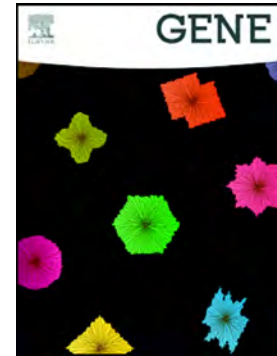


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## **Whole sequence of the mitochondrial DNA genome of Kearns Sayre Syndrome patients: identification of deletions and variants**

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## Whole sequence of the mitochondrial DNA genome of Kearns Sayre Syndrome patients: identification of deletions and variants

Mitochondria both produce the energy of the cell as ATP via respiration and regulate cellular metabolism. Accordingly, any deletion or mutation in the mitochondrial DNA (mtDNA) may result in a disease. One of these diseases is Kearns Sayre syndrome (KSS), described for the first time in 1958, where different large-scale deletions of different sizes and at different positions have been reported in the mitochondrial genome of patients with similar clinical symptoms. In this study, sequences of the mitochondrial genome of three patients with clinic features of KSS were analyzed. Our results revealed the position, heteroplasmy percentage, size of deletions, and their haplogroups. Two patients contained deletions reported previously and one patient showed a new deletion not reported previously. These results display for the first time a systematic analysis of mtDNA variants in the whole mtDNA genome of patients with KSS to help to understand their association with the disease.

Keywords: mitochondrial disease; large-scale deletion; heteroplasmy; haplogroup; phylogenetic analysis.

Subject classification code: SCIENTIFIC RESEARCH

### 1. Introduction

Mitochondrial diseases result from alterations in either the mitochondrial or nuclear genome (Gorman et al. 2015; Chinnery, 2015) due to the close relationship of the genomes coding for the proteins that constitute the respiratory chain, which is the main source of cellular energy. The human mitochondrial genome is a double-stranded DNA molecule (mtDNA) of 16569 bp that encodes 13 subunits of the oxidative phosphorylation system and 24 RNAs for intra-mitochondrial protein synthesis (Gorman et al. 2016). Most of the estimated 1500 mitochondrial proteins are encoded by the nuclear genome (Calvo and Mootha 2015). However, mutations or

rearrangements in mtDNA, such as deletions and duplications, disturb multiple organ systems, particularly the brain and spinal cord, peripheral nerve, heart, muscle and endocrine organs (Wallace, 2015; Stewart and Chinnery 2015).

There are several mutations, rearrangements, or deletions in the mitochondrial genome that cause well-known mitochondrial diseases, such as mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes syndrome (MELAS), myoclonic epilepsy with ragged red fibers (MERRF), neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP), Leber hereditary optic neuropathy (LHON), Pearson Syndrome, chronic progressive external ophthalmoplegia (CPEO) and Kearns Sayre Syndrome (Gorman et al. 2016). Furthermore, different variants have been also associated with other diseases such as like Alzheimer and Parkinson (Wu et al. 2018; Shoffner et al. 1993; Coskun et al. 2012), cancer (Bai et al. 2007), metabolic syndrome (Juo et al. 2010) and diabetes (Jiang et al. 2017).

Among these diseases, the present study focused on Kearns-Sayre Syndrome (KSS) with an onset before 20 years, as defined by the presence of ptosis and/or ophthalmoparesis due to mtDNA single large-scale deletion and at least one of the following features: retinopathy, ataxia, cardiac conduction defects, hearing loss, short stature, cognitive involvement, tremor and cardiomyopathy (Mancuso et al. 2015). Since KSS was first reported by Kearns and Sayre in 1958, there have been many reports of deletions in the mtDNA at different positions and size among patients (Holt et al. 1988; Nelson et al. 1989; Montiel-Sosa et al. 2013). In addition, a direct repeat of 13 bases flanking a common pathogenic large-scale deletion of 4977 bp within nucleotides 8470-8482 and 13447-13459 has been identified (Schon et al. 1989; Samuels et al. 2004). Approximately 60% of mtDNA deletions are flanked by direct repeat sequences (class I deletions), 30% are flanked by imperfect repeats (class II deletions) and 10%

have no flanking repeats (class III repeats) (Mita et al. 1990; Johns et al. 1989; Samuels et al. 2004).

Whole sequences have not been reported for this mitochondrial disease that may give us a clue to explain the mechanisms of KSS origin. Consequently, to contribute to the knowledge of mtDNA regulation and the cause of the disease (Carelli and Chan 2014; Latorre-Pellicer et al. 2016; Kang et al. 2016), the complete mtDNA sequences of 3 patients were obtained and analyzed. Our results showed the size of the large-scale deletions, heteroplasmic percentage, haplotype/haplogroup identification and the phylogenetic analysis compared with other representative haplogroups. It is important to notice that for first time in this area of research, a systematic analysis of mtDNA variants in the whole mtDNA genome of patients with KSS was developed. It is expected that this analysis will help to understand the mtDNA variants relationships with KSS.

