Segregation of Naturally Occurring Mitochondrial DNA Variants in a Mini-Pig Model

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ABSTRACT The maternally inherited mitochondrial genome (mtDNA) is present in multimeric form within cells and harbors sequence variants (heteroplasmy). While a single mtDNA variant at high load can cause disease, naturally occurring variants likely persist at low levels across generations of healthy populations. To determine how naturally occurring variants are segregated and transmitted, we generated a mini-pig model, which originates from the same maternal ancestor. Following next-generation sequencing, we identified a series of low-level mtDNA variants in blood samples from the female founder and her daughters. Four variants, ranging from 3% to 20%, were selected for validation by high-resolution melting analysis in 12 tissues from 31 animals across three generations. All four variants were maintained in the offspring, but variant load fluctuated significantly across the generations in several tissues, with sex-specific differences in heart and liver. Moreover, variant load was persistently reduced in high-respiratory organs (heart, brain, diaphragm, and muscle), which correlated significantly with higher mtDNA copy number. However, oocytes showed increased heterogeneity in variant load, which correlated with increased mtDNA copy number during *in vitro* maturation. Altogether, these outcomes show that naturally occurring mtDNA variants segregate and are maintained in a tissue-specific manner across generations. This segregation likely involves the maintenance of selective mtDNA variants during organogenesis, which can be differentially regulated in oocytes and preimplantation embryos during maturation.

KEYWORDS mitochondrial DNA; segregation; variants; generations; embryo

WHILE the nuclear genome is inherited from both parents, the mitochondrial genome (mtDNA) is only inherited from the population present in the oocyte at fertilization (Chinnery *et al.* 2000). The porcine mitochondrial genome is ~16.7 kb in size and encodes 13 of the subunits of the electron transport chain, which drives ATP synthesis through the biochemical process of oxidative phosphorylation (OXPHOS) (Ursing and Arnason 1998). It also encodes

22 transfer RNAs (tRNAs), which are interspersed between the encoding genes, and 2 ribosomal RNA (rRNA) complexes. mtDNA is located in the mitochondrial matrix and is tethered to proteins, which collectively form the mitochondrial nucleoid (Kucej and Butow 2007). mtDNA is present in multiple copies within cells and these genomes can be polymorphic. Consequently, cells can inherently harbor variant and wild-type (WT) sequences, *i.e.*, heteroplasmic populations, of mtDNA at different frequencies (Wallace and Chalkia 2013).

Most mtDNA variants are nonpathogenic (Ramos *et al.* 2013) but specific nucleotide mutations and large-scale deletions can lead to molecular defects, resulting in a wide range of clinical conditions, known as mitochondrial diseases (McFarland *et al.* 2007). Within the spectrum of mitochondrial diseases, the onset of symptoms can vary according to

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the nature and level of the mtDNA variation and the requirement for OXPHOS among tissues. A mother affected by mitochondrial disease can be diagnosed for mtDNA heteroplasmy to determine whether she would be at risk of transmitting the disease to her child (Hellebrekers *et al.* 2012). Moreover, healthy mothers can be carriers and transmit a mtDNA defect (Chinnery *et al.* 1998; Spikings *et al.* 2006). In both cases, the defect would have originated from the population of mtDNA that segregated to the primordial germ cells during early fetal development (Cree *et al.* 2008; Rebolledo-Jaramillo *et al.* 2014). The primordial germ cells are the first identifiable germ cells and their constituent population of mtDNA copies increases exponentially during oogenesis (Spikings *et al.* 2006; Cotterill *et al.* 2013). Consequently, mtDNA variants have the propensity to populate oocytes at differing levels, which can give rise to mitochondrial disease in the offspring (Shoubridge and Wai 2007).

Apart from the occurrence of pathogenic mtDNA variants, naturally occurring variants have also been reported in humans (Li *et al.* 2010; Goto *et al.* 2011; Payne *et al.* 2013), which also have the potential to affect the health of the individual and lead to disease (Kirches *et al.* 2001; Coon *et al.* 2006; He *et al.* 2010; Ye *et al.* 2014). A recent study has shown the effect of maternal age on heteroplasmic transmission between the mother and child (Rebolledo-Jaramillo *et al.* 2014). However, it is still unclear whether naturally occurring mtDNA variants segregate through multiple generations and whether this differs between tissues (Stewart and Larsson 2014).

To understand the transmission of naturally occurring mtDNA variants within and across generations, we have developed a mini-pig model. There are several significant advantages to using a mini-pig model. Mini-pig embryology and development are very similar to that of the human (Humpherson *et al.* 2005; Bode *et al.* 2010). Mini pigs also have a longer period of gestation than the mouse, which lasts ~114 days (Bode *et al.* 2010). This allows mtDNA mass to accumulate during longer periods of fetal development, which gives rise to more cell divisions and hence the potential for more variants to accumulate during this critical stage of development (Sato *et al.* 2005; St John and Campbell 2010; St John *et al.* 2010). Mini pigs are also an excellent model of human physiology and pathophysiology because many of their organ systems and physiological and pathophysiological responses are similar to those of the human (Larsen and Rolin 2004). Finally, mtDNA replication and reduction events have been mapped in porcine oocytes and embryos (El Shourbagy *et al.* 2006; Spikings *et al.* 2007), which are very similar to human oocytes and embryos (Humpherson *et al.* 2005; El Shourbagy *et al.* 2006; Santos *et al.* 2006; Spikings *et al.* 2007; Bode *et al.* 2010).

Here, we investigate whether naturally occurring mtDNA variants, identified by next-generation sequencing and validated by high-resolution melting (HRM) analysis, are transmitted from one generation to the next. We have analyzed a series of tissues from 31 animals across three generations,

which all originated from one founder female. While there were differences within generations, the levels of variants remained similar across the generations. However, we observed gender- and tissue-specific segregation that correlated with changes in mtDNA copy number.

Materials and Methods

All chemicals were obtained from Sigma-Aldrich (Sydney, Australia), unless stated otherwise.

Animal ethics

Approval for the use of animals was granted by Monash Medical Centre Animal Ethics Committee A (MMCA), approval no.: MMCA/2012/84.

