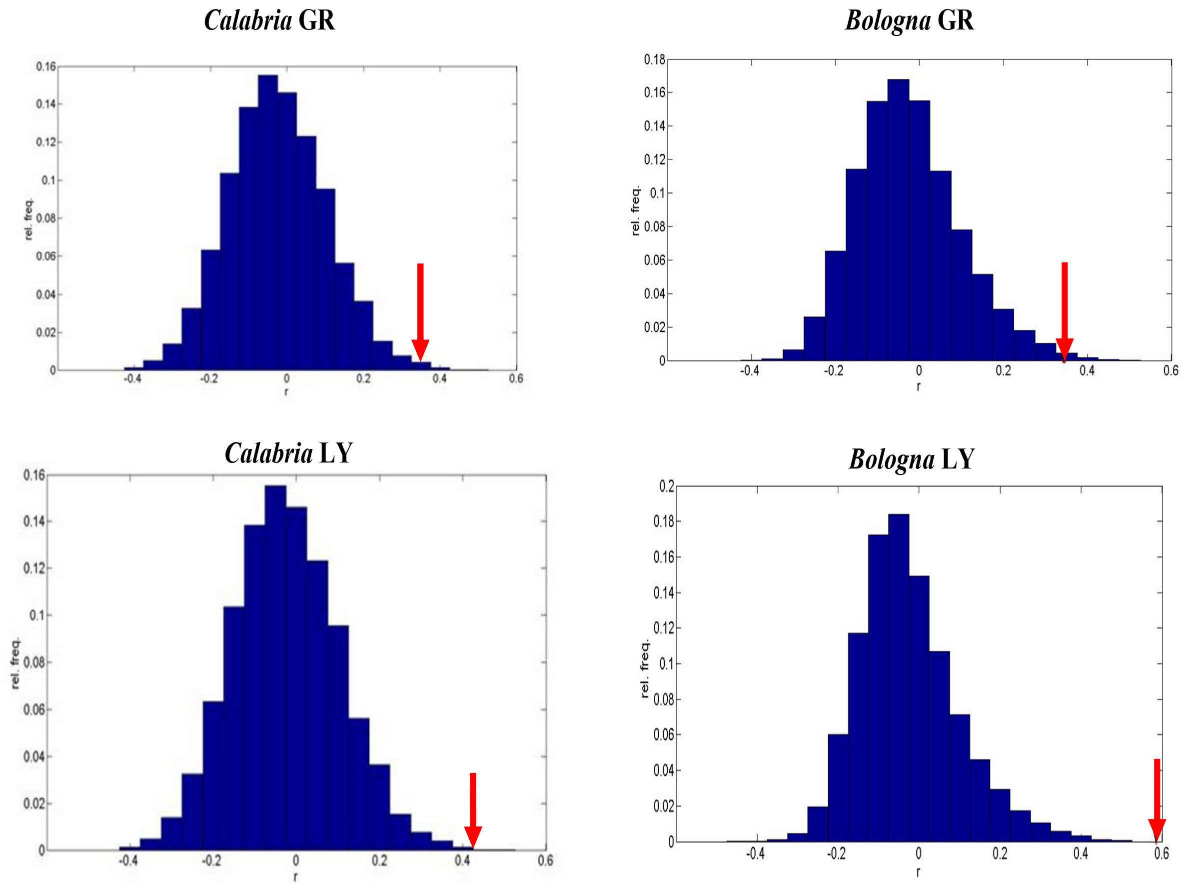


Figure 16. Empirical distribution of the *r* values obtained by permutation procedure

The arrows show the correlation coefficient (r) observed in GEHA sib-pairs. The fraction of simulated *r* values which exceeds the value observed in sib pairs is: 1.1% in Calabria LY, < 0.1% in Bologna LY, 1.6% in Calabria LY, 0.8% in Bologna GR.

To verify if the genetically controlled occurrence/accumulation of mtDNA CR heteroplasmy is a phenomenon shared by different populations, we applied the same methodological approach in 65 sib pairs from Finland (Tampere). In this case, DNAs from buffy coats (BC) were analyzed. Fig. 17 shows the distribution of mtDNA CR heteroplasmy levels.

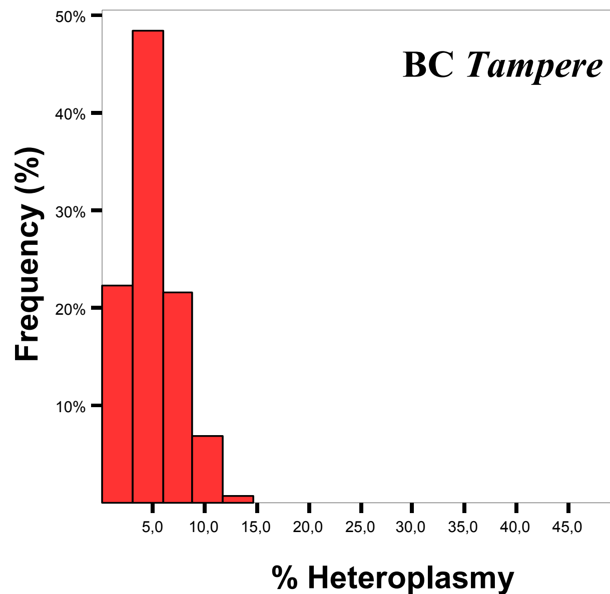


Figure 17. Heteroplasmy distribution in Finnish sib-pairs
The histogram shows the levels of heteroplasmy in buffy coats (BC) of sib-pairs from *Tampere*. The percentages of heteroplasmy are estimated on the DHPLC reference curve of Fig.13.

A significant correlation of the heteroplasmy levels was found also in Finnish sib-pairs (Fig. 18a); also in this case such a correlation was lost by performing the above mentioned permutation procedures (Fig. 18b).

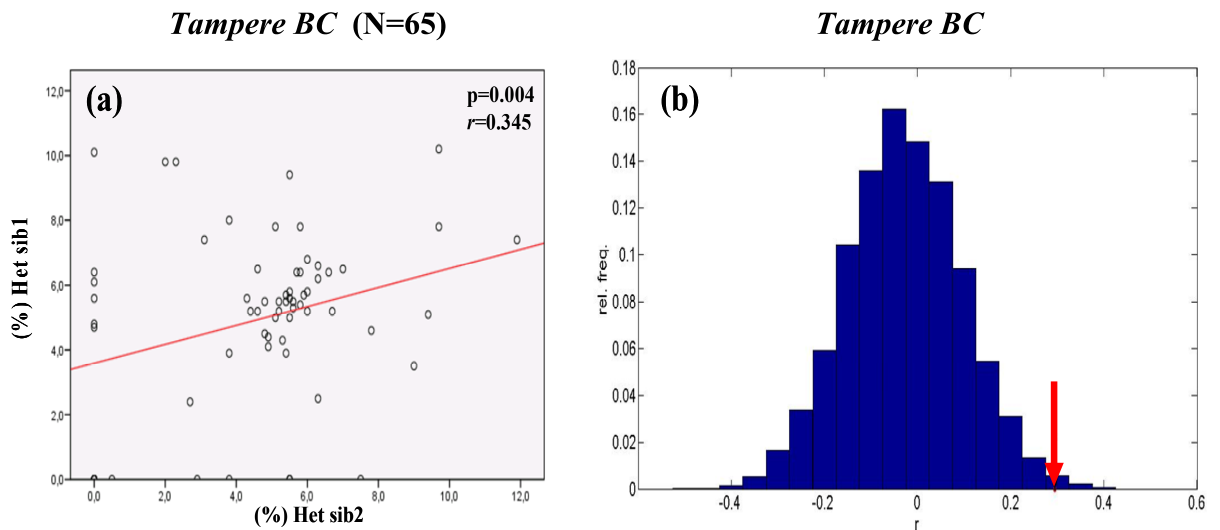


Figure 18. Analysis of correlation in sib pairs (a) and empirical distribution of the *r* values obtained by permutation procedure (b)
The arrow shows the correlation coefficient (r) observed in GEHA sib-pairs. The fraction of simulated *r* values which exceeds the value observed in sib pairs is 1.0%.

On the whole, in agreement with the results obtained by studying families of centenarians (Rose et al., 2007), evidences were obtained that the correlation between heteroplasmy levels in sib pairs is a genetically controlled phenomenon. In addition, such a genetic control is present in different populations (Italians and Finns).

Screening of the C150T transition in GEHA sib-pairs

Quantitative DHPLC showed to be very efficient in detecting mtDNA heteroplasmy. Moreover, combined data from DHPLC and sequence analyses showed that different point mutations can be revealed by quantitative DHPLC (Fig 19). However, this approach was limited to samples with a minimum level of heteroplasmy of 25%, as sequence analysis does not detect lower levels of heteroplasmy.