## **2. Materials and Methods**

### **2.1 Clinical information**

Molecular analyses of three unrelated patients with clinical information and the diagnosis of KSS are included in this study. The first patient was a 10-year-old girl previously reported by our group (Montiel-Sosa et al. 2013). To complete the analysis of this patient (KSS-1P), the full sequence was obtained. Unfortunately, this patient deceased in 2013. Furthermore, the mtDNA sequence of her mother was also obtained and analyzed. The second patient (KSS-2P) was a 19-year-old presenting with diplopia, progressive external ophthalmoplegia, palpebral bilateral ptosis and sporadic muscle cramps. The third patient (KSS-3P) was 18 years old and had had progressive bilateral ptosis palpebral since he was 8 years old, ophthalmoplegia, hearing loss, intolerance of

exercise, occasional occipital cephalic pain, weight loss, and decreased visual acuity since he was 11 years old; he also has a cardiac pacemaker because he suffers from an auricular-ventricular blockade. This study was performed according to an Institutional Review Board protocol for research on human subjects at Centro Médico Nacional Siglo XXI, IMSS.

## **2.2 mtDNA analysis**

After receiving informed consent from the parents, total genomic DNA was extracted from 50 mg of frozen biopsied skeletal muscle from each patient using a DNeasy Blood and Tissue kit and Handbook (Qiagen, Hilden, Germany).

## **2.3 Detection and mapping of mtDNA deletions**

Long-range PCR for mtDNA was performed on the muscle sample using the GeneAmp XL PCR kit (PerKin-Elmer, Boston, MA) according to previously described protocols (Yakes and VanHouten 1997; Santos et al. 2002). The PCR amplification was performed using the long-range PCR enzyme mix (Takara, Shiga, Japan) with the “Forward” and “Reverse” primers (Supplementary Table 1). The conditions for PCR were an initial denaturation at 95°C for 2 min, followed by 9 cycles of 30 s at 92°C, and 10 min at 68°C; 19 cycles of 92°C for 40 s, 68°C for 12 min, 72°C for 10 min and 95°C for 2 min; an additional 9 cycles of 30 s at 92°C and 10 min at 68°C; and then 19 cycles at 92°C for 40 s and 68°C for 12 min; and finally an extension at 72°C for 10 min. Products were separated and visualized by electrophoresis on a 0.8% agarose gel with ethidium bromide.

Total genomic DNA (5 µg) was digested with the restriction endonuclease *Pvu II*, which cleaves human mtDNA at a single position (nucleotide 2652). The digested DNA

was electrophoresed on a 0.7% agarose gel and transferred to nylon membranes. The nylon membranes were hybridized with the 16S region of a human mtDNA probe labelled with the non-radioactive precursor digoxigenin-UTP (Dig-High Prime, Roche catalogue #1585606) by PCR amplification following the manufacturer's protocol. Labelling efficiency was tested using the control labelling kit Dig-labelled control DNA from Roche. The deleted mtDNA was mapped by long-range PCR of the whole mtDNA genome (Tengan and Moraes 1996), which allowed us to determine both the best primers to use to amplify the sequence that contained the deleted region and the exact limits of this deletion by sequencing. The measurement of the percentage of deletion was calculated from the signal intensities of the deletion mutant and the full-length molecules using densitometry (Edris et al. 1994).

After identifying mtDNA deletions in the sample of each patient, the amplification of fragments around nucleotides 8470 to 13447 of 7158 bp size was examined using the primers L7148(nt.7148) and L14268(nt.14268) to determine if the genome of both patients contained the common deletion. Next, the whole mtDNA in two fragments was amplified using two pairs of primers, L644/H8982 and L8789/H877 (Supplementary Table 1), which provided two overlapping fragments of 8381 and 8703 bp, respectively. This strategy also permitted an approximation of both the localization and, by sequencing, the exact sites of the deletion.

#### **2.4 Sequencing of whole mtDNA**

The whole mtDNA of all samples was amplified and sequenced by using 24 pairs of primers previously described (Rieder et al. 1998), with the following PCR conditions: 3 min at 95°C for an initial denaturation and 35 cycles of 95°C for 30 s, 60°C for 45 s, 72°C for 55 s, and a final extension at 72°C for 5 min. The 24 overlapping fragments were visualized by electrophoresis on a 0.7% agarose gel and purified with ExoSap-IT

before the sequencing reaction with the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit, following the manufacturer's instructions (Applied Biosystems). The products of this reaction were purified and analyzed on an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Comprehensive contig assembly and sequence alignment was performed using the Sequencher Software version 4.1 (Gene Codes Corporation, New York City), and SNPs relative to the revised Cambridge Reference Sequence (rCRS) were scored and confirmed by manual checking of electropherograms.