The mini-pig colony

The colony was founded by one female (Y1) and three males (G1, 69, and 71; Figure 1), which produced three branches. Based on SNP array analysis, the mini pigs were developed from Hampshire pigs with a minor contribution from Berkshire and/or Yorkshire breeds, as demonstrated in Supporting Information, Figure S1, A and B. From the red branch, Red 17 was chosen as the genitor of the second generation of the yellow lineage and for the third generation of the red lineage.

Sample collection

Blood was sampled from the female founder Y1, male founder G1, the first yellow generation (Y2–8) as well as W1, R1, and R1's offspring (R2–R4 and R17). For tissue collection, animals were killed and weights recorded. All animals were monitored daily and those included in the study had normal development. From each animal, 12 different tissues (heart, lung, testis or ovary, brain, liver, muscle, diaphragm, kidney, fat, duodenum, spleen, and colon) were collected from the same anatomical locations for each animal and frozen at -80° .

DNA extraction

DNA extraction was performed using the Isolate II Genomic DNA kit (Bioline, London) including proteinase K and RNase treatments, according to the manufacturer's instructions. Individual oocytes and embryos were dissolved in 50 μ l of H₂O and prepared for PCR and HRM, as described (El Shourbagy *et al.* 2006).

In vitro maturation and embryo culture

Granulosa cells and cumulus-oocyte complexes (COCs) were collected by syringe aspiration (21-gauge needle) from the ovaries of 6-month-old culled gilts ($n = 6$). After aspiration, total COCs were washed and incubated for 44 hr of *in vitro* maturation (IVM) at 39° , 5% CO₂ at maximum humidity, as described (Spikings *et al.* 2007). After IVM, metaphase II oocytes were inseminated by intracytoplasmic sperm injection (ICSI) and cultured *in vitro* for 7 days at 39° , 5% CO₂, 5% O₂ at maximum humidity, as described (Spikings *et al.* 2007). The granulosa cells and the immature oocytes (denuded from

Figure 1 Mini-pig genealogy tree. Circles and squares represent female and male founders. One female (Y1) and three males (G1, male 69, and male 71) are the founders of the colony. The yellow branch of mini pigs extends for two generations while the red branch extends for three generations.

cumulus cells) were collected at the time of ovary collection. Maturing oocytes were collected after 44 hr of IVM. Following insemination, preimplantation embryos were harvested at different stages of development during *in vitro* culture.

SNP array

The extracted DNA from the mini-pig samples was run on an Illumina PorcineSNP60 v2 BeadChip, containing 61,565 SNPs, delivering uniform genome-wide coverage with an average probe spacing of 43.4 kb. Image processing and SNP calling were performed using the genotyping module of GenomeStudio software (GenCall cutoff for failed genotypes of 0.15, software versions 1.9.4 and 2011.1, respectively).

Genotypic data for pedigreed pigs were from the Illumina PorcineSNP60 v2 BeadChip. The DNA for purebred pigs ($n = 65$) was extracted from semen samples purchased from commercial boar studs or kindly provided by the National Swine Registry (West Lafayette, IN). These boars were unrelated and selected to represent the breed. All crossbred pigs ($n = 60$) were produced at the US Department of Agriculture and DNA was extracted from tail tissue. A principal component analysis (PCA) was then computed using the SNPs from the pigs with known pedigree with the EIGENSOFT package (Price *et al.* 2006), and then the mini-pig SNP data were overlaid on that PCA (Figure S1B).

Hierarchical clustering analysis and the resulting dendrogram were also produced from these SNP data using a distance measure based on the number of SNPs different between individuals (Figure S1A).

Long-PCR amplification of the mitochondrial genome and next-generation sequencing

Next-generation sequencing of mtDNA was performed on amplified mtDNA template generated by long PCR using two sets of overlapping primers (Table S2). Each reaction

of 50 μ l consisted of 50 ng genomic DNA, 1 $\times$ High Fidelity PCR buffer, 100 mM MgSO₄, 1 mM dNTPs (Bioline, London), 10 μ M of the forward and reverse primers, and 1 unit of Platinum Taq High Fidelity (Invitrogen, Carlsbad). Reaction conditions were 94° for 2 min, 35 cycles of 94° for 15 sec, 63° for 30 sec, and 68° for 8 min 45 sec. Products were purified using the Isolate II PCR kit (Bioline), as described in the manufacturer's instructions.

Next-generation sequencing reactions were performed, as described (Sobinoff *et al.* 2014; Yeung *et al.* 2014). Briefly, purified long-PCR fragment pairs were combined at equal concentrations, prior to generation of the amplicon libraries. Amplicon libraries were generated using the recommended workflow procedures from the Ion Fragment Library kit and Ion Xpress Template kit (Life Technologies). mtDNA was sheared using the Covaris Adaptive Focused Acoustics system. Fragments of ~200 bp were selected following electrophoretic separation with the E-gel system (Life Technologies). Confirmation of product and the quality of the mtDNA were assessed by the Agilent Bioanalyzer using the Agilent High Sensitivity DNA kit (Agilent, Santa Clara, CA). For multiplexing of the samples, each DNA library was barcoded using different ligation adaptors. Libraries were then pooled at equal concentrations and loaded onto 318 chips for sequencing. Sequence alignment to the *Sus scrofa* reference genome (NCBI accession no.: AJ002189) was performed using CLC Genomics Workbench V7.5 (Qiagen, Aarhus A/S, Denmark).

Variant selection was also performed using CLC Genomics Workbench (Version 7.5), as described (Sobinoff *et al.* 2014; Yeung *et al.* 2014). For quality control, reads were filtered to exclude those of a nucleotide length of <15 bp, with one nucleotide being trimmed from each end of all reads. All reads that were accepted into the analysis surpassed a Phred quality score of 15. The following parameters were applied to score reads during the selection process for inclusion into the final alignment: a mismatch cost of 2 and an insertion/deletion cost of 3 were set; reads that had a minimum of 80% identity to the reference sequence were accepted; and all duplicate reads were excluded. For variant (single nucleotide variant, insertion, and deletion) analysis, we used the CLC's Low Frequency Variant Detection Program. This ensures that sequencing error rates are minimized by statistically determining if the nucleotides observed in the reads are due to sequencing errors or if there is more than one allele at the particular site being called. We set the most rigid level of required significance. A minimum mutation threshold of 3% was also applied to any variant, therefore eliminating the possibility of false positive calls.

Determining regions of the mitochondrial genome susceptible to variants

Susceptibility was determined by normalizing the number of variants identified in each mtDNA region to the size of the region (in base pairs) on which the variant was located, as described (Yeung *et al.* 2014).