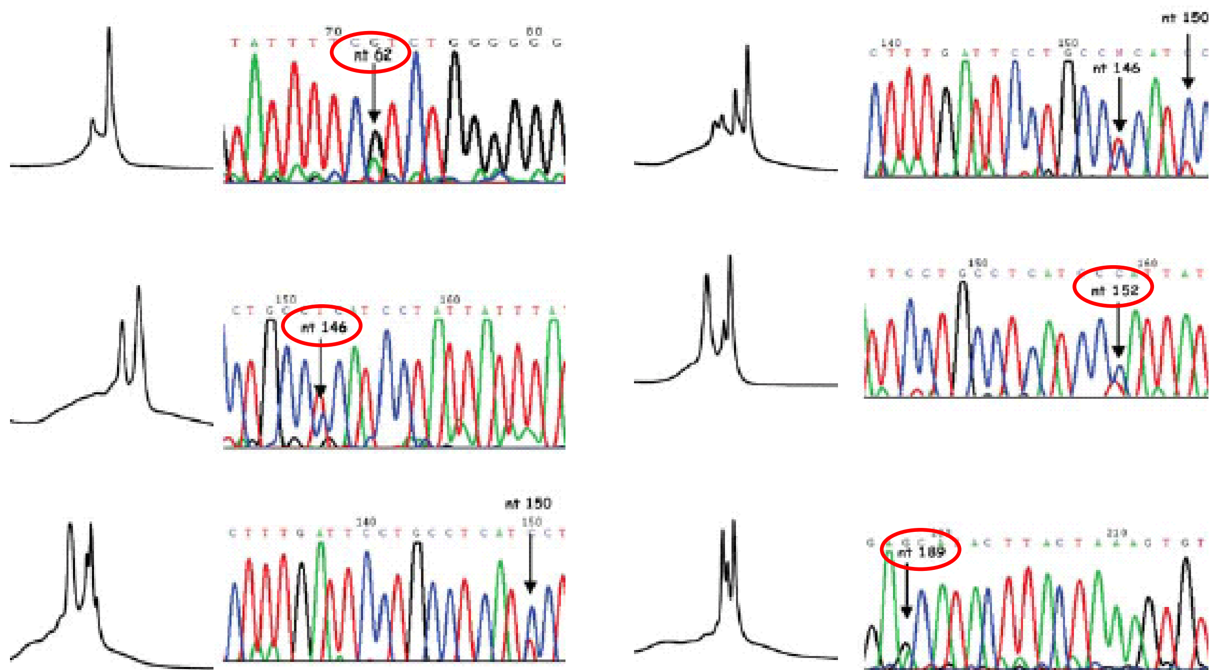


Figure 19. Combined data from DHPLC and sequence analyses
DHPLC profiles and sequence data observed in the samples having levels of heteroplasmy equal to or higher than 25%.

Thus, to specifically detect the presence of the C150T transition in samples with low heteroplasmy, we applied the PARFAH technology (Procaccio et al., 2006), a method by which we could not only determine the presence of the C150T transition, but also estimate the percentage of mutant DNA.

Fig. 20 shows the chromatogram patterns obtained for the wild-type (C150), the mutant (150T) and the heteroplasmic (50% C150 and 50% 150T) amplicons.

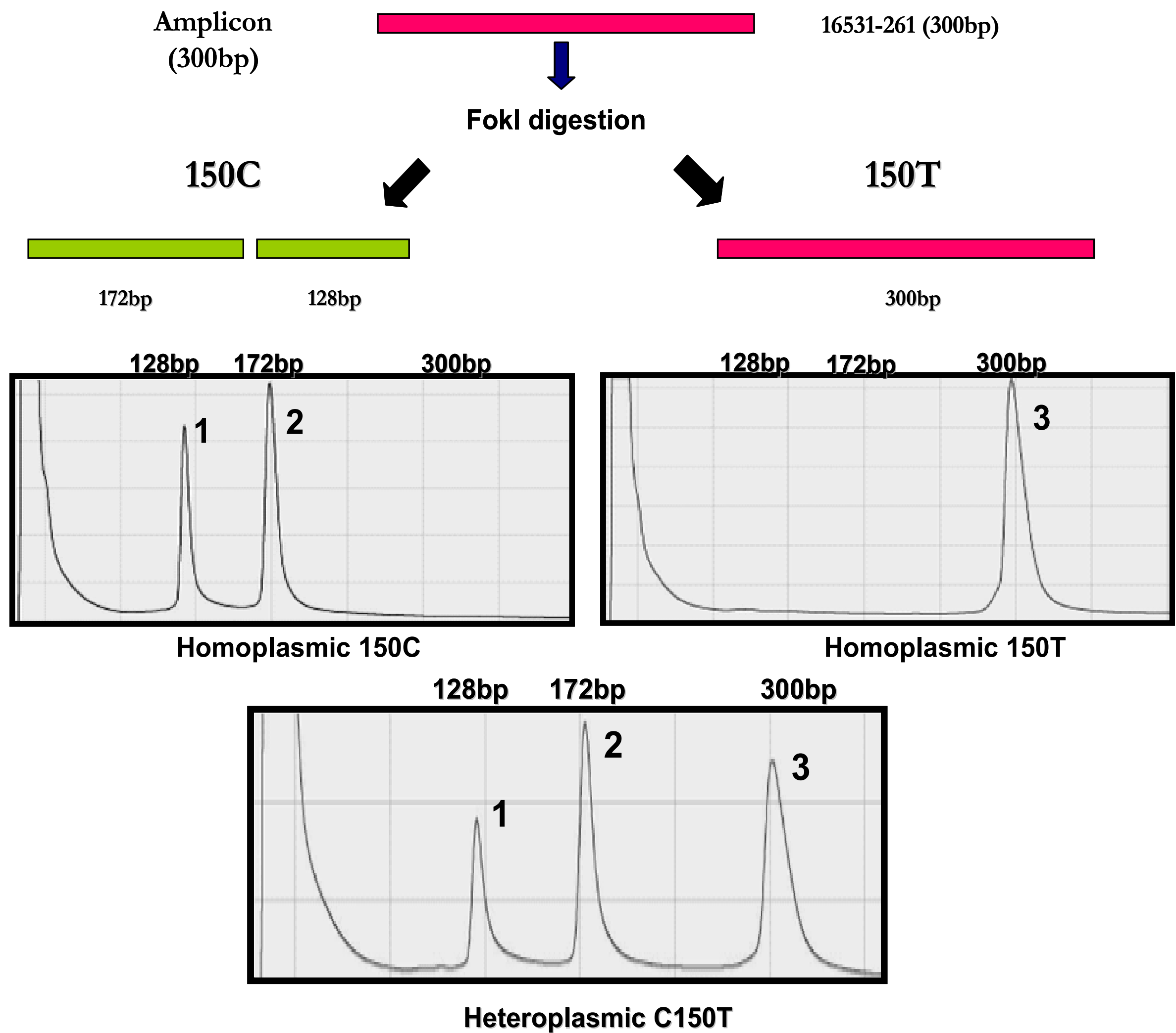


Figure 20. Schematic representation of the PARFAH method
Amplification, endonuclease digestion, HPLC separation and chromatogram analysis of DNA fragments submitted to the PARFAH method. The HPLC separation in non-denaturing conditions provide a "gel-like" separation: digested fragments eluate at different times depending on their molecular weigh. The peaks 1 and 2 refer to the homoplasmic 150C allele; the peak 3 refers to the homoplasmic 150T allele; the presence of the peaks 1, 2 and 3 refers to an heteroplasmic situation (50% of heteroplasmy): the levels of heteroplasmy can be estimated by calculating the percentage of the peak areas.

To test the sensitivity of the PARFAH method, we analyzed a series of samples with known heteroplasmy levels. These samples were obtained by mixing cloned sequences differing for the nucleotide in 150 position only (see Materials and Methods). As we can see in Fig. 21, this method is able to detect heteroplasmy levels as low as 2.5%.

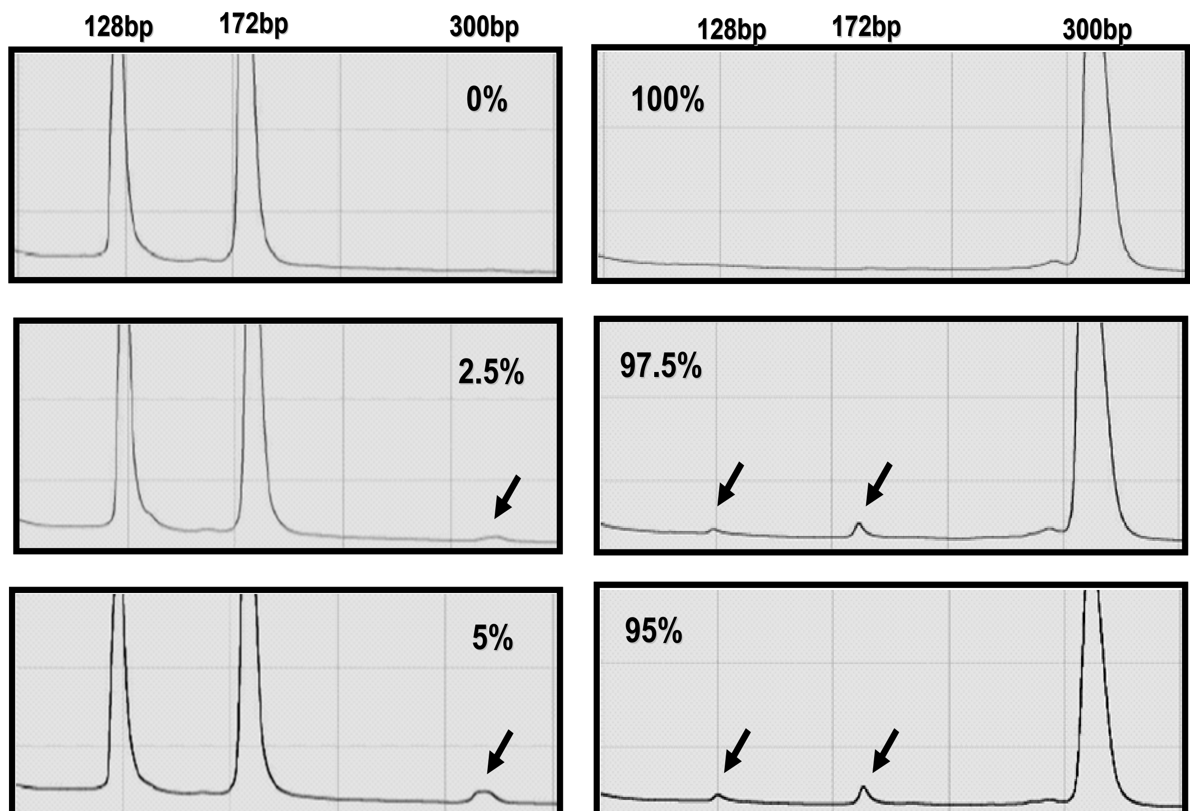


Figure 21. Chromatograms obtained for HPLC separation of digested samples with known heteroplasmy levels

The percentage on the chromatograms indicates the percentage of mutant allele (150T) in the sample. We found that the heteroplasmic peaks are clearly visible and can be reliably detected by the chromatogram analysis software (WAVE system's Navigator software, Transgenomic) up to the 2.5% of heteroplasmy.