## **2.5 Mutation impact analysis**

The impact of the non-synonym mutations was assessed by using the predictors MitImpact 2.9 (Castellana et al. 2015; <http://mitimpact.css-mendel.it/>), Polymorphism Phenotyping (PolyPhen v2; Adzhubei et al. 2010), and MutationAssessor (Reva et al. 2011). MitImpact provided a view of the pre-computed pathogenicity score of the amino acid change as a pathogenic predictor; PolyPhen predicted the potential impact of the amino acid substitution in the protein structure and function by using physical and comparative considerations; and MutationAssessor predicted the functional effect due to amino acid substitution in the protein based on evolutionary conservation.

## **2.6 Phylogenetic analysis**

Sequences of the patients of this study were aligned with 51 and 84 representative Amerindian and Asian mtDNA complete sequences of haplogroups B (KSS-P2) and C (KSS-P1 and KSS-P3), respectively, using the Clustal W program (Thompson et al. 1994). The phylogenetic tree was then constructed using maximum likelihood reconstruction, the Neighbor Joining algorithm (Nei et al. 1985), and the HKY85 nucleotide substitution model (Hasegawa et al. 1985). The TN93RV algorithm included



in the Hypothesis Testing using Phylogenies software package was used to obtain the genetic distance with gamma correction 1 (Tamura and Nei, 1993) (HyPhy, Pond et al. 2005). Finally, a bootstrap of 1000 repetitions was performed.

### **3. Results**

#### **3.1 Clinical features of patients**

Kearns-Sayre Syndrome is a mitochondriopathy with an onset of symptoms before 20 years of age. Accordingly, the three unrelated patients reported here presented with palpebral bilateral ptosis, progressive external ophthalmoplegia and cardiomyopathy. In addition, patient KSS-P2 had sporadic muscle cramps, and patient KSS-P3 suffered from left hearing decreases, fatigue on exercise, occasional occipital headaches, weight loss and progressive decreases in visual acuity.

#### **3.2 Identification of mtDNA deletion**

To identify the size and location of the deletions in the mitochondrial genome of KSS, long PCR amplification, southern blot assays (Figure 1A) and sequencing (Figure 1B-D) were performed. The large-scale deletion of 7629 bp from nucleotides 7437 to 15065 of the mtDNA (Figure 1B) of KSS-P1 was reported previously (Montiel-Sosa et al. 2013). Sequence analysis showed that the mother and her daughter had the same full sequence; however, the patient's mother did not show the large-scale deletion.

The amplification of the whole mtDNA of KSS-P2 and KSS-P3 by long-range PCR and southern blot analysis showed two bands of approximately 16.5 and 11.2 Kb, indicating the presence of a heteroplasmic deletion of approximately 5 Kb in each patient (Figure 1A). Then, to determine if the patients displayed a common large-scale deletion, the mtDNA was amplified using the primers displayed in Supplementary

Table 1. KSS-P2 showed a deletion between nucleotides 7148 and 14268 because the amplicon obtained was 2100 bp instead of 7158 bp. Digestion of this fragment with *Hinc II* restriction endonuclease displayed the expected two bands of 630 and 709 bp, indicating that enzyme cuts at nucleotides positions 7857 and 13638 were present. Primers from nucleotide 8150 to 13650 were used to obtain an expected amplicon of 5500 bp; however, we obtained a fragment of 500 bp. The sequence of this fragment revealed that the deletion starts at nucleotide position 8469 and ends at 13446, indicating a deletion of 4977 bp. KSS-P2 contained a large-scale deletion flanked by a perfect direct repeated of 13 bp, which was reported previously as flanking the common deletion (Samuels et al. 2004). The genes deleted in KSS-P2 are ATPase 6 and 8, COIII, tRNA<sup>G</sup>, ND3, tRNA<sup>R</sup>, ND4, tRNA<sup>H</sup>, tRNA<sup>S</sup>, tRNA<sup>L</sup> and part of ND5 (Figure 1C).