Preparation of HRM standard curves

To quantify variant load, synthesized mtDNA oligomers representative of WT and variant sequences were annealed to produce double-stranded DNA standards (Table S2). WT and variant standards were mixed at specific ratios (WT:variant 0:100; 10:90; 20:80; 30:70; 40:60; 50:50; 60:40; 70:30; 80:20; 90:10; and 100:0). HRM curves were plotted, as described below, to determine the degree of displacement between each WT:variant ratio.

HRM analysis

Genomic DNA samples from all 12 tissues from each offspring from the 31 mini pigs were loaded into 96-well plates (Bio-Rad Laboratories, Hercules, CA) with each sample analyzed in triplicate. The 10- μ l reactions contained 10 ng DNA template from tissue or 2 μ l of oocyte or embryo product, 1 $\times$ HRM master mix containing LC Green Plus+ (TrendBio, Preston, VIC, Australia), and 2.5 μ M of forward and reverse primers (Table S2), overlaid with 20 μ l mineral oil. Amplification conditions were 95° for 2 min, 45 cycles of 94° for 30 sec, and 62° for 30 sec, followed by 1 cycle at 94° for 30 sec, and cooling to 25° for heteroduplex formation.

Products underwent melt analysis on the LightScanner (Idaho Technologies, Salt Lake City) with analysis performed using the LightScanner Instrument and Analysis software with Call-IT 2.0 (V.2.0.0.1331). Data acquisition began at 70° and increased incrementally by 0.1° up to 96°. After melt analysis, PCR products were loaded onto agarose gel and purified products were prepared for capillary sequencing to confirm product specificity.

Determination of mtDNA copy number by real-time PCR

Real-time PCR was performed on all 12 tissues from each offspring from the 31 mini pigs using external standards for mtDNA and β -globin (see Table S2 for primer details). A total of 2 μ l of DNA template (10 ng/ μ l) was added to a master mix containing 10 μ l SYBR green (Bioline), 6 μ l of H₂O, and 1 μ l of each forward and reverse primer. Real-time PCR was conducted in a 72-well Rotorgene-3000 (Corbett Research, Cambridge, UK), according to the following conditions: 95° for 5 min, 45 cycles of the annealing temperature (55°) for 30 sec, and acquisition temperature (72°) for 15 sec, followed by 1 cycle of ramping temperature from 72° to 95° with continuous fluorescence acquisition. Real-time PCR was also performed on individual oocytes and embryos that had been dissolved in 50 μ l of H₂O with 2 μ l added directly to the master mix, as described (El Shourbagy *et al.* 2006). For each sample, mtDNA copy number analysis was performed in triplicate and values were averaged.

Statistical analysis

For real-time PCR and HRM analysis, statistically significant differences were determined by one-way or two-way ANOVA followed by Bonferroni *post hoc* test using GraphPad v5.0c (GraphPad Software, San Diego). Analysis of dependence

between variables was performed by linear regression and Pearson correlation. Statistical significance is expressed as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Data availability

Reference numbers for data available in public repositories are as follows: GEO submission: GEO series record: GSE73276. GEO accession nos. are: GSM1889978 - Y1_1; GSM1889979 - Y2_1; GSM1889980 - Y3_1; GSM1889981 - Y4_1; GSM1889982 - Y5_1; GSM1889983 - Y6_1; GSM1889984 - Y7_1; GSM1889985 - Y8; GSM1889986 - R1_1; GSM1889987 - R2; GSM1889988 - R3; GSM1889989 - R4; GSM1889990 - R17; GSM1889991 - G1_1; GSM1889992 - W1; GSM1889993 - Y1_2; GSM1889994 - G1_2; GSM1889995 - R1_2; GSM1889996 - Y2_2; GSM1889997 - Y3_2; GSM1889998 - Y4_2; GSM1889999 - Y5_2; GSM1890000 - Y6_2; GSM1890001 - Y7_2. Whole mitochondrial genome sequence for Y1 is deposited at BankIt1847677 Y1; accession no.: KT372134. Next-generation mitochondrial genome sequencing is deposited at Sequence Read Archive. BioSample accession nos. are: Y1 (SAMN03945395); G1 (SAMN04158750); Y2 (SAMN04158751); Y3 (SAMN04158752); Y4 (SAMN04158753); Y5 (SAMN04158754); Y6 (SAMN04158755); Y7 (SAMN04158756); W1 (SAMN04158757); R1 (SAMN04158758); R2 (SAMN04158759); R3 (SAMN04158760); R4 (SAMN04158765); R17 (SAMN04158766); and Y8 (SAMN04158767).

Results

Identification of mtDNA variants by next-generation sequencing

To identify and determine the frequency of mtDNA variants in a maternally related mini-pig population, we established a mini-pig colony by mating the female founder (Y1) with the male founders G1, 69, and 71 (Figure 1). Using the Illumina PorcineSNP60 Array, we determined that these pigs originated from Hampshire pigs with a minor contribution from Berkshire and or Yorkshire crossing, as demonstrated in Figure S1, A and B. From this colony, we have successfully generated three generations of offspring, which we have interrogated here.

Two overlapping regions of the whole mitochondrial genome (16,675 bp) from blood DNA of 15 mini pigs (Y1, female founder; G1, male founder; the first yellow generation (Y2–8; W1, daughter of Y1, R1, daughter of Y1; and R1's offspring, R2–R4 and R17; Figure 1) were amplified by long PCR and processed for next-generation sequencing (coverage >1000). From the multiple reads obtained, the mtDNA sequences showed 99% similarity to the *Sus scrofa* mitochondrial genome but exhibited serial nucleotide variants to the consensus sequence derived from Y1 (accession no. KT372134; Table S1). In total, 81 variant positions were detected when a 3% threshold was applied to the analysis. These were mainly deletions (Del) (52) but some substitutions (29) were also observed. On average, each mini pig harbored 28 variants

(range = 19–54) with 9 identical variants present in each mini pig. The male founder G1, who is unrelated to Y1, harbored 15 single nucleotide polymorphisms (SNPs). Susceptibility, which is defined as the overall probability of deriving a mutation within a region of the mitochondrial genome, was assessed for coding and noncoding regions. Susceptibility to mtDNA variants in the coding genes and noncoding regions ranged from 1.2×10^{-3} in the *16S rRNA* gene to 15.2×10^{-3} in the *12S rRNA*.