Therefore, the PARFAH method provides a fast, reliable and sensitive strategy to analyse specifically the presence of the C150T transition in all the samples previously analysed by quantitative DHPLC.

In table 4 the results relevant to the heteroplasmic samples are summarized. We found that only a part of heteroplasmic samples carried the heteroplasmic form of the C150T transition, while the majority of the samples were heteroplasmic for other point mutations.

Sample group	**Heteroplasmic samples (DHPLC-based analyses)**	**Samples heteroplasmic for the C150T transition (PARFAH-based analyses)**
Calabria **GR**	124/126 (98.4%)	25/124 (20.2%)
Calabria **LY**	121/126 (96%)	21/121 (17.3%)
Bologna **GR**	121/134 (90.3%)	29/121 (24%)
Bologna **LY**	133/134 (99.2%)	24/133 (18%)
Tampere **BC**	108/130 (83.1%)	30/108 (27.8%)

Table 4. Proportion of samples heteroplasmic for the C150T mutation.

Then, we analyzed the distribution of the C150T transition in the Italian and Finnish sib-pairs. Fig. 22a shows the distribution of the C150T mutation in both GR and LY of Calabrian sib-pairs. Only 23 of 63 sib pairs exhibited the mutation at least in one sib (either in GR or in LY). For the majority of them we can observe that, when the 150T allele was present in one sib in homoplasmic or near homoplasmic form, high levels of heteroplasmy were present in the other. Conversely, when the 150C allele was present in one sib in homoplasmic or near homoplasmic form, low levels of heteroplasmy were present in the other. Indeed, correlation analyses of mutation levels between sibs of each pair indicated a very strong correlation ($r>0.98$, $p<0.001$) for both cell types (Fig. 22b). In addition, by looking at the distribution of the C150T transition in granulocytes (GR) and lympho-monocytes (LY) of each subject (Fig. 22a), a clear somatic contribution in the occurrence/accumulation of the mutation emerged. In fact, although a strong correlation of intra-individual heteroplasmy was observed ($r=0.991$, $p<0.001$) (Fig. 22c), some samples were found to be heteroplasmic in one cell type only.

Fig. 23a shows the distribution of the C150T mutation in GR and LY of sib-pairs from Bologna. Only 28 of 67 sib pairs exhibited the C150T mutation at least in one sib (in GR or in LY). Also in this case, we found a strong correlation in mutation levels between sibs ($r>0.673$, $p<0.001$)(Fig. 23 a and b). Again, as shown in Fig. 23c, the analysis of the intra-individual heteroplasmy reveals a somatic contribution to the occurrence/accumulation of the transition, and a strong correlation of heteroplasmy levels between the two blood cell types (GR and LY) of the same individual ($r=0.774$, $p<0.001$).

The analysis of Finnish sib-pairs showed that only 20 of 65 sib pairs exhibited the mutation at least in one sib (Fig.24a). It is clear, by looking at Fig. 24a, that in the majority of cases the mutation is somatically acquired, while only in two cases (number 4 and 7) it seems to be inherited. Also for the Finnish sib-pairs, a strong correlation in mutation levels was found ($r=0.885$, $p<0.001$)(Fig 24b).

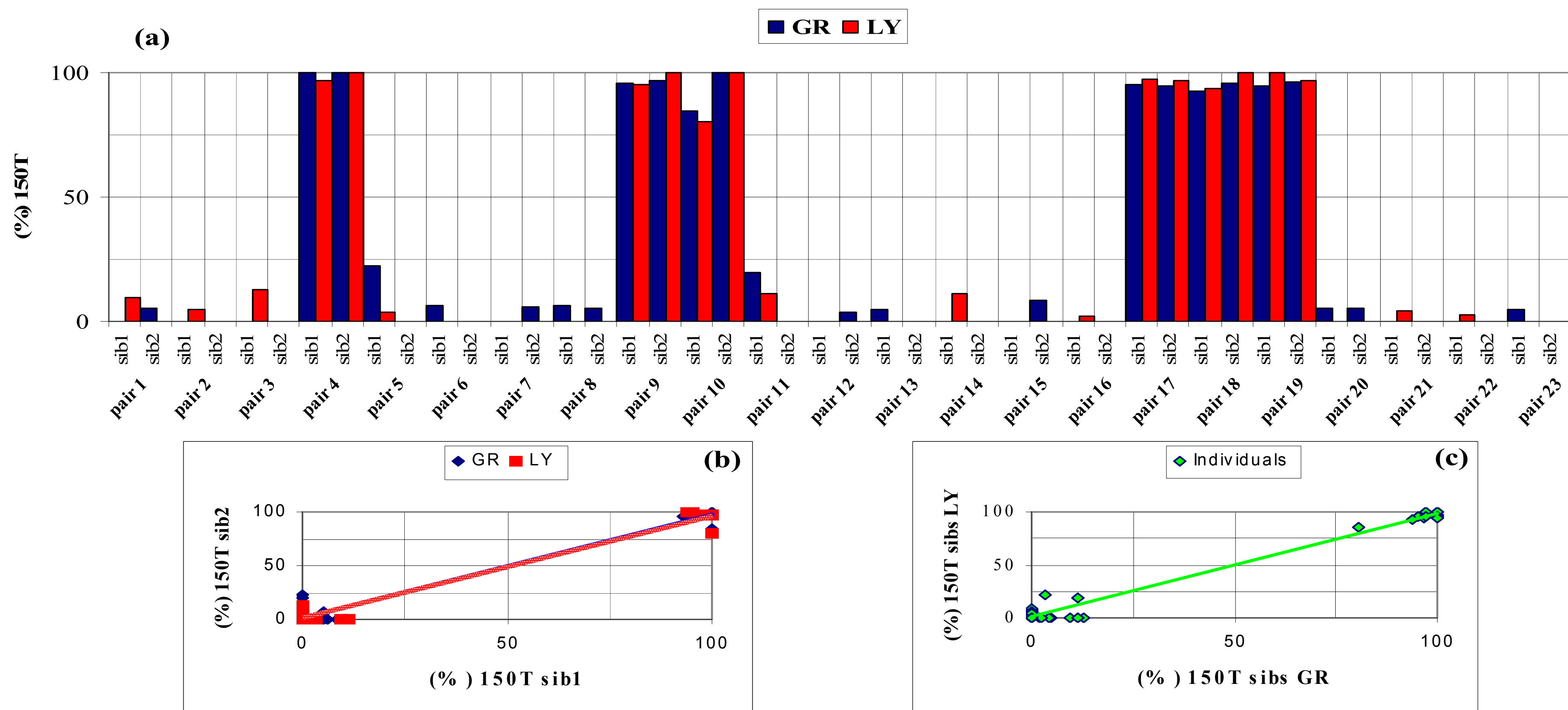


Figure 22. Analysis of the C150T mutation in sib-pairs from *Calabria.*
a) Bar graph summarizing the frequency and the distribution of the C150T in GR and LY. Red bars, mutation levels in LY; blue bars mutation levels in GR. In the x-axis is indicated each sib pair carrying the mutation; sib1 and sib2 refers respectively to the older and the younger sib in the pair. b) Correlation analyses for mutation levels in sib-pairs (GR and LY). Mutation levels are indicated as blue points in GR, and as red points in LY. Regression lines for LY (red, $r=0.99$ and $p<0.001$) and GR (blue, $r=0.986$ and $p<0.001$) are also indicated. c) Correlation analyses for mutation levels in GR and LY from the same individual. Mutation levels are indicated as green points. The regression line is also indicated ($r=0.991, p<0.001$).