The mtDNA of KSS-P3 was amplified using two pairs of primers L644/H8982 and L8789/H877 (Supplementary Table 1) to obtain two overlapping fragments of 8381 and 8703 bp, respectively. The fragments obtained were 8381 bp and 3100 bp, which correspond to a large-scale deletion of 5387 nucleotides. To map the location of this deletion, the 3100 bp was digested with the restriction enzymes as follows: *Pst I*, *Apa I*, *Hinc II*, *Xba I*, *Bam HI*, and *Xho I*, with the restriction sites at positions determined by NEBcutter V2.0 (Vincze et al. 2003, <http://nc2.neb.com/NEBcutter2/>) in a fragment from nucleotides 7148-877. *Pst I*, *Apa I*, *Xba I*, and *Hinc II* cut at nucleotide sites 9024, 9269, 10256, 10016, 16458, indicating the absence of the restriction sites at positions 12409, 13262, 13637, 14,259, and 14956 and, therefore, the lack of a region that contains these sites. To continue mapping the deletion, a region between nt 9989 and 15978 was amplified, obtaining an amplicon of 600 bp that was sequenced, which established that the deletion involved nucleotides 10371 to 15758. This result indicated a deletion of 5387 bp (Figure 1D). The sequence of KSS-P3 had no flanking repeated

bases. The genes deleted in KSS-P3 were tRNA<sup>R</sup>, ND4, tRNA<sup>H</sup>, tRNA<sup>S</sup>, tRNA<sup>L</sup>, ND5, ND6 and part of *cyt b* (Figure 1D).

Quantification of heteroplasmic mtDNA deletions using densitometry was 84%, 40%, and 60% for KSS-P1 (Montiel-Sosa et al. 2013), KSS-P2, and KSS-P3, respectively.

### 3.3 Analysis of whole genome sequence

To search for common variants relevant to the pathology and identification of haplogroups, whole mitochondrial genome sequencing of KSS-P1, KSS-P2, and KSS-P3 was determined, and the GenBank numbers are MG652750, MG652752 and MG652751, respectively. Table 1 shows all of the polymorphisms identified in the three sequences of the patients. Sequences of KSS-P1, KSS-P2, and KSS-P3 displayed 28 synonym variants (Table 2) and a total of 7 non-synonym changes for each one (Table 3). Among these, m.6340C>T has been associated with cancer (Scott et al., 2012); m.7444G>A has been reported to be associated with Leber's hereditary optic neuropathy (LHON; Yuan et al. 2005) and sensorineural hearing loss (SNHL; Yang et al. 2016); and m.10398A>G has been related to Parkinson's disease (PD; Otaegui et al. 2004), altered mitochondrial matrix pH (Kazuno et al. 2006), metabolic syndrome (Juo et al. 2010) and has been considered a risk factor for breast cancer (Bai et al. 2007).

### 3.4 Haplogroup identification

Haplogroups of the KSS patients were determined to define whether they have any relevance to this disease because mitochondrial haplogroups have been reported to be associated with other diseases such as Alzheimer and breast cancer, (Santoro et al. 2010; Ma et al. 2018 respectively), or influence the mitochondrial function (Kenney et al. 2014). Consequently, haplogroups for each patient were identified according to

Achilli et al. 2008, Derenko et al. 2010, Kumar et al. 2011, Rieux et al. 2014 and MitoMaster (Brandon et al. 2009; <http://www.mitomap.org>). The KSS patient 1 and her mother were haplogroup C1b14, KSS-P2 was haplogroup B2 and patient KSS-P3 was haplogroup C1d. Variants m.3552T>A, m.11914G>A and m.16327C>T specific for haplogroup C were common in KSS-P1 and KSS-P3. Polymorphisms m.493A>G, m.5894A>G, m.10397A>G and m.16181A>G were specific for C1b14 (Table 1) and exclusively found in KSS-P1 and her mother; and m.194C>T, m.6340C>T, m.9545A>G, m.13263A>G, m.16051A>G and m.16325T>C variants (Table 1) were specific for haplogroup C1d, as observed in KSS-P3. The haplogroup B2 of KSS-P2 was defined by the presence of variants m.827A>G, m.3547A>G, m.4820G>A, m.4977T>C, m.6473C>T, m.9950T>C, m.11177C>T, m.13590G>A, m.15535C>T, m.16217T>C, and the deletion of 9 bp at nucleotide positions 8281-8289 (Table 1).

### 3.5 Phylogenetic analysis

The phylogenetic tree of the KSS patients was constructed directly from the entire mtDNA sequences and representative sequences obtained from GenBank to understand the genetic ancestry through the direct maternal line. Figures 2-3 show the phylogenetic tree of the 3 mtDNA sequences from the KSS patients (deposited in GenBank under Accession No. MG652750 - MG652752). According to the analysis of variants obtained by MitoMaster (Brandon et al. 2009; Lott et al. 2013) and previous haplogroup classifications (Kumar et al. 2011), the mtDNA phylogenetic trees of KSS-P1, KSS-P3 and KSS-P2 displayed haplogroups C1b14, C1d (Figure 2) and B2 (Figure 3), respectively.