Offspring share maternal mtDNA variants

From the mtDNA variants identified in the colony founders (Table S1), we determined whether the maternally inherited variants would be present at the same frequency in the offspring. None of the 15 mtDNA SNPs identified in male G1 was observed in his offspring, which is expected, as the matings were indicative of intraspecific breeding. On the other hand, the majority of the maternal mtDNA variants were present in Y1's offspring, with some variants above or below the 3% threshold of accurate detection. Consequently, only variants at or above 3% were included (Table S1). The frequency of the mtDNA variants differed slightly among the offspring but this was minimal when a variant was identified in each of the offspring. However, we observed that particular maternal variants, such as Del A3880, were not detected in all of the offspring, while other variants were *de novo* in the offspring, for example Del A1394. This, therefore, implies that some mtDNA variants can be progressively acquired and others lost in successive generations.

Determination of the presence of mtDNA variants within and among generations

Four mtDNA variants, located at nucleotide positions 376, 1302, 1394, and 9725 (highlighted in Table S1), were selected for further analysis over subsequent generations of mini pigs. The yellow branch was analyzed over two generations and the red branch over three generations. These variants were present in the coding (*NADH3*) and noncoding regions (*12S rRNA* and *16S rRNA*). Del A9725 in the *NADH3* gene induces a frameshift mutation that results in a truncated protein at amino acid position 90/115. Specific primer sets were used to amplify the mtDNA region containing a single variant. The resulting PCR products were then assessed by HRM analysis for the frequency and percentage of variant and wild-type molecules against incremental standard curves (Figure S2A) for each of the offspring's tissues ($n = 12$; Figure S2B). When tissue variant loads were averaged and values expressed as total mean, there were significant differences in variant load among the offspring for the four mtDNA variants analyzed (Figure 2, A–D). However, there were no significant changes when presented as total mean per generation (Figure S3, A–D). Nevertheless, variant load correlated significantly with body weight for three of the variants, either negatively (Del A376 and Del A1302) or positively (Del A1394). The R^2 values were 0.04452 (Del A376), 0.02218 (Del A1302), and 0.02403 (Del A1394).

Pearson's correlation was also performed between variant load in tissues and animal weight, which showed significance for Del A376 ($P < 0.05$, $R^2 = 0.1230$; Figure S4). These results demonstrate that, although individually variable within a generation, variant load remains stable among generations.

Variant load differs between genders

To determine whether mean variant load was affected by gender, we compared the frequency and level of variant load in male ($n = 15$) and female ($n = 16$) offspring. There were position-specific significant differences for distribution of frequency based on gender for Del A376, Del A1302, and Del A1394, while Del A9725 was present at a similar frequency in both sexes (Figure S5). Interestingly, differences in variant load were bidirectional, being either higher in males for Del A376 ($P < 0.01$) and Del A1302 ($P < 0.05$) or females for Del A1394 ($P < 0.05$). At the tissue level, Del A376 was significantly higher in male heart ($P < 0.001$) and liver ($P < 0.05$) tissues compared to females (Figure 3A). The other variants did not show tissue-specific differences between the genders due to high variability between samples and offspring (Figure 3, B–D).

Variant load is significantly reduced in specific tissues and correlates with mtDNA copy number

Within the offspring, specific tissues showed lower variant load compared to other tissues. Variant load in muscle and diaphragm was consistently reduced for each variant analyzed (Figure 4, A–D). Moreover, liver, fat, heart, and brain were lower for Del A376 (Figure 4A), as were fat and brain for Del A1302 (Figure 4B). Across the generations, muscle, diaphragm, liver, fat, heart, and brain showed significant differences for Del A376 (Figure 5).

As the reduction in variant load mainly affects tissues with high OXPHOS-requiring functions, namely, brain, heart, diaphragm, and muscle, we investigated whether changes in variant load correlated with mtDNA copy number. Determination of the mtDNA copy number in each tissue ($n = 12$) and offspring ($n = 31$) showed significantly higher mtDNA copy number for diaphragm, heart, and brain (Figure 6), which did not reflect a gender bias (Figure S6A). Nevertheless, mtDNA copy number correlated with weight for heart ($P < 0.05$) and kidney ($P < 0.001$) tissues (Figure S6B). By applying the values for mean variant load and mtDNA copy number, linear regression analysis determined that there was a significant relationship between a decrease in variant load for each of the four variants and increases in mtDNA copy number. However, a Spearman's correlation was also performed between variant load in tissues and mtDNA copy number (Figure 7), which showed significance for Del A376 ($P < 0.0001$, $r = -0.8791$), Del A1302 ($P < 0.01$, $R^2 = -0.7802$), and Del A1394 ($P < 0.05$, $R^2 = -0.5330$).

In a tissue-specific manner, there was a significant correlation between variant load and mtDNA copy number for heart, lung, testis, ovary, liver (Del A1302), brain (Del

Figure 2 Mean variant load ($\pm$ SEM) in offspring across generations. Variant load was determined for the deletions at positions 376 (A), 1302 (B), 1394 (C), and 9725 (D). Data represent the mean variant load from the tissues of each animal across three generations. $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$ (ANOVA).

A9725), and muscle (Del A376 and Del A1302; Figure S7). Altogether, these results suggest that the regulation of mtDNA copy number plays a role in reducing specific variant load among high OXPHOS-requiring tissues.

Heterogeneous variant load among oocytes

As oogenesis and early embryo development are characterized by dynamic changes in patterns of mtDNA copy number (Spikings *et al.* 2007) (Figure S8), we determined the variant load in granulosa cells, which support oocyte development prior to fertilization, immature (GV - germinal vesicle) and maturing (post *in vitro* maturation) oocytes, and preimplantation embryos (four and eight cell, morula and blastocyst). We observed three different patterns (Figure 8A). The first pattern produced a stable variant load across samples for Del A1302 and Del A1394 at similar levels to those observed in somatic tissues. A second pattern of variant load exhibited a heterogeneous distribution among the oocyte populations with high levels maintained in the embryos for Del A376. Finally, the third pattern showed similarity with the second, except that variant load was stabilized in preimplantation embryos for Del A9725.