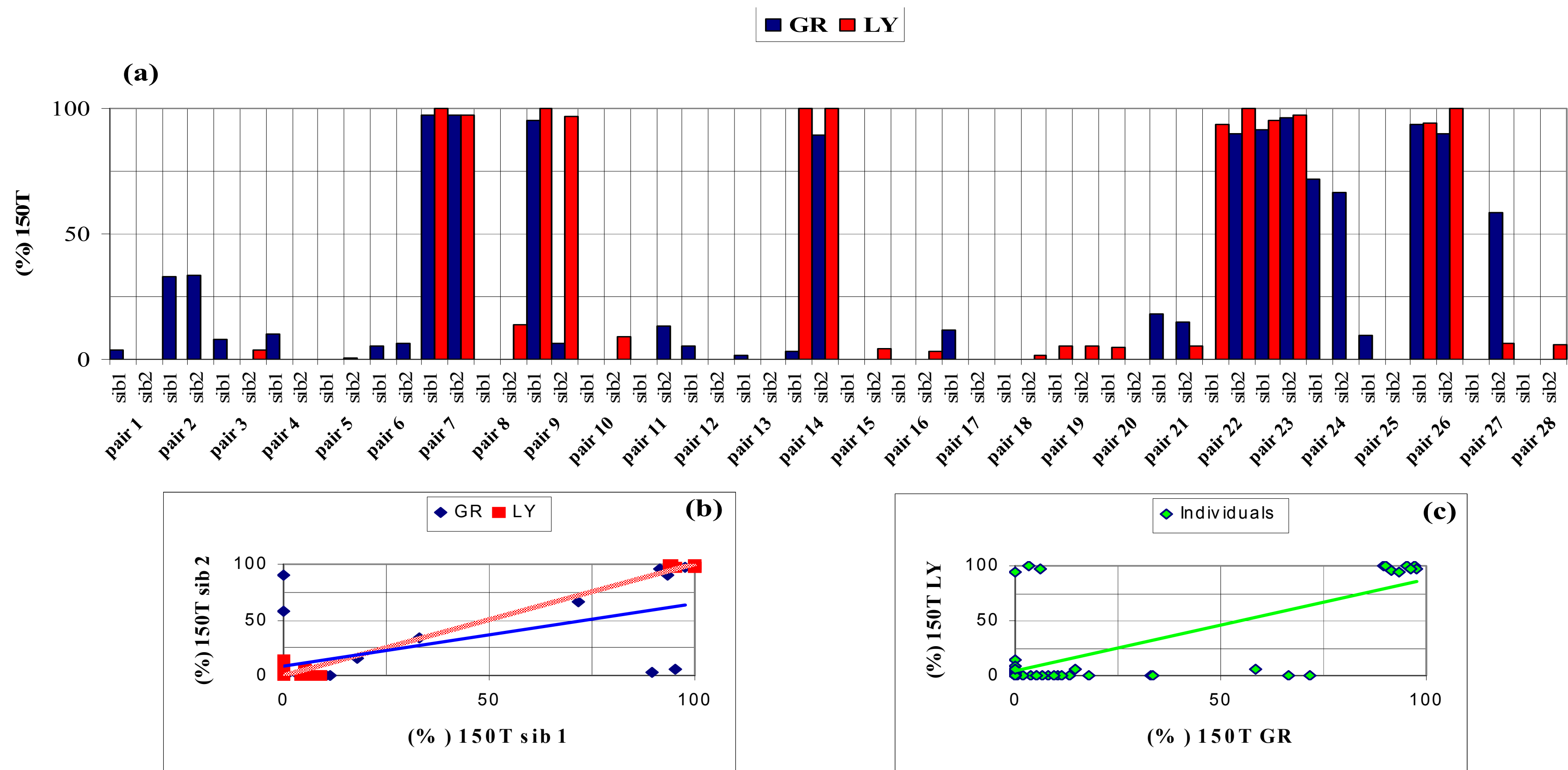


Figure 23. Analysis of the C150T mutation in sib-pairs from *Bologna.*
a) Bar graph summarizing the frequency and the distribution of the C150T in GR and LY. Red bars, mutation levels in LY; blue bars mutation levels in GR. In the x-axis is indicated each sib pair carrying the mutation; sib1 and sib2 refers respectively to the older and the younger sib in the pair. b) Correlation analyses for mutation levels in sib-pairs (GR and LY). Mutation levels are indicated as blue points in GR, and as red points in LY. Regression lines for LY (red, r=0.995 and p<0.001) and GR (blue, r=0.673 and p<0.001) are also indicated. c) Correlation analyses for mutation levels in GR and LY from the same individual. Mutation levels are indicated as green points. The regression line is also indicated (r=0.774, p<0.001).

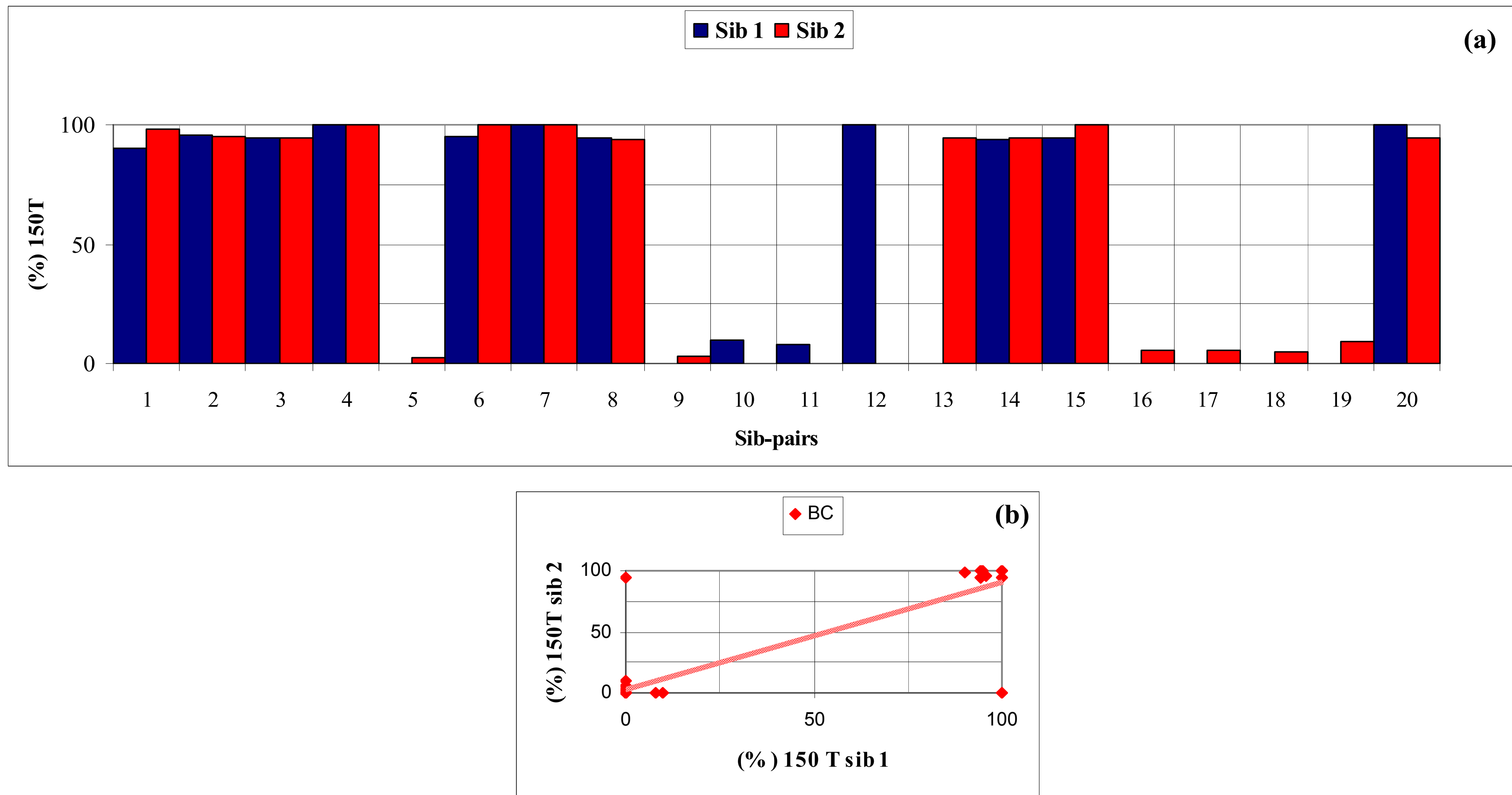


Figure 24. Analysis of the C150T mutation in sib-pairs from *Tampere.*
a) Bar graph summarizing the frequency and the distribution of the C150T in BC. Blue bars mutation levels in sib1; red bars, mutation levels in sib2;. In the x-axis each sib-pair carrying the mutation is indicated. b) Correlation analyses for mutation levels in sib-pairs (BC). Mutation levels are indicated as red points. The regression line (red, r=0.885 and p<0.001) is also indicated.

In summary, the correlation analysis of the C150T heteroplasmy in sib-pairs from different populations, as well as correlation analysis in different blood cell types (GR and LY) from a single individual, consistently suggest a nuclear genetic control on the occurrence/accumulation of such a mutation. This finding is in line with the results obtained by Zhang and co-workers (Zhang et al., 2003), showing that the levels of the C150T heteroplasmy are more concordant in MZ than in DZ twins. However, we can not exclude that the inherited form of the transition and/or a possible somatic acquisition of the C150T mutation in stem cells during haematopoiesis can play an important role in the observed correlation.

Heteroplasmy due to mutations different from C150T

As shown in table 4, by combining the data of DHPLC and PARFAH analyses, we found that only a part of heteroplasmic samples carried the heteroplasmic form of the C150T transition, while the majority of the samples were heteroplasmic for other point mutation(s). We then investigated whether CR heteroplasmy not due to the C150T transition was correlated between sibs. Interestingly, as shown in Fig. 25, a significant correlation was observed in the Bologna and in the Tampere groups ($p<0.05$ in both GR and LY), but not in the Calabria group ($p>0.05$ in both GR and LY).

These results suggest that in sib-pairs from Bologna and from Tampere, in contrast to the sib-pairs from Calabria, other heteroplasmic mtDNA CR mutation(s) (different from the C150T transition) may play a role in the genetically controlled occurrence/accumulation of the mtDNA CR heteroplasmy. Therefore, our data suggest that other mutations in addition to the C150T transition have a role in the remodelling of mtDNA replication in order to compensate functional deterioration at old age. On the other hand, the discordance between different samples suggests that these mutations can be population-specific. In fact, differences between populations have been already recognized not only for the C150T mutation (Iwata et al., 2007), but also for mtDNA inherited variants (Dato et al., 2004; Shlush et al., 2008). It is likely that environmental factors, the nuclear genetic pool and/or their interaction may play a role in the occurrence/accumulation of mtDNA CR heteroplasmy.