#### 4. Discussion

An in depth genetic characterization of whole mtDNA genome was carried out in this research, which will help to understand pathogenic mechanisms of mtDNA diseases like KSS. Previous research have shown that a better understanding of the location, length and percentage of a single mtDNA deletion explains mitochondrial syndromes (López-Gallardo et al. 2009; Grady et al. 2014; Mancuso et al. 2015; Rocha et al. 2018). The effect of mtDNA population genetic variation might be very important in patients with single mtDNA deletions. Because, it is well known that mtDNA genetic background can affect mitochondrial gene expression, disease susceptibility and severity of disease-causing mtDNA mutations (Wallace. 2015; Cohen et al. 2016; Wei et al. 2017). Thus, these findings will support future meta-analyses in order to refine the association between single mtDNA deletions and mitochondrial syndromes.

Mitochondrial diseases have been continuously studied because of their complicated diagnosis and treatment. Kearns Sayre Syndrome is associated with large-scale mtDNA deletions that differ among patients. It is important to extend the sequence analysis beyond the large-scale deletion and the flanking regions to find and establish haplogroup-related and mitochondrial pathogenic variants. Recently, Latorre-Pellicer et al. (2016) used conplastic animals to reveal that mitochondrial variations have an important influence on the physiology, phenotype and longevity of organisms, suggesting that the same effect occurs in humans.

Importantly, it also has been demonstrated by using cybrid cells that mtDNA haplogroup J has increased transcription and replication and, as a result, there are more mtDNA molecules in haplogroup J than there are in haplogroup H (Suissa et al. 2009). Therefore, mitochondrial haplogroups of patients with mitochondrial disorders could be very relevant. Consequently, it is very important to determine the mtDNA haplogroups

when treating mitochondrial diseases, e.g., with mitochondrial replacement therapy in human oocytes (Latorre-Pellicer et al. 2016).

Whole mtDNA sequences in patients displayed twelve non-synonym changes (Table 3); among them, m.8584G>A, m.8701A>G, and m.14318T>C were shared between KSS-P1 and KSS-P3, and m.8860A>G, m.14766C>T, and m.15326A>G were shared among the 3 patients. All of these non-synonyms changes were previously reported in MITOMAP (<http://www.mitomap.org>). Some of these variants have been associated with other disease conditions (Rollins et al. 2009; Ebner et al. 2011; Bai et al. 2007; Tommasi et al. 2014; Rad et al. 2016; Venkatesan et al. 2014; Wallace. 2015) such as a) m.6340C>T and m.7444G>A variants in CO1 gene identified in the KSS-P3 and KSS-P2 patients respectively, which were previously detected in patients that suffered prostate cancer (Petros et al. 2005; Scott et al. 2012), Leber's hereditary optic neuropathy (LHON), or sensorineural hearing loss (SNHL) (Zhu et al. 2006; Yang et al. 2016); b) m.827A>G variant located at the A-site of the mitochondrial 12S rRNA gene identified in KSS-P2 patient, these were previously associated with non-syndromic and aminoglycoside-induced hearing loss (Li et al. 2004; Xing et al. 2006), a sign in KSS patients, probably due to alteration of the rRNA structure leading to mitochondrial dysfunction (Chaig et al. 2008; Xing et al. 2006; Nivoloni et al. 2010; Barbarino et al. 2016); and c) m.10398A>G variant within the ND3 gene identified in KSS-P1 and KSS-P3 has been identified as a risk factor with the metabolic syndrome (Juo et al. 2010), and it is considered a predictor for T2D, which is present in some of the KSS patients (Ho et al. 2014). In addition, since 153A>G and 152T>C variants are located close to the replication site in KSS-P1 and KSS-P3 respectively, it would be important to study their function in cybrids, since it has been reported that m.150C>T variant

close to the replication site changes the replication site in the mtDNA (Chen et al. 2012).

Kazuno et al. (2006, 2008) found in cybrid cell lines that mitochondrial metabolism can be altered by m.8701G/10398G variants identified in the mtDNA deleted region of KSS-P1 and KSS-P3 patients, which has been reported to play a role in the pathophysiology of complex diseases by affecting mitochondrial matrix pH and intracellular calcium dynamics. Cybrids with 10398G and 8701G variants did not respond to treatment with valproate to stabilize calcium levels; contrary cybrids with 10398A and 8701A variants responded to the treatment (Kazuno et al. 2008). These differences are important since the KSS-P2 patient had 8701A/10398A variants, which make the patient susceptible to some kind of medical treatments and KSS-P1 and KSS-P3 patients containing m.8701G/10398G variants will be resistant to the treatment of some compounds. Some KSS patients may present cyclic vomiting syndrome (Boles et al. 2007), previously associated with the variant m.16519T (Venkatesan et al. 2014; Boles et al. 2010) identified in KSS-P1 and KSS-P2. Accordingly, the treatment for these patients usually include mitochondrial supplements like co-enzyme Q10, riboflavin and L-carnitine (Boles et al. 2007). These findings highlight the importance of mitochondrial genetic background that also may influence the patient response to medical treatments.