As mtDNA is replicated in the oocyte prior to fertilization, we investigated whether each of the variants was correlated with changes in mtDNA copy number from immature oocytes to the blastocyst stage (Figure 8, B–E). As a result, variant load correlated positively with increases in mtDNA copy number for Del A376 ($P < 0.0001$) and Del A1302 ($P < 0.005$) (Figure 8, B–E).

Discussion

We have identified a number of mtDNA variants in a mini-pig model derived from one female ancestor. These mtDNA variants were dispersed across the genome, in both coding and noncoding regions. Four variants located in the 12S and 16S rRNA and the NADH3 region were selected for further investigation by HRM analysis to confirm their presence, prevalence, and segregation in tissues across three successive generations and for further analyses. The 12S rRNA (Shoffner *et al.* 1993; Ballana *et al.* 2008), 16S rRNA (Crispim *et al.* 2005; Seibel *et al.* 2008), and ND3 (Canter *et al.* 2005) regions have been previously reported to have potential effects on cellular bioenergetics (Ruiz-Pesini and Wallace 2006).

Figure 3 Comparison between genders for mean variant load ($\pm$ SEM) across tissues. Variant load was determined for the deletions at positions 376 (A), 1302 (B), 1394 (C), and 9725 (D). Data represent the mean variant load ($\pm$ SEM) from the tissues of male and female offspring. * $P < 0.05$ and *** $P < 0.001$, respectively (ANOVA).

In cattle, the radical drift of mtDNA genotypes across generations of maternally related cows has been reported (Olivo *et al.* 1983; Laipis *et al.* 1988). In this respect, we observed significant differences among the mini-pig siblings. However, there was no evidence of significant drift in total variant load among the generations. In a mouse model generated from two mtDNA haplotypes that underwent multiple generations of backcrossing, the progeny from heteroplasmic females had consistent enrichment for one haplotype relative to their mothers (Sharpley *et al.* 2012). This high level of variant load (50%) resulted in functional consequences for cellular metabolism, which was reflected in altered behavior and cognition in the offspring. In our study, the interindividual differences in variant load were not related to any visible phenotypes at the whole animal level, most likely due to the low level of variants maintained in the offspring. Interestingly, there was a significant drift across the generations for one mtDNA variant present in the 12S rRNA region in six tissues, namely the brain, diaphragm, liver, heart, muscle, and fat. However, this mtDNA variant never replaced the wild-type population. This pattern of segregation suggests that there is strict regulation of the maintenance of low levels of mtDNA variant across generations, which protects the offspring against the potential impact of mtDNA variants on cellular function by ensuring sufficient wild-type mtDNA is present.

Contrary to the genetic diversity that the two parental nuclear genomes contribute at fertilization, mtDNA variants present in the oocyte provide the only source of mtDNA

diversity for the progeny, especially as the paternal mitochondrial genome is either eliminated prior to fertilization or before embryonic genome activation (Sato and Sato 2013). Such diversity may serve as the framework for new adaptive responses, at the genetic and epigenetic levels, to challenge bioenergetic conditions (Ruiz-Pesini *et al.* 2004; Rodell *et al.* 2013). Here, the mini-pig colony had stable lifestyle conditions in terms of food and a sheltered environment, which would likely not have burdened mitochondrial adaptation and could explain the relative stability of mtDNA variant load across generations. However, they would have been exposed to the large range in temperature experienced in southern Australian (range = $+10^{\circ}$ in winter to 41° in summer). Indeed, variation in temperature can restrict the transmission and prevalence of heat-sensitive mtDNA variants across generations by preferentially replicating the nonheat sensitive variant in the female germline in some species, as described in *Drosophila melanogaster* (Hill *et al.* 2014). Nevertheless, such external conditions may have a differential impact on male and female physiology (Rand *et al.* 2001; Innocenti *et al.* 2011) and could be related to the gender-specific differences in variant load that we observe in offspring tissues. For example, variant load was higher in male livers than for females. The liver is regarded as the detoxification center and plays a major role in metabolism, including hormone production, which is sex specific and regulates glycogen storage. It is well documented, for example, that heat stress can affect liver function, hormone production, and reproductive function (Federman 2006; De Marinis *et al.* 2008).

Figure 4 Mean variant load ($\pm$ SEM) in offspring tissues. Variant load was determined for deletions at positions 376 (A), 1302 (B), 1394 (C), and 9725 (D). Data represent the mean variant load from all offspring across each specific tissue. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$, respectively (ANOVA).

Somatic mtDNA variants have been shown to recur in a tissue-specific pattern in humans (Samuels *et al.* 2013), and here we show that it is also the case for maternally inherited variants. This tissue-specific segregation was maintained across generations, although we observed slight but significant variations for one mtDNA variant present in the 12S rRNA region in six tissues, namely the brain, diaphragm, liver, heart, muscle, and fat. Tissue-specific segregation has been described in the mouse harboring different mitochondrial or nuclear DNA genotypes. Dependent on the nuclear background, the selected mtDNA haplotype exhibited differential segregation in specific tissues, namely blood, spleen, and liver, while other tissues remained stable (Jenuth *et al.* 1997; Battersby *et al.* 2003). These studies demonstrate the influence of the nucleus, whether through genetic or epigenetic means, on mtDNA segregation (Dunbar *et al.* 1995) and tissue-specific regulation of mtDNA variants. As the variations in our mini-pig model were mainly restricted to high-respiratory tissues (brain, diaphragm, muscle, liver, heart, and fat), this suggests that there is epigenetic regulation of variant load to ensure the regulation of OXPHOS performance, which is reflected by higher mtDNA copy number (Kelly *et al.* 2012; St John 2014). For example, for adipose tissue, this would link OXPHOS regulation to adipogenesis (Hofmann *et al.* 2012), insulin metabolism (Ryu *et al.* 2013), and thermogenesis (Duteil *et al.* 2014).

Cells can regulate the replication of their mtDNA copy number to maintain a specific set point for biosynthesis of

OXPHOS subunits, which, together with the nuclear-encoded subunits, support the production of ATP by the electron transport chain (Kelly *et al.* 2012). Among the mini-pig samples, high-respiratory tissues showed lower variant load correlated with increased mtDNA copy number. This correlation was significant for each of the variants analyzed across the tissues. Moreover, within specific tissues, namely heart, lung, testis, ovary, brain, liver, and muscle, increased mtDNA copy number was associated with reduced variant load for at least one of the four variants. This suggests that mtDNA copy number could regulate mtDNA variant segregation, which is in agreement with a recent mouse study (Burgstaller *et al.* 2014) that reported on tissue-specific segregation over time, especially for the heart, brain, and muscle. Dependent on mtDNA haplotype, different patterns of segregation were documented, demonstrating pre- or postnatal regulation. This emphasizes how large-scale mtDNA replication events during development can result in loss of neutrality for some mtDNA variants in specific tissues.