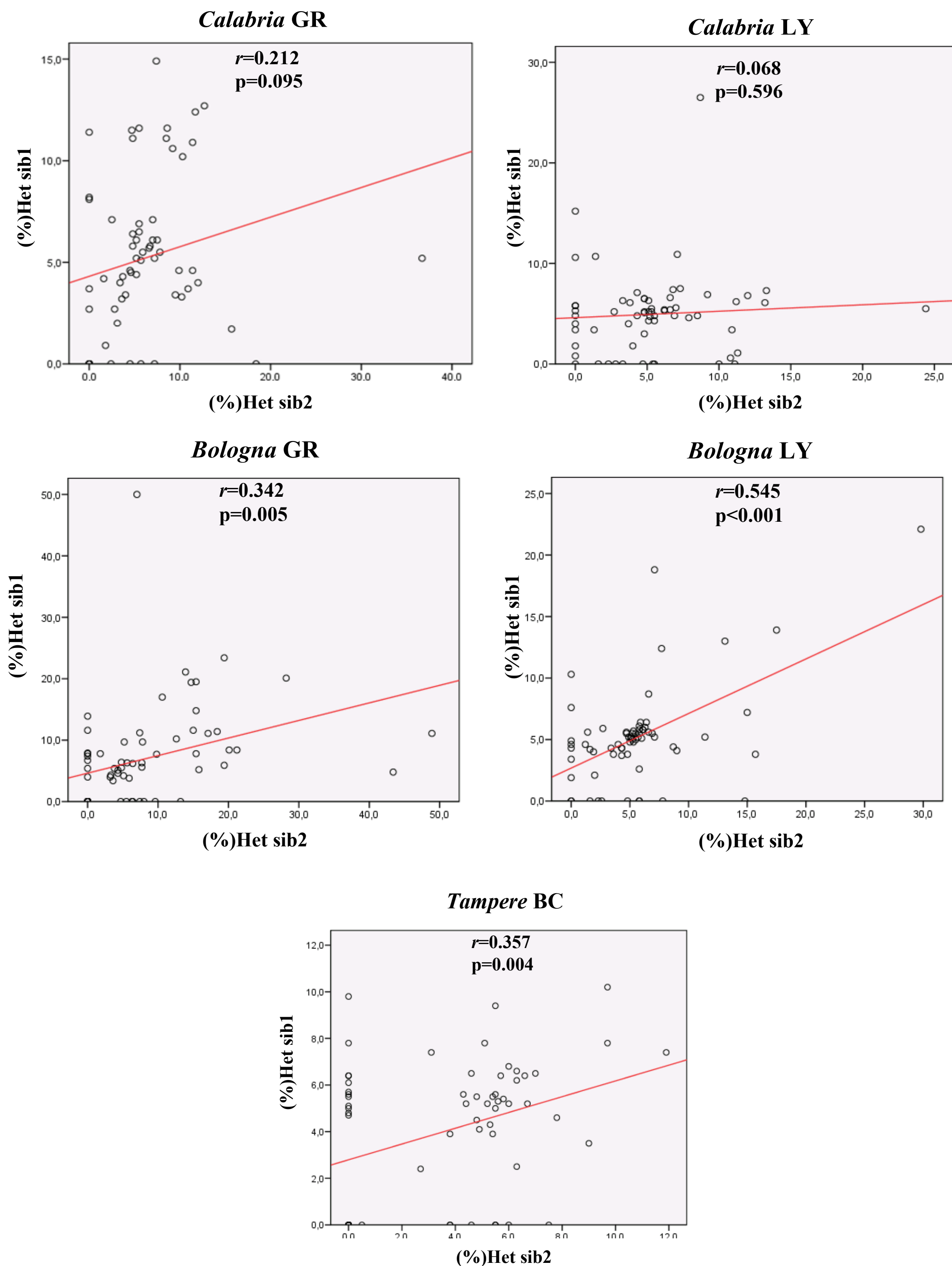


Figure 25. Correlation analysis of other mtDNA CR mutation(s) in GEHA sib-pairs.

For each sample group are indicated regression lines and correlation coefficients (r). The p-values are obtained by the 2-tailed Pearson test.

In conclusion, this study confirms the high incidence of somatic point mutation and the consequent heteroplasmy in the mtDNA CR of long lived subjects. On the other hand, we showed that heteroplasmy may be due to the previously described C150T mutation, but also to other mutations, which may be population-specific. In any case, the genetic control of the mtDNA CR heteroplasmy suggests that the occurrence of mtDNA somatic mutations may represent an important strategy for the age-related remodelling of the organismal functions.

5. Concluding remarks

The majority of the current data agree with a detrimental effect of mtDNA somatic mutations on the cell activities. However, recent findings on the occurrence/accumulation of somatic mutations in the mtDNA CR in Italian centenarians are challenging this idea: the inherited or somatically acquired C150T transition, by remodelling a mtDNA replication origin (OH2), can restore the replication machinery, providing a survival advantage (Zhang et al., 2003).

To study mtDNA CR heteroplasmy in relation to aging and longevity, we took advantage of large population samples collected in the frame of the European Challenge for Healthy Aging (ECHA) and the GEnetics of Healthy Aging (GEHA) projects, where centenarians and their descendants and ultra-nonagenarians sib pairs were respectively collected.

The analysis of mtDNA CR heteroplasmy in leukocytes from families of centenarians and unrelated controls (ECHA samples) showed that the observed occurrence/accumulation of heteroplasmy in the mtDNA CR is not a phenotypic consequence of extreme aging, but a genetically controlled phenomenon (which is likely due to the nuclear genome) that provides a survival advantage. In fact, centenarians and their descendants, despite the different ages, showed similar levels of heteroplasmy which were significantly higher than levels in controls who were age matched with centenarian's descendants.

To further investigate such a genetic control, we performed a study on a population sample collected in the frame of the GEHA project (http://www.geha.unibo.it/). The hypothesis of our work was that, if the mtDNA CR heteroplasmy is genetically modulated, sib-pairs selected for longevity will be correlated in mtDNA CR heteroplasmy levels. In fact, we found that the mtDNA CR heteroplasmy levels are significantly correlated in ultra-nonagenarians sib-pairs sampled in different European populations from northern and southern Europe (Finns and Italians). By permutation procedures, we demonstrated that such a correlation is due to the kinship between sibs. Moreover, the strong correlation of heteroplasmy levels between sibs of a single pair and between different leukocytic cells (GR and LY) of a single subject suggested that

the occurrence/accumulation of the C150T mutation in sib-pairs selected for longevity is a somatic event under a nuclear genetic control.

These results, in agreement with those found by Zhang (Zhang et al., 2003), add intriguing clues to the contribution of the T allele to the longevity trait. It is likely that the C150T mutation, providing a new replication site to the mtDNA, could be related to the regulation of the of the mitochondrial genome replication through a copy number control mechanism. This remodelling could accelerate the mtDNA replication to compensate a functional deterioration occurring at old ages. Interestingly, we also found that sib pairs from northern Italy and Finland, but not sib pairs from Calabria, are correlated also for the mtDNA CR heteroplasmy not due to the C150T transition. This suggests that, in addition to the C150T transition, other population-specific somatic mutations could be involved in such a remodelling.

The nuclear genetic control on the occurrence/accumulation of mtDNA CR heteroplasmy in sib-pairs selected for longevity highlights the relation between the nuclear and the mitochondrial genomes which interplay in complex phenotypes, like longevity. The involvement of the mitochondrial fission in the remodelling of the mitochondrial network morphology associated with the cell proliferation (Chen et al., 2004; Chan et al., 2006), the cell differentiation (e.g. neuronal development) (Park et al., 2001), and with the regulation of apoptotic events (Youle et al., 2005; Chan et al., 2006) could be genetically determined also through a coordinate regulation of the mtDNA replication.

On the whole, our study has provided new clues on the relevance of mtDNA somatic mutations, and of the consequent heteroplasmy, for aging and longevity. Surprisingly, if one considers previous literature maintaining that mtDNA mutations are detrimental, we observed that the occurrence and the accumulation of somatic mtDNA CR mutations are one of the strategies to remodel cell biological functions in aging.

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END SECTION

Reprint of the published paper *"The mitochondrial DNA control region shows genetically correlated levels of heteroplasmy in leukocytes of centenarians and their offspring".*

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The mitochondrial DNA control region shows genetically correlated levels of heteroplasmy in leukocytes of centenarians and their offspring

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Abstract

Background: Studies on heteroplasmy occurring in the mitochondrial DNA (mtDNA) control region (CR) in leukocytes of centenarians and younger subjects have shown that the C150T somatic transition is over-represented in centenarians. However, whether the occurrence/accumulation of heteroplasmy is a *phenotypic consequence* of extreme ageing or a *genetically controlled event* that may favor longevity is a question that deserves further attention. To clarify this point, we set up a Denaturing High Performance Liquid Chromatography (DHPLC) protocol to quantify mtDNA CR heteroplasmy. We then analyzed heteroplasmy in leukocytes of centenarians (100 subjects), their offspring and nieces/nephews (200 subjects, age-range 65–80 years, median age 70 years), and in leukocytes of 114 control subjects sex- and age-matched with the relatives of centenarians.

Results: The centenarians and their descendants, despite the different ages, showed similar levels of heteroplasmy which were significantly higher than levels in controls. In addition we found that heteroplasmy levels were significantly correlated in parent-offspring pairs ($r = 0.263$; $p = 0.009$), but were independent of mtDNA inherited variability (haplogroup and sequence analyses).

Conclusion: Our findings suggest that the high degree of heteroplasmy observed in centenarians is genetically controlled, and that such genetic control is independent of mtDNA variability and likely due to the nuclear genome.

Background

Mitochondrial DNA (mtDNA) is much more exposed to mutagenic events than nuclear DNA (nDNA) due to its high replication rate, lack of histone-like proteins, scarcity of repair enzymes, and production of Reactive Oxygen Species (ROS) which results from Oxidative Phosphorylation (OXPHOS) in mitochondria. The fate of heteroplasmic mutations depends on several factors, including type and location of the variation, replication rate of the cell, and also to chance since the mutant molecules can be randomly lost as a consequence of mitochondria replicative segregation. In any case, since mutations are stochastic events, mtDNA heteroplasmy tends to increase with age. Usually, a low level of heteroplasmy does not impair mitochondrial function, but once the level of mutant mtDNA exceeds a certain threshold, OXPHOS dysfunction may arise [1,2]. The cell tries to cope with such a stressful condition by increasing OXPHOS, and therefore producing ROS, in a vicious circle that may become lethal to the cell itself.

As a rule, the age-related accumulation of mtDNA somatic mutations leads to a decline in mitochondrial function, which contributes to ageing and degenerative diseases [3,4]. In fact, most of the literature on mtDNA somatic mutations reports data on the role played by mtDNA heteroplasmy on age-related diseases, but recent findings open a new perspective. Zhang et al. [5] carried out a large-scale screening of the mtDNA main control region in leukocytes from centenarians and younger controls. They found that the C150T mutation is significantly more represented in centenarians than in younger controls, and provided evidence that somatic events, probably under nuclear genome control, contribute to the striking selective accumulation of this mutation in centenarians. In the same report, using fibroblast longitudinal studies the authors showed an age-related somatic expansion of the mutation up to homoplasmy. Finally, 5' end analysis of nascent heavy mtDNA strands revealed a new replication origin at position 149, substituting the one at 151, only in fibroblasts or immortalised lymphocytes carrying the C150T mutation. On the whole, the data showed that a high level of C150T heteroplasmy, possibly up to a new homoplasmy arrangement, might be favourable for longevity. In agreement, a significant association between the inherited C150T mutation and longevity has been observed in both Finnish and Japanese populations [6].