Haplogroup identification was also developed because variants in the whole mtDNA genome primarily define haplogroup, which has been found to be associated with certain human diseases (Wu et al, 2018). KSS-P1 and her mother belonged to the C1b14 haplogroup (Figure 2), which is uncommon, and there are only three sequences reported in the database of mtDNA sequences (GenBank). One of them pertained to a Zapotec individual (GenBank number: KJ923846), and the other was from a Mexican

American individual (GenBank number: HQ012208) (Gómez-Carballea et al. 2015, Kumar et al. 2011). There is also one sequence with the C1b14 haplogroup from the 1000 genomes project (NA19773). Furthermore, there is a partial sequence reported in a pre-Hispanic Mayan individual that belongs to the C1b14 haplogroup (Ochoa-Lugo et al. 2016). This haplogroup was confirmed by the phylogenetic analysis displayed in Figure 2. Haplogroups C1b, C1c and C1d are of Beringian origin, as are the haplogroups A2, B2 and D1 (Achilli et al. 2008). The estimated age of the C1b14 on the American continent is approximately 15 ky (Gómez-Carballea et al. 2015). Moreover, one infant burial (USR1) from a common interment at the Upward Sun River Site in central Alaska dating to ~11,500 cal B.P. was determined to possess variants that define mitochondrial lineage C1b (Tackney et al. 2015). The presence of the C1b14 haplogroup in a Mayan ancient individual clearly shows that this haplogroup was present in Mexican pre-Hispanic populations, and it has been maintained in contemporary Mexican populations such as the Zapotec, although in very low frequencies suggesting that this haplogroup has not been expanded in contemporary populations successfully.

The KSS-P3 displayed haplogroup C1d (Figure2), which is a founding Native American lineage that is defined by transitions at nucleotides 194 and 16051 and entered the continent from Beringia at the end of the Last Glacial Maximum ( $18.7 \pm 1.4$  kya) (Achilli et al. 2008; Perego et al. 2010). The C1d haplogroup is distributed along the American continent (Perego et al. 2010) and has been identified in a Mexican mestizo population with a frequency of 18.8% in a cohort of 270 individuals (Guardado-Estrada et al. 2009). KSS-P3 sequence was grouped with sequences from Mexican individuals living in USA (Figure 2).



Finally, KSS-P2 belongs to haplogroup B2 of a Native American ancient haplogroup, with coalescence time estimated at 19 ky (Achilli et al. 2008). This lineage was also identified in one infant burial (USR2) from a common interment at the Upward Sun River Site in central Alaska dating to ~11,500 cal B.P. (Tackney et al. 2015). In addition, this haplogroup has been associated with a risk of cervical cancer in a Mexican mestizo population (Guardado et al. 2012), but it is not associated with prostate cancer in Colombian patients (Cano et al. 2014). There is also one MELAS case of a Mexican patient reported with haplogroup B2c (Delgado-Sanchez et al. 2007).

The MXKSS-P2 sequence is more closely related to the sequences with haplogroups B2u and B2k, with very low frequency in Mexican American individual residents of the USA (Figure 3). The B2o1 haplogroup is also in the same branch and corresponds to sequences from Bolivian and Colombian individuals. The MXKSS-P2 sequence is more closely associated with haplogroup B2u and is a rare haplogroup in Mexico because no identical sequences have been reported in the mitochondrial databases.

The haplogroup of patients with mitochondrial diseases in Mexicans has rarely been reported in Mexican populations. Therefore, it is recommended to sequence and determine both the haplogroup and haplotype to establish their importance for pathology and to trace the migration and evolution of populations. Two of the KSS patients reported in this study belong to haplogroup C, and one belongs to haplogroup B; the latter has been associated with susceptibility to diseases such as hearing loss (Ying et al. 2015), Alzheimer's disease (Bi et al. 2015), high-altitude pulmonary edema (Luo et al. 2012) and T2D (Liou et al. 2012).