Existence of a selective mechanism for mtDNA variants is fiercely debated, likely because it is a multifactorial process that does not follow a simple model. It has been proposed that a selective replication process, both at the cellular and organelle level, is unlikely to bias variant segregation but rather factors involved in mtDNA maintenance, such as constituents of the mtDNA nucleoid (Battersby and Shoubridge 2001). Others have hypothesized the implication of mitochondrial fusion as well as mitophagy in the fate of mtDNA variants

Figure 5 Tissue-specific variant load (±SEM) across three generations. The mean variant load for four mtDNA variants and among three generations of offspring was analyzed in lung (A), ovary (B), testis (C), muscle (D), heart (E), fat (F), colon (G), liver (H), diaphragm (I), brain (J), kidney (K), duodenum (L), and spleen (M). * $P < 0.05$, ** $P < 0.01$, respectively (two-way ANOVA).

(Chen *et al.* 2010; Lackner *et al.* 2013). A replicative disadvantage could also be due to nucleotide differences in the D-loop region of the mitochondrial genome (Takeda *et al.* 2000). While the variants we analyzed by HRM are not located in the D-loop, we do not rule out the possibility of

polyplasmies, *i.e.*, the different combination of multiple variants within a single mtDNA molecule (Smeitink *et al.* 2001). Indeed, we observed a total of 81 mtDNA variants, which is at similar levels to the 98-point heteroplasmies observed in 39 healthy human mother-child pairs of European ancestry

Figure 6 Mean mtDNA copy number ($\pm$ SEM) in tissues from offspring. In all, 12 tissues per offspring from 31 offspring were analyzed for mtDNA copy number. *** $P < 0.001$, **** $P < 0.0001$, respectively (two-way ANOVA).

(Rebolledo-Jaramillo *et al.* 2014). A polyplasmic combination of D-loop variants could affect the replication of accompanying mtDNA variants and explain the diverse patterns of segregation (Takeda *et al.* 2000; Zsurka *et al.* 2005). Indeed, the failure of these molecules to replicate efficiently would suggest that fewer of these molecules would be present for transmission and segregation.

When a mtDNA variant induces a detrimental effect due to high loading, it is likely to be lethal at the cellular level, resulting in selective purification among oocytes (Fan *et al.* 2008). The mtDNA mutator mouse demonstrated the differential purification between synonymous and nonsynonymous mtDNA variants within the mouse germline (Stewart *et al.* 2008). Moreover, the extent of heteroplasmy was primarily determined in the oocyte population with the level of mtDNA variant modified in the next generation (Freyer *et al.* 2012). Oocyte metabolism could cope with slightly inefficient OXPHOS and, because of the mitochondrial genetic bottleneck in the germline (Cree *et al.* 2008), different levels of mtDNA variants can be found among the oocyte population (Frederiksen *et al.* 2006). This was the case in our study for two variants with increasing range of segregation in maturing oocytes in correlation with increasing mtDNA copy number. This is similar to the finding of a homopolymeric region of mtDNA showing heteroplasmic patterns in human oocytes (Marchington *et al.* 1998). Moreover, increased mtDNA copy number has been correlated with increased variant load for a particular respiratory chain mutation in the preimplantation window (Monnot *et al.* 2013). These findings suggest an uncoupled relationship between mtDNA variant load and OXPHOS metabolism and could be related to the relaxed metabolic state in the preimplantation stages, also known as “quiet” metabolism (Leese 2002; Leese *et al.* 2007). In addition, selective replication of different mtDNA populations during oocyte maturation (Chinnery *et al.* 2000; Wai *et al.* 2008) could also explain the differential pattern of mtDNA variant load that we observed between respective variants.

In our model, the increases in variant load were restricted to either the oocyte or preimplantation embryo or both.

Figure 7 Correlation of variant load with mtDNA copy number in offspring tissues. Tissue-specific data for mtDNA variant load and mtDNA copy number were analyzed by linear regression and significant dependence was determined by Spearman's correlation (* $P < 0.05$, ** $P < 0.01$, and **** $P < 0.0001$, respectively).

However, the reduction in mtDNA copy number that takes place during preimplantation development in larger mammals, due to there being no expression of the key mtDNA replication factors (Lloyd *et al.* 2006; Bowles *et al.* 2007; Spikings *et al.* 2007), and persists in the inner cell mass (Spikings *et al.* 2007) and up to gastrulation, most likely filters the levels of variants persisting in the offspring. This process is aided by the shedding of mtDNA during preimplantation development (Stigliani *et al.* 2014). This suggests that these variants provide a positive functional role during early development but, as they may be potentially lethal later in life, they are subsequently reduced. Indeed, parallels can be drawn with undifferentiated tumors, where reduced OXPHOS and a greater reliance on glycolysis promotes aerobic glycolysis, *i.e.*, the Warburg effect to mediate cell proliferation. As with developing tumors, which also harbor mtDNA variants (Yeung *et al.* 2014), this would be advantageous for the developing embryo when its prime role is to divide to increase cellular number in an exponential manner (Cagnone *et al.* 2011; Krisher and Prather 2013). Indeed, the use of OXPHOS inhibitors promotes cell proliferation in dividing preimplantation pig embryos (Machaty *et al.* 2001).