The study by Zhang et al. [5] is of great value because it indicates a possible beneficial effect on longevity by an mtDNA somatic mutation able to restore the mitochondrial replication machinery. Therefore, it seemed worthwhile to further investigate possible links between mtDNA CR heteroplasmy and longevity. Since longevity shows clear patterns of familiarity [7,8], the study of such a heteroplasmy in relatives of centenarians may help to clarify the role of mtDNA somatic variability in longevity.

We set up a Denaturing High Performance Liquid Chromatography (DHPLC) protocol by which the heteroplasmy of an mtDNA CR fragment encompassing the C150T mutation could be quantified. Then we compared the levels of heteroplasmy between relatives of centenarians (offspring and nieces/nephews) and age-matched controls. Indeed, if heteroplasmy accumulates because of age-related stochastic events, it should be similar between age-matched groups regardless of the genetic relationship with centenarians. On the contrary, if the heteroplasmy is under genetic control, it should be higher in relatives of centenarians than in controls. In this case, the heteroplasmy occurring in the mtDNA region under study may be regarded as a contributing factor to familial recurrence of longevity.

Results

DHPLC reference curve and sensitivity of the method

In order to quantify the levels of heteroplasmy in the biological samples under study, we applied DHPLC to artificial heteroplasmic samples and assembled the curve shown in Fig. 1. The reference curve was used for estimating the levels of heteroplasmy in the biological samples.

In order to compare DHPLC and sequencing sensitivity in revealing heteroplasmy, we submitted all the artificial samples reported in Fig. 1 to sequence analysis. We found that DHPLC is able to reveal up to 5% of heteroplasmy, while the minimum level of heteroplasmy detectable by sequence analysis is roughly 20–25%.

Before applying DHPLC to the biological samples, we verified that the PCR conditions were specific for the mtDNA fragment under study (16531 nt-261 nt). No signal indicating DNA amplification was observed by applying our PCR protocol to DNA extracted from rho-zero cells (cells depleted of mitochondria). Furthermore, the PCR primers gave negative results to BLAST search.

Heteroplasmy in families of centenarians

DHPLC was then applied to PCR products of DNA extracted from leukocytes of four sample groups: centenarians, offspring and nephews/nieces of centenarians, and controls. Fig. 2 shows the distribution of the levels of heteroplasmy in the four groups, as estimated using the reference curve in Fig. 1.

Fig. 2 shows that approximately 15% of both centenarians and their children, approximately 20% of nephews/nieces of centenarians and more than 35% of controls display levels of heteroplasmy lower than 2.5%. Therefore, not

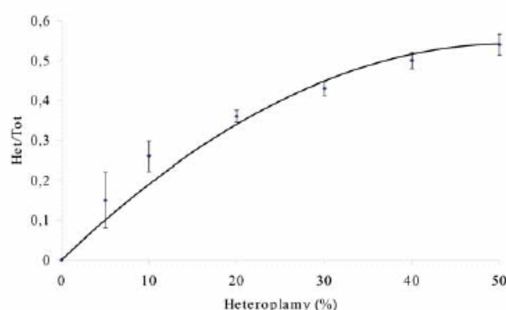

Figure 1
DHPLC reference curve assembled from clones of mtDNA CR (16531 nt -261 nt) including either C150 or T150. The two clones were combined to generate samples having heteroplasmy levels of 0%, 5%, 10%, 20%, 30%, 40%, 50 %. Het/Tot is the ratio between the height of the Heteroplasmic peak and the Total height of homoplasmic plus heteroplasmic peaks. Bars denote the standard deviation in triplicate experiments. The observed values were used to fit a 2nd degree polynomial function $y = \beta_1 x + \beta_2 x^2$.

only do rather few centenarians have low heteroplasmy, but the same occurs in their younger relatives.

We checked the statistical significance of the differences shown in Fig. 2 by comparing the patterns of heteroplasmy between pairs of samples. Centenarians differed from younger controls ($p = 0.001$) while they did not from their relatives ($p = 0.699$ and $p = 0.944$ by comparing centenarians with offspring and nieces/nephews, respectively). What is more, the comparison between age-matched groups revealed that heteroplasmy differed between groups according to the presence/absence of a centenarian in the family ($p = 0.666$ between offspring and nieces/nephews of centenarians; $p = 0.006$ between offspring of centenarians and controls; $p = 0.003$ between nieces/nephews of centenarians and controls). The above differences remained significant when the level of significance was reduced to $\alpha = 0.009$ (six independent comparisons).

The finding that the centenarians' offspring displayed a heteroplasmic pattern similar to that of their very old parents suggested that the level of mtDNA CR heteroplasmy could be under genetic control. By linear regression analysis we confirmed a genetic control on this trait, as the levels of heteroplasmy resulted significantly correlated in parent-offspring pairs (Fig. 3; $r = 0.263$; $p = 0.009$).

Which genome (mtDNA or nDNA) could account for the above results? In order to answer this question we partitioned the group of parents according to sex (Fig. 4), and found that the levels of heteroplasmy were significantly correlated in mother-offspring pairs ($r = 0.456$; $p = 0.001$) while there was no correlation in father-offspring pairs ($r = -0.053$; $p = 0.704$).

The above result, which indicated a maternal genetic control, prompted us to search for a possible association between inherited and epigenetic (somatic) mtDNA variability. Thus, we screened haplogroup and haplotype variability in both centenarians and controls, and analyzed the distribution of heteroplasmic subjects within each haplogroup/haplotype category. The data are reported in Tables 1 and 2.

Table 1: Inherited and epigenetic mtDNA variability. The number of subjects classified within a specific haplogroup is reported together with the number of subjects showing heteroplasmy levels higher than 5%. MtDNA haplogroups are classified according to Torroni et al., [27].

MtDNA Haplogroup	Centenarians		Controls	
	Absolute frequency	Heteroplasmic subjects	Absolute frequency	Heteroplasmic subjects
H	35	15	34	10
I	2	2	0	0
J	11	8	17	7
K	10	6	16	6
T	6	3	10	3
U	13	7	11	4
V	1	1	1	0
W	2	1	4	3
X	9	8	5	3
Other	11	4	16	6
Total	100	55	114	47

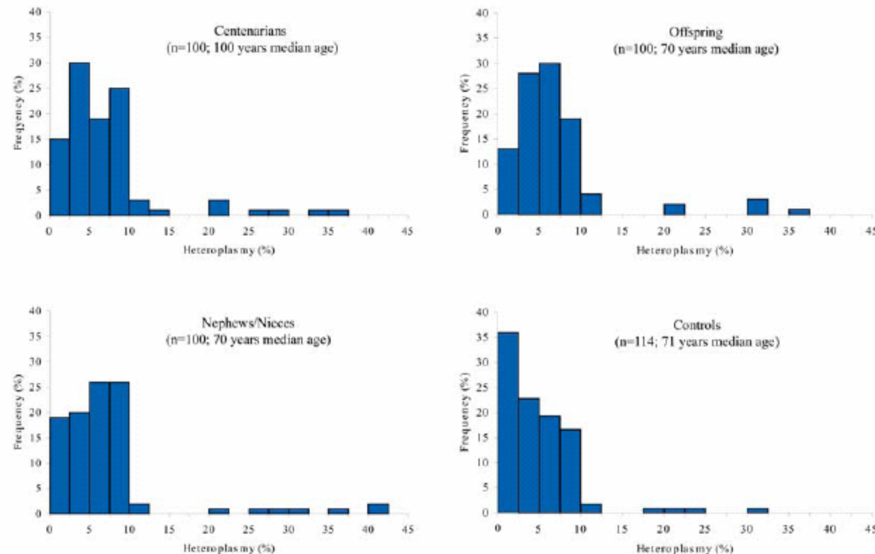

Figure 2
Histograms showing mtDNA CR heteroplasmy in the four sample groups. Heteroplasmy is estimated from the DHPLC reference curve reported in Fig.1.

By comparing the distribution of heteroplasmic subjects within haplogroups (Table 1) or haplotypes (Table 2) with the distribution expected under random association between epigenetic and inherited variability, no significant difference was found either in centenarians or in controls ($p > 0.2$ by permutation tests).

In addition, we compared the pools of haplotypes (Table 2) between centenarians and controls by considering as inherited the variant whose sequence peak was higher than 80%. No significant difference was evident between the groups ($p = 0.999$ by permutation test); therefore the high heteroplasmy observed in centenarians was not due to one or few haplotypes which could be particularly prone to somatic mutations. On the whole, haplogroup and haplotype analyses consistently indicated that mtDNA CR heteroplasmy is independent of mtDNA inherited variability.

The C150T somatic mutation

The DHPLC protocol used for assembling the reference curve of Fig. 1 had been set up using mtDNA cloned fragments that only differed in the C150T position. However, when we deal with biological samples, we cannot exclude that the DHPLC patterns of heteroplasmy we obtain might be due to other heteroplasmic sites. To highlight this point, first of all we carried out a careful visual inspection of every DHPLC profile in centenarians, their offspring, and controls. More than 60% of the samples showed DHPLC profiles comparable with those obtained with the different mixtures of C150 and T150 clones; however other profiles were also observed, suggesting the presence of additional heteroplasmic mutations in the region under study. Thus we compared DHPLC profile and sequence data whenever possible (requirement: minimum of 25% heteroplasmy). Out of 16 samples satisfying the requirement, we identified the six profiles reported in Fig. 5, where the corresponding heteroplasmic mutations detected by sequence analysis are also shown. Further data on sequence analyses on DHPLC fractions collected with a fraction collector are ongoing at present.