## 5. Conclusions

Our results support the importance of performing full sequencing of mitochondrial genomes of patients with multisystem disorders to discover new variants and their functional impact as part of complex protein suppliers of ATP by the phosphorylation system. Furthermore, the three patients have different classes, positions, lengths and grades of heteroplasmy of the large-scale deletion, suggesting that size and heteroplasmy are related to the grade of this pathology. We can also suggest that when a family has any family history of mitochondrial disease, it is important to perform full mtDNA sequencing to diagnose any mutation associated with the disease and suggest an effective treatment or a preventative strategy with a mitochondrial replacement procedure.

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## Disclosure statement

The authors declare that they have no competing interests.

## Ethical approval and consent to participate

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#### Figure Legends

Figure 1. Characterization of the mtDNA deletion. (A) Southern blot analysis of mtDNA from KSS-P2 and KSS-P3; lines 1 and 3 display the molecular ladder DNA  $\square$  Hind III; lines 2 and 5 display the long range PCR from KSS-P2 and KSS-P3 showing the 16.5 kb bands with no deletion and the DNA containing the large deletions of approximately 11.2 kb for KSS-P2 and KSS-P3, respectively; line 4 corresponds to a control mtDNA with no deletion; sequence across the mtDNA deletions breakpoint showing the site of the flanking sequence in (B) KSS-P1 reported previously (Montiel et al. 2013) and (C and D) KSS-P2 and KSS-P3, respectively.

Figure 2. Molecular phylogenetic analysis by the maximum likelihood method of haplogroup C. The evolutionary history was inferred by using the maximum likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa et al. 1985). The tree

with the highest log likelihood (-24486.9906) is shown and Akaike's informative criterion (49750.9). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with the superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (3 categories (+G, parameter = 0.0500)). The rate variation model allowed for Tamura and Nei. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 76 nucleotide sequences (Supp. Table 2). All positions containing gaps and missing data were eliminated. There were a total of 16,560 positions in the final dataset. The bootstrap = 1000. Sequences in bold are from the KSS patients, and the ancient sequences are labeled with an asterisk (\*).

Figure 3. Molecular phylogenetic analysis by the maximum likelihood method of haplogroup B. The evolutionary history was inferred by using the maximum likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa et al. 1985). The tree with the highest log likelihood (-24486.9906) is shown with Akaike's informative criterion (49750.9). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with the superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (3 categories (+G, parameter = 0.0500)). The rate variation model allowed for

Tamura and Nei (Tamura and Nei. 1993). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 76 nucleotide sequences (Supp. Table 2). All positions containing gaps and missing data were eliminated. There were a total of 16,560 positions in the final dataset. The bootstrap = 1000. Sequences in bold are from the KSS patients, and the ancient sequences are labeled with an asterisk.

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Table 2. Synonym variants identified in the KSS patients and the mother of KSS-1P. Analysis was performed by MitoMaster.

<b>Nucleotide position</b>	<b>KSS patient*</b>	<b>Locus</b>	<b>Mutation type</b>	<b>Amino acid</b>	<b>Codon position</b>	<b>GB frequency %</b>
T3552A	1, 3	MT-ND1	transversion	Ala82	Third	3.24
G3915A	2	MT-ND1	Transition	Gly203	Third	1.34
A4715G	1, 3	MT-ND2	Transition	Gly82	Third	3.92
A4769G	1, 2, 3	MT-ND2	Transition	Met100	Third	97.26
A5894G	1	MT-NC5	Transition	Non-coding	-	0.35
C7028T	1, 2, 3	MT-COI	Transition	Ala375	Third	78.89
C7196A	1, 3	MT-COI	Transversion	Leu431	Third	3.83
T7885C	2	MT-COII	Transition	Ile100	Third	0.03
T9540C	1, 3	MT-COIII	Transition	Leu112	First	32.92
A9545G	1, 3	MT-COIII	Transition	Gly113	Third	4.00



T9950C	2	MT-COIII	Transition	Val248	Third	2.86
A10397G	1	MT-ND3	Transition	Trp113	Third	0.93
C10400T	1, 3	MT-ND3	Transition	Thr114	Third	20.42
G10646A	3	MT-ND4L	Transition	Val59	Third	0.33
T10873C	1, 3	MT-ND4	Transition	Pro38	Third	32.88
G11719A	1, 2, 3	MT-ND4	Transition	Gly320	Third	75.36
G11914A	1, 3	MT-ND4	Transition	Thr385	Third	11.02
G12007A	2	MT-ND4	Transition	Trp416	Third	6.48
A12172G	3	MT-TH	Transition	tRNA p38	Anticodon loop	0.78
C12705T	1, 3	MT-ND5	Transition	Ile123	Third	41.74
T13174C	2	MT-ND5	Transition	Leu280	First	0.08
A13263G	1, 3	MT-ND5	Transition	Gln309	Third	3.47

G13590A	2	MT-ND5	Transition	Leu418	Third	5.39
T14783C	1, 3	MT-CYB	Transition	Leu13	First	20.29
G15043A	1, 3	MT-CYB	Transition	Gly99	Third	22.77
G15301A	1, 3	MT-CYB	Transition	Leu185	Third	27.55
A15487T	1, 3	MT-CYB	Transversion	Pro247	Third	3.85
C15535T	2	MT-CYB	Transition	Asn263	Third	2.01

\*KSS patient 1, 2, and 3 represent patient KSS-P1, KSS-P2, and KSS-P3 respectively. KSS-P1M has the same synonyms variants as KSS-P1.

<b>Nucleotide position</b>	<b>KSS Patient</b>	<b>Locus</b>	<b>Amino acid</b>	<b>PolyPhen</b>	<b>Mutation Assessor Impact</b>	<b>Codon position</b>	<b>GB frequency (%)</b>	<b>Disease reported</b>	<b>Reference</b>
A3547G	2	MT-ND1	I81V	Benign	Low	First	1.44		
C6340T	3	MT-CO1	T146I	Benign	Medium	Second	0.16	Prostate cancer	Scott et al., 2012
G7269A	2	MT-CO1	V456M	Benign	Neutral	First	0.16		
G7444A	2	MT-CO1	TERM514K	-	-	Second	0.37	LHON, SNHL, DEAF	Gorman et al. 2016
G8584A	1 and 3	MT-ATP6	A20T	Benign	Neutral	First	4.64		
A8701G	1 and 3	MT-ATP6	T59A	Benign	Neutral	First	32.82		
A8860G	1, 2 and 3	MT-ATP6	T112A	Benign	Medium	First	98.34		
A10398G	1	MT-ND3	T114A	Benign	Neutral	First	43.68	PD, longevity,	Otaegui et al.

								altered mitochondrial pH, metabolic syndrome, breast cancer risk	2004; Dato et al. 2004; Kazuno et al. 2006; Juo et al. 2010; Bai et al. 2007 respectively
C11177T	2	MT-ND4	P140S	Benign	Medium	First	1.44		
T14318C	1 and 3	MT-ND6	N119S	Benign	Neutral	Second	3.27		
C14766T	1, 2 and 3	MT- CYB	T7I	Benign	NA	Second	75.1 5		
A15326G	1, 2 and 3	MT- CYB	T194A	Benign	Neutral	First	98.29		

**Table 3.** Analysis of nonsynonymous variants identified in the KSS patients and the mother of patient 1 by PolyPhen and MutationAssessor programs. Predicting functional effect analysis in MitImpact 2.9 in the 4 whole sequences of this study by PolyPhen and MutationAssessor for pathogenicity predictions.

PD: Parkinson's disease.

## Abbreviation List

ATP: Adenosine triphosphate

KSS: Kearns Sayre syndrome

mtDNA: mitochondrial DNA

MELAS: Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes syndrome

MERRF: Myoclonic epilepsy with ragged red fibers

NARP: Neurogenic muscle weakness, ataxia and retinitis pigmentosa

LHON: Leber hereditary optic neuropathy

CPEO: Chronic progressive external ophthalmoplegia

KSS-1P: First patient

KSS-2P: Second patient

KSS-3P: Third patient

bp: base pair

nt: nucleotide

SNP: Single nucleotide polymorphism

rCRS: Revised Cambridge Reference Sequence

PD: Parkinson's disease

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**Research Highlights:**

1. Mitochondrial DNA genome of Kearns Sayre Syndrome patients displayed haplogroups with low frequency in contemporary populations.
2. Two patients contained deletions reported previously and one patient showed a novel deletion not reported previously.
3. Mitochondrial DNA polymorphism were displayed in other diseases.

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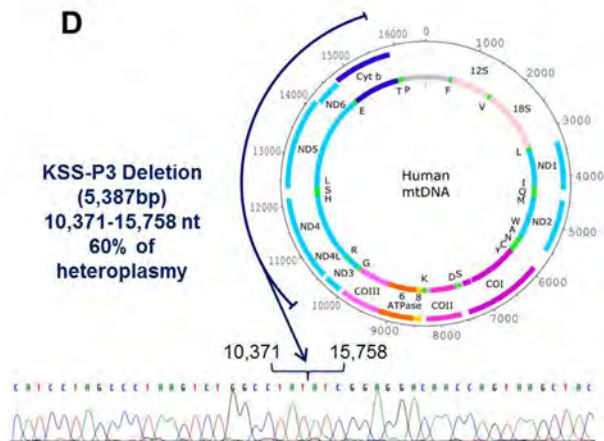