In conclusion, we have demonstrated that naturally occurring variants are maintained at low levels across generations, with variant-specific tendencies associated with tissue type and gender. These different patterns highlight the complex nature of mtDNA segregation that can occur pre- and postnatally (Burgstaller *et al.* 2014). Specifically, mtDNA variant load was consistently reduced in muscle and diaphragm,

Figure 8 Distribution of mtDNA variant load and correlation with mtDNA copy number in oocytes and early preimplantation embryos. Variant load (A) was determined for the deletions at positions 376, 1302, 1394, and 9725 in oocytes (before $n = 8$ and after $n = 10$ *in vitro* maturation) and embryos (four cells to blastocyst, $n = 6$) from 6-month-old gilts ($n = 6$). Variant load was also measured in oocyte-matched granulosa cells. $*P < 0.05$, $**P < 0.01$, and $****P < 0.0001$, respectively (ANOVA). Oocyte and embryo data for mtDNA variant load and mtDNA copy number were analyzed by linear regression (B–E) and significant dependence was determined by Pearson's correlation.

which share a common ancestor during development: the lateral dermomyotome (Birchmeier and Brohmann 2000). It is, therefore, likely that the segregation of mtDNA variants is determined postgastrulation during organogenesis, in line within the onset of increased mtDNA replication. On the other hand, tissue-specific segregation could also depend on different mtDNA replication rates between individual tissues and the differential purification of mtDNA variants at the cellular, organelle, or DNA levels. Replication of mtDNA is a

dynamic process and maternal variant load can potentially increase with age (Yao *et al.* 2013) and promote mtDNA heteroplasmy (Ross *et al.* 2013). Moreover, mtDNA copy number also increases during oocyte maturation and early embryo development, with different patterns of variant load compared to somatic tissues. These findings imply the existence of tissue-specific selection for mtDNA variant load during development, especially as each tissue will have specific requirements for OXPHOS to undertake its specialized function.

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Supporting Information

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Segregation of Naturally Occurring Mitochondrial DNA Variants in a Mini-Pig Model

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Gary Rohrer, Lynsey Cree, Ian A. Trounce, and Justin C. St. John

Cluster Dendrogram

Figure S1 Hierarchical clustering of mini-pig founder and representative offspring genotypes with complete linkage methods. Chromosomal DNA was extracted from the founder's blood and hybridized to the Illumina PorcineSNP60 Array. The result shows the hierarchical clustering of DNA sequences between the mini-pig members and other breeds (A). From the microarray data, Principal Component Analysis displays the genetic location of the mini-pig members compared to other breeds (B).

Figure S2

Figure S2 Determination of mtDNA variant load in offspring tissues. Difference curves from HRM analysis of mtDNA standards containing incremental ratios of the variant sequences (del A1302 is shown as an example) compared to the WT (A). The mtDNA extracted from mini-pig tissues (red/grey/blue line) were run against variant/WT standards (del 1394 is shown as an example) in order to determine their variant loads (B).

Figure S3

Figure S3 Variant load in mini-pig tissues amongst generations. Variant load was determined for deletions at positions 376 (A), 1302 (B), 1394 (C) and 9725 (D). Data

represent the mean variant load across generations (left) and across tissues (right) from all offspring across 3 generations.

Figure S4

Figure S4 Variant load in mini-pig tissues correlated with body weight. Tissue-specific data for mtDNA variant load and body weight were analysed by linear regression and significant dependence was determined by Pearson's correlation (* $p < 0.05$).

Figure S5

Figure S5 Distribution of variant load between genders. Variant load was determined for deletions at positions 376 (A), 1302 (B), 1394 (C) and 9725 (D). Data represent the distribution of variant load in frequency intervals from the tissues of male and female offspring. For each mtDNA variant, total variant load was compared between males and females (E). *, ** represent $p < 0.05$ and 0.01 , respectively (t-test).

Figure S6

Figure S6 Comparison of mean mtDNA copy number in tissues between genders (A) and correlation with body weight (B). Tissue-specific data for mtDNA copy number and body weight were analysed by linear regression and significant dependence was determined by Pearson's correlation (*, *** represent $p < 0.05$ and 0.001 , respectively).

Figure S7

Figure S7 Correlation between variant load and mtDNA copy number for each tissue. Data for each offspring for 4 mtDNA variants and mtDNA copy number were analysed by linear regression and significant dependence was determined by Pearson's correlation (* indicates $p < 0.05$).

Figure S8

Figure S8 Distribution of mtDNA copy number in oocytes and early preimplantation embryos. MtDNA copy number was assessed in oocytes (before $n=8$ and after $n=10$ *in vitro* maturation) and embryos (4 cells to blastocyst, $n=6$) from 6 month old gilts ($n=6$). *, **, *** represent $p < 0.05$, 0.01 and 0.001, respectively (ANOVA).

Table S1 List of mtDNA variants detected in mini-pig founders and offspring. Next generation sequencing of long PCR mtDNA products resulted in high coverage (> 1000) of the mitochondrial genome. Description and frequencies of variant positions detected in next generation sequence reads (threshold $> 3\%$) are listed for each animal.