Table 3 summarises the proportion of different mutations found in centenarians, their offspring and controls. The C150T somatic mutation was present in 4 out of 7 centenarians, 4 out of 6 children of centenarians, and, in association with the T146C mutation, in 2 out of 3 controls.

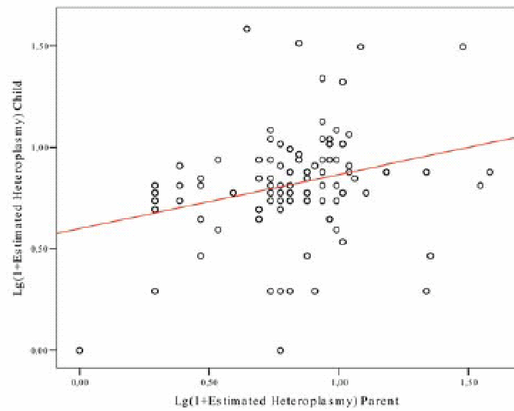

Figure 3
Linear regression between heteroplasmy levels in centenarians (x axis) and their offspring (y axis). Log transformed values were used in order normalise the distribution. The regression line showed r = 0.263 (p = 0.009).

Discussion

The aim of the present study was to reconsider an intriguing observation: a strikingly high frequency of the C150T mutation in mtDNA CR in centenarians [5]. The observation was at odds with the consolidated idea that heteroplasmy is detrimental for attaining longevity, considering that a variety of mtDNA deletions and mutations accumulate with age [9] and that mitochondrial function declines with age [10]. The novelty of our approach was to compare mtDNA CR heteroplasmy in descendants of centenarians (both offspring and nephews/nieces) and age-matched controls unrelated to centenarians. The analysis of offspring of centenarians is a valuable tool in searching for susceptibility genetic factors in longevity [11]; however, it has never been used to investigate a putative role of mtDNA somatic variability on longevity.

The first requirement for our study was to set up a fast and reliable method to screen mtDNA heteroplasmy, and DHPLC met our requirement. Till now DHPLC has been applied to detect mutations on the entire mtDNA molecule in samples of rather limited size [12-16]. In our case, we needed to carry out quantitative comparisons of the level of heteroplasmy in a sole mtDNA region, the control region, but in a large population sample (414 subjects in total). The DHPLC protocol we set up provided reliable results (see standard deviations of the reference curve in Fig. 1) and was reasonably sensitive.

A further critical point was to establish that the PCR protocol was specific for the amplification of the 16531 nt-261 nt mtDNA fragment. In fact, it was recently shown that the pseudo-mitochondrial genome can induce errors in heteroplasmy interpretation [17]. The negative results we obtained both by processing rho-zero cells and by a BLAST search excluded that nuclear pseudogenes contaminated mtDNA PCR amplifications.

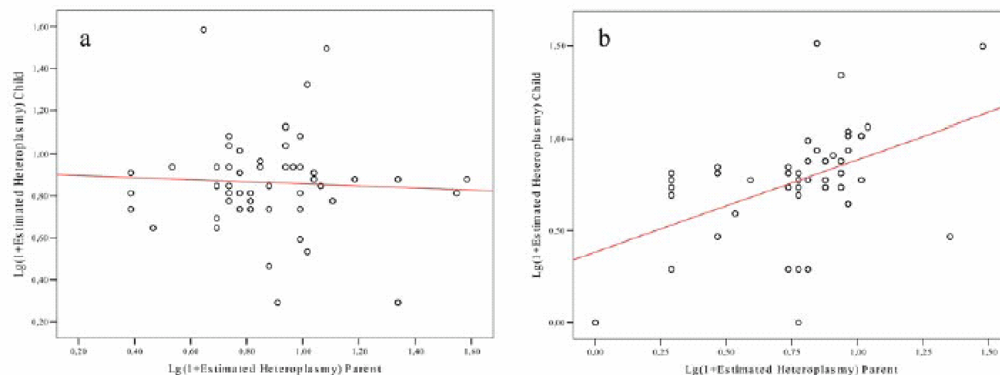

Figure 4
Linear regression of heteroplasmy levels in parent-offspring pairs according to the sex of the parent: (a) male centenarian parents; (b) female centenarian parents. The regression lines showed r = -0.053 (p = 0.704) and r = 0.456 (p = 0.001) in (a) and (b), respectively.

Table 2: Variant sites found in mtDNA CR (16531 nt – 261 nt). For each haplotype the absolute frequency in centenarians (A) and controls (B) is reported. The number of subjects showing DHPLC heteroplasmy levels higher than 5% is reported in parenthesis. rCRS refers to the revised Cambridge Reference Sequence [28].

	Nt	20	55	57	64	72	73	93	114	143	146	150	151	152	153	182	185	188	189	191	194	195	198	199	200	204	207	217	225	226	227	228	235	239
	rCRS	A	T	T	C	T	A	A	C	G	T	C	C	T	A	C	G	A	A	A	C	T	C	T	A	T	G	T	G	T	A	G	A	T
A	B																																	
27(14)	18(3)	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2 (1)	0	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3 (3)	0	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-
21(8)	24(6)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5 (3)	7 (4)	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 (0)	0	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
1 (1)	2 (0)	-	-	-	-	-	G	-	-	-	-	T	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
1 (1)	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-
1 (1)	0	-	-	-	-	-	G	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 (1)	7 (6)	-	-	-	-	-	G	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 (1)	0	-	-	-	-	-	G	-	-	-	-	T	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 (1)	0	-	-	-	-	-	G	-	-	-	C	-	-	-	G	-	-	-	-	-	-	C	-	-	-	-	-	-	A	C	-	-	-	-
4 (4)	7 (1)	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
1 (1)	0	-	-	-	-	-	G	-	-	A	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	A	C	-	-	G	-
1 (1)	2 (0)	-	-	-	-	-	G	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 (0)	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-
2 (2)	0	-	-	-	-	-	G	-	-	-	-	-	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3 (1)	2 (2)	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-
1 (0)	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 (0)	0	-	-	-	-	-	G	-	-	-	-	-	-	-	G	-	-	-	-	-	-	C	-	-	-	-	-	-	A	C	-	-	-	-
1 (0)	0	-	-	-	-	-	G	-	-	A	C	-	-	C	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
2 (1)	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-
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1 (1)	6 (5)	-	-	-	-	-	G	-	-	-	-	T	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 (1)	0	-	-	-	T	-	G	-	-	-	-	-	-	-	G	-	-	-	-	-	-	C	-	-	-	-	-	-	A	C	-	-	-	-
1 (0)	0	-	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
2 (1)	0	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C
2 (0)	7 (1)	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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1 (1)	2 (2)	-	-	-	-	-	G	-	-	-	-	-	-	C	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
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1 (1)	0	-	-	-	-	C	-	G	-	-	-	T	-	-	-	-	-	-	-	-	-	C	T	-	-	-	-	-	-	-	-	-	-	-
1 (1)	4 (2)	-	-	-	-	-	G	-	-	-	C	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 (1)	0	-	-	-	-	-	G	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 (0)	1 (0)	-	-	-	-	-	G	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 (1)	0	-	-	-	-	-	G	-	-	-	-	T	-	-	-	-	-	-	-	-	-	C	-	-	G	-	-	-	-	-	-	-	-	-
0	2 (1)	-	-	-	-	C	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-
0	1 (0)	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	A	-	G	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-
0	1 (0)	-	-	-	-	C	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0	3 (3)	-	-	-	-	-	G	-	-	-	-	-	-	C	-	-	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0	1 (1)	-	-	-	-	-	-	-	-	-	C	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0	1 (1)	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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0	5 (2)	-	-	-	-	-	G	-	-	-	C	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
0	2 (1)	-	-	-	-	-	G	-	-	-	C	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0	1 (0)	-	-	-	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
0	1 (0)	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	A	-	-	G	-	-	-	-	-	-	-	-	-	-	-	A	-	-
0	1 (0)	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-
0	2 (2)	-	-	-	-	-	G	-	-	-	-	T	-	C	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
0	1 (1)	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	G	-	T	C	-	-	-	-	-	-	-	-	-	-	-	-
0	1 (1)	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0	1 (1)	-	-	-	-	-	G	-	-	-	C	T	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

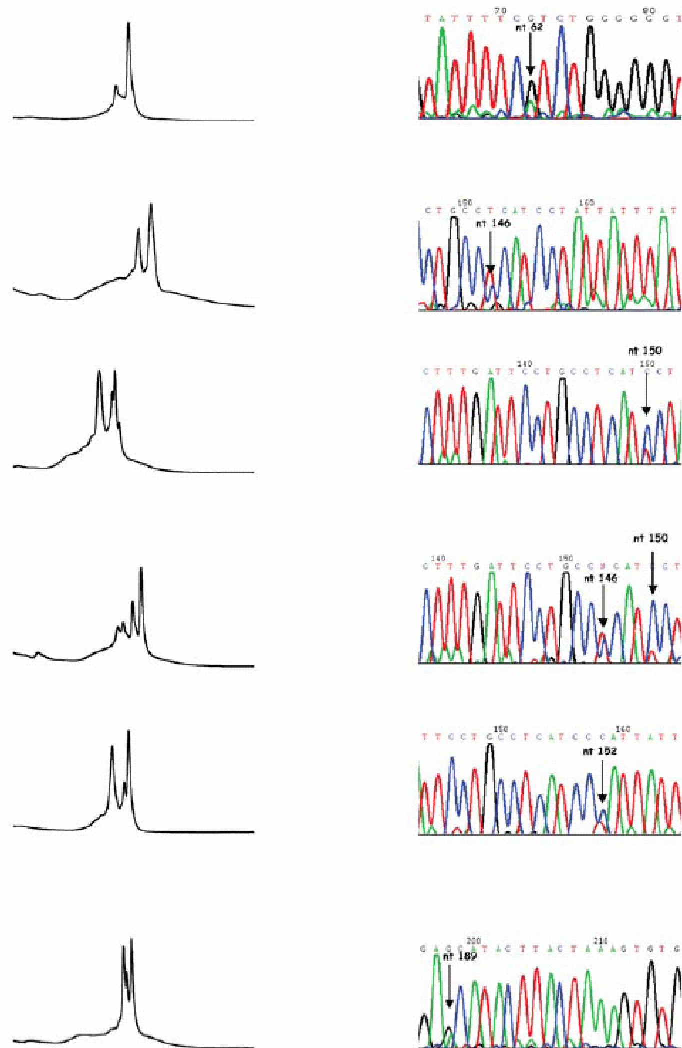

Figure 5
DHPLC profiles observed in the samples having levels of heteroplasmy equal to or higher than 25%. The sequence which characterizes each profile is shown on the right.