Table S1

Reference Position	Type	Length	Reference	Alt	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8	R1	R2	R3	R4	R17	W1	Q1	Overlapping annotations	Coding region change	Amino acid change	
176 Deletion	1 A	-	-	-	3.25	4.5	3.54	3.61	4.64	4.84	4.05	3.29	4.36	3.93	5.17	3.21	4.37	3.91	34.76				
143 Deletion	1 A	-	-	-	-	-	-	-	3.95	3.18	3.65	-	3.51	-	-	3.25	3.34	-	1.31	rRNA: rRNA, Gene: 125 rRNA			
178 Deletion	1 A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.45	-	rRNA: rRNA, Gene: 125 rRNA			
139 Deletion	1 C	-	-	-	6.57	-	-	-	-	-	7.2	-	-	-	-	-	-	-	-	rRNA: rRNA, Gene: 165 rRNA			
1303 Deletion	1 A	-	-	-	14.54	15.55	14.41	14.82	13.96	13.39	14.71	11.31	12.87	14.35	15.45	12.14	11.92	11.8	14.3	rRNA: rRNA, Gene: 165 rRNA			
1185 Deletion	1 C	-	-	-	-	-	-	-	-	-	3.12	3.27	-	-	-	-	-	-	-	rRNA: rRNA, Gene: 165 rRNA			
1197 SNV	1 C	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	rRNA: rRNA, Gene: 165 rRNA			
1194 Deletion	1 A	-	-	-	3.21	-	-	-	3.26	-	3.41	-	-	-	-	-	-	-	-	rRNA: rRNA, Gene: 165 rRNA			
1121 Deletion	1 T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	rRNA: rRNA, Gene: 165 rRNA			
2632 Deletion	1 C	-	-	-	-	-	-	-	-	-	-	4.63	-	-	-	-	-	-	-	rRNA: rRNA, Gene: 165 rRNA			
2756 SNV	1 T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	rRNA: rRNA, Gene: 165 rRNA			
3125 SNV	1 T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	rRNA: rRNA, Gene: 165 rRNA			
1332 Deletion	1 C	-	-	-	4.58	6.79	4.19	5.36	4.81	5.12	5.95	5.01	5.27	6.38	7.15	5.74	7	6.46	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.127-C		
3527 Deletion	1 A	-	-	-	-	-	-	-	-	-	-	11.27	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.4717-C		
3880 Deletion	1 A	-	-	-	11.05	13.23	-	11.11	12.4	10.57	13.88	12.2	11	4.98	12.5	12.2	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.5386-A	CAA05228.1:p.Pro158Rfs	
4515 Deletion	1 A	-	-	-	4.66	5.74	4.2	5.57	8.48	4.65	5.23	5.53	4.78	5.89	7.19	8.52	6.52	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S	
4878 Deletion	1 A	-	-	-	3.13	-	-	3.19	-	-	-	3.31	-	-	3.08	3.71	4.24	3.47	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
4992 SNV	1 C	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S	
5270 Deletion	1 A	-	-	-	8.76	-	-	-	8.92	8.14	7.88	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
5702 Deletion	1 G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
5810 Deletion	1 G	-	-	-	5.8	6.79	4.87	4.95	6.05	6.54	7.07	5.3	6.06	5.83	5.9	5.78	6.13	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
6007 SNV	1 T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
6286 Deletion	1 A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
6766 Deletion	1 A	-	-	-	4.55	5.42	4.86	4.7	4.15	4.68	5.4	4.78	-	5.64	5.49	4.03	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
7844 Deletion	1 T	-	-	-	6.85	7.53	4.6	8.14	6.17	5.36	6.85	7.25	7.59	6.55	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
7972 Deletion	1 C	-	-	-	44.56	51.39	51.2	-	45.95	49.05	46.68	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
8486 Deletion	1 C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
8872 SNV	1 T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
8995 Deletion	1 C	-	-	-	42.65	45.45	48.29	-	38.94	44.04	41.94	42.13	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
9113 SNV	1 C	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
9409 Deletion	1 T	-	-	-	3.88	4.47	5.2	4.69	3.78	4.95	4.8	3.68	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
9446 Deletion	1 C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
9725 Deletion	1 A	-	-	-	11.95	16.22	11.8	13.98	14.09	14.86	15.11	14.52	18.56	15.17	14.43	15.98	17.25	16.18	15.15	CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S	
10336 Deletion	1 A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
10274 Deletion	1 T	-	-	-	12.13	12.42	9.77	12.97	13.96	12.89	12.13	12.8	14.33	12.28	11.86	15.13	16.24	12.21	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
10185 Deletion	1 A	-	-	-	3.61	4.34	3.92	4.9	3.13	5.11	3.89	3.53	3.96	4.11	3.89	4.66	5.69	4.38	4.76	CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S	
10788 Deletion	1 C	-	-	-	51.56	44.33	-	46.52	47.76	45.78	41.12	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
10434 Deletion	1 A	-	-	-	5.25	5.01	3.26	3.13	5.63	-	-	-	4.11	5.12	3.77	4.16	4.93	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
10452 Deletion	1 A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
11071 SNV	1 A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
11469 SNV	1 T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
11483 Deletion	1 C	-	-	-	-	8.15	-	-	7.9	6.69	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
11604 Deletion	1 A	-	-	-	-	-	-	-	3.44	-	3.29	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
11628 Deletion	1 A	-	-	-	3.31	-	-	3.74	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
11726 Deletion	1 A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
11889 SNV	1 C	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
12521 Deletion	1 G	-	-	-	-	4.76	4.47	5.28	5.71	5.4	5.78	-	4.43	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
12719 Deletion	1 C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
13089 Deletion	1 A	-	-	-	11.35	5.67	9.52	9.33	11.11	8.33	10.53	10.11	9.06	9.15	4.49	14.58	11.29	9.18	9.18	CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S	
13096 Deletion	1 C	-	-	-	22.92	-	19.59	17.31	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
13444 Deletion	1 A	-	-	-	-	14.85	15.56	17.37	15.67	14.82	14.58	13.66	14.6	15.2	17.49	11.03	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
13464 Deletion	1 A	-	-	-	-	-	-	-	8.45	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
13662 Deletion	1 C	-	-	-	3.04	3.47	-	3.53	5.92	4.26	3.22	3.07	3.71	3.6	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
13718 Deletion	1 C	-	-	-	9.33	-	-	-	10.81	10.37	7.38	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
13910 Deletion	1 A	-	-	-	-	-	-	-	7.77	5.83	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
14348 SNV	1 T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
14378 SNV	1 A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
14381 SNV	1 T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
14391 SNV	1 G	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
14399 SNV	1 C	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
14402 SNV	1 T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
14468 SNV	1 T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
14420 SNV	1 A	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
14426 SNV	1 A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98.77 CDS: NADH1, Gene: NADH1	CAA05228.1:c.7836A	CAA05228.1:p.Leu451S
14429 SNV	1 C	T	-																				

Table S2 List of primers used for long PCR amplification, and HRM and mtDNA copy number analysis.

ID	Forward	Reverse	Product length	Annealing temperature
Long-PCR primers A	ATAGGACTCGAACCTAAACCTGAGAA	GACGAATAGTGCTACGGGAATGAATA	8272	60
Long-PCR primers B	TTCTACCACTACTACTGACCTTA	AGAATATAGGAGGTTGATGATGATGG	9262	60
HRM-nt 376	CGGCGTAAAGAGTGTTTAAGA	ACTAGGGCTTTTTACAGC	73	62
HRM-nt 1302	CCCCTTCTACCTTTTGCAT	CTGGTT TCG GGG TAT CTA GC	78	62
HRM-nt 1394	AAC CAA CTC ATC TAT GTG GC	GGCTTTTCACCTCTACCT	57	62
HRM-nt 9725	CTACCATGAGCATCCCAAAC	GTA GGA TAA GAA GGA ATA GTG C	81	62
mt-DNA copy number	CTC AAC CCT AGC AGA AAC CA	TTA GTT GGT CGT ATC GGA ATC G	254	55
nuclear-DNA copy number (B-globin)	GTCTAAGCTGGTCCTCTACT	GAGCCAGAACAACCACTATC	93	60