Table 3: Heteroplasmic sites identified by sequencing mtDNA CR (16531 nt_261 nt) in subjects showing DHPLC heteroplasmy levels equal to or higher than 25%.

	Mutations				
	G62A	T146C	C150T	T152C	G189A
Centenarians	1	1	4		1
Offspring of centenarians		1	4#	1	
Controls			2*	1	

One of the 4 children showing C150T heteroplasmy was the son of one of the 4 centenarians displaying C150T heteroplasmy.
* Subjects showing heteroplasmy for both T146C and C150T mutations.

The most important finding presented here is that the patterns of mtDNA CR heteroplasmy do not differ between centenarians and their descendants, but differ between relatives of centenarians and age-matched controls (Fig. 2). This result ruled out that heteroplasmy was exclusively due to age-related stochastic mutations, however indicated that it was genetically controlled. In agreement, the levels of heteroplasmy were significantly correlated in parent-offspring pairs (Fig. 3). The observation that the correlation was significant in mother-offspring pairs while not in father-offspring pairs (Fig, 4a and 4b) suggested that a possible genetic control on heteroplasmy was due to the mitochondrial genome. However, other clues indicated a different, and probably more complicated, genetic control pattern. First, the lack of association between mtDNA haplotypes and heteroplasmy (Tables 2 and 3) rules out that there are mtDNA molecules more prone than others to somatic mutations. Second, both the tissue specificity of mtDNA CR point mutations [18] and the concordance of heteroplasmy higher in monozygotic than in dizygotic twins [5] denote that heteroplasmy is not related to the mtDNA haplotype the offspring inherited from the mother. Thus, although the genetic mechanism modulating the occurrence/accumulation of the mtDNA CR heteroplasmy needs further work to be elucidated, all the data suggest the involvement of nuclear sex specific factors.

It should also be noted that the heteroplasmy revealed by DHPLC does not refer to the sole C150T variability, but to additional possible mutations occurring in the entire 16531 nt-261 nt mtDNA fragment. In fact, the C150T mutation was found to be present in 10 out of 16 subjects (Table 3), while the remaining subjects showed other heteroplasmic mutations [5]. Interestingly, most of the observed heteroplasmic positions were either replication origins (position 146, see Ref. [19]) or contiguous to replication origins (positions 150 and 152 that flank the 151 replication origin; position 189 which is 2 bp from the 191 replication origin). Since the C150T transition is able to provide alternative replication origins [5], a similar effect could be hypothesized for the other mutations.

The results reported in Fig. 2, show that mtDNA CR heteroplasmy cannot be accounted for only by to age-related stochastic mutations. What is more, the finding that mtDNA CR heteroplasmy is greater in descendants of centenarians than in age-matched controls suggests a beneficial role of mtDNA heteroplasmy for attaining longevity. In fact, several data show that the offspring of centenarians have a better chance to attain longevity than the general population [20,21]. How could this apparent paradox be explained from a biological point of view? The well known mitochondrial theory of ageing proposes that age-associated mitochondrial dysfunction is a consequence of age-associated accumulation of somatic mutations in the mtDNA population. However, recent findings suggest that at least some aspects of the above theory require reconsideration [22]. In fact, a key for explaining the paradox that mtDNA heteroplasmy could be beneficial for longevity may be the new emerging concept of mitochondria complementation, which suggests that human cells are protected from mitochondrial dysfunction by complementation of mtDNA products in fused mitochondria [23]. The beneficial effect of complementation may be enhanced by efficient mtDNA replication, as provided by CR mutations which introduce alternative replication sites. In fact, multiple replication origins falling in this DLoop region could play a major role in accelerating mtDNA synthesis to satisfy developmental, physiological, or aging-related demands [19]. However, neither the replicative advantage of some variants nor the mitochondrial complementation can explain, by themselves, the heteroplasmy patterns of Fig. 2. By contrast, it is likely that the interplay among new replication origins, mitochondrial complementation and nuclear factors might provide an advantage for pursuing longevity by counteracting age-related mitochondrial damages. In this frame, the subjects who are genetically predisposed to mtDNA CR heteroplasmy would be clearly favoured in the demographic selection as defined by Perls et al. [24].

Conclusion

By studying offspring and nephews/nieces of centenarians we have shown that mtDNA CR heteroplasmy is genetically controlled and it recurs in families of centenarians. This observation suggests a beneficial role of mtDNA heteroplasmy for attaining longevity and it may provide indirect evidence to the complementation of mitochondria in coping with age-related mitochondrial dysfunction.

Methods

Biological samples

A total of 414 subjects were analyzed: 100 trios composed of one centenarian, his/her child, his/her nephew/niece *plus* a control group of 114 unrelated subjects with no centenarian in the family. The age range in each of the four sample groups was as follows: 100–108 years in centenarians (median age 100 years, 53 males and 47 females); 65–80 years in offspring of centenarians (median age 70 years; 42 males and 58 females) as well as in nephews/nieces of centenarians (median age 70 years, 51 males and 49 females) and in the control group (median age 71 years, 50 males and 64 females). All subjects lived in Calabria (southern Italy) and their Calabrian ancestry was ascertained up to the grandparents' generation. The sampling was carried out in the frame of the ECHA research project [25]. All the subjects provided written informed consent for the use of their phenotypic and genetic data in studies on human ageing.

Molecular analyses

Total DNA was extracted from blood buffy-coats following standard procedures.

1. PCR amplification

A 300 bp region of mtDNA encompassing the C150T site (region 16531-261) was amplified by 5'-AAT-AGCCCACACGTTCCCCTTA-3' forward primer and 5'-GCTGTGCAGACATTCAATTG-3' reverse primer (0,4 μM each) in a final volume of 25 μl, containing 100 ng DNA, 1.5 mmol/L $MgCl_2$, 200 μmol/L of each dNTP, and 1 U of EuroTaq DNA polymerase (EuroClone). Amplification was performed in a Perkin Elmer Cetus 9600 PCR system. The amplification conditions were as follows: initial denaturation at 93°C for 30s, followed by 35 cycles at 93°C for 15s, 64°C for 20s, 72°C for 1 m. PCR products were checked by 2% agarose gel electrophoresis in TBE buffer with ethidium bromide staining.

2. DHPLC

After PCR fragments had been denatured for 3 m at 95°C, and gradually re-annealed from 95°C to 65°C in 30 m, 15 μl of each sample were injected onto a DNASep™ column of a Transgenomic Wave Nucleic Acid Fragment Analysis System (Transgenomic, San Jose, CA). The amplicons were eluted in 0.1 M triethylammonium acetate, pH 7, with a linear acetonitrile gradient at a flow rate of 0.9 ml/min. Temperature conditions were chosen according to the online program provided by Stanford University [26]. Mismatches were recognised by the appearance of two or more peaks in the elution profiles. The heights of DHPLC peaks were measured by using WAVEMAKER 4.0 software (Transgenomic San Jose).

3. Quantification of heteroplasmy levels

PCR products containing common (C150) and mutant (150T) sequences were cloned into a plasmid vector pGEM-T Easy by using the TA cloning kit (Invitrogen, USA) according to the manufacturer's protocol. The correct insertion of the PCR product was verified by sequence analysis.

In order to build a reference curve for measuring the levels of heteroplasmy in the biological samples, plasmids containing the common (C150) and the mutant (150T) sequences were mixed in different proportions (0% C with 100% T; 5% C with 95% T; 10% C with 90% T; 20% C with 80% T; 30% C with 70% T; 40% C with 60% T; 50% C with 50% T) and again subjected to PCR amplification. Using this approach, artificial samples having controlled conditions of C150T heteroplasmy were created. These samples were then subjected to DHPLC and a reference curve was assembled where the ratio between the height of the heteroduplex peak and that of the total peak was reported as a function of heteroplasmy, which varied according to the proportion between the two categories of cloned plasmids. The levels of heteroplasmy in the biological samples were then estimated on the reference curve.

4. Sequencing

PCR-amplified fragments were purified by QIAquick PCR purification Kit (Qiagen), and sequenced by fluorescence-based automated direct sequencing using a BigDye Terminator Cycle Sequencing Ready Reaction Kit in a 310 DNA sequencer (PE Applied Biosystems). Sequencing reaction mixtures contained 4 μl of Terminator Ready Reaction Mix, 200 ng of template, 3.2 pmol of each primer in a total volume of 20 μl. Cycle sequencing was carried out for 25 cycles at 96°C for 10s, 50°C for 5s, 60°C for 4 m in GeneAmp PCR system 9600. The extension products were purified using Centri-Sep™ spin columns (Princeton Separations).

Statistical analysis

SPSS v.10 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Non-parametric two-sided Mann-Whitney-U test was used to verify if the patterns of heteroplasmy were different between sample groups. The level of significance was adjusted to $\alpha = 1\text{-}0{,}95^{1/n}$, where *n* represents the number of independent comparisons.

Permutation tests were used to verify if the population pools of mtDNA haplotypic sequences (region 16531-261) differed between the sample of centenarians and that of younger controls with no centenarian in the family.

Authors' contributions

GR, GP, VS and RM : DHPLC analyses; DB: cloning. VM, EF and AB: sampling. VS, GR and SD: mtDNA genotyping.

GP, GR, CF and GBB: work hypothesis, study design, and coordination. All the authors discussed the data and participated to the draft of the manuscript, which was finalized by GP and GDB.

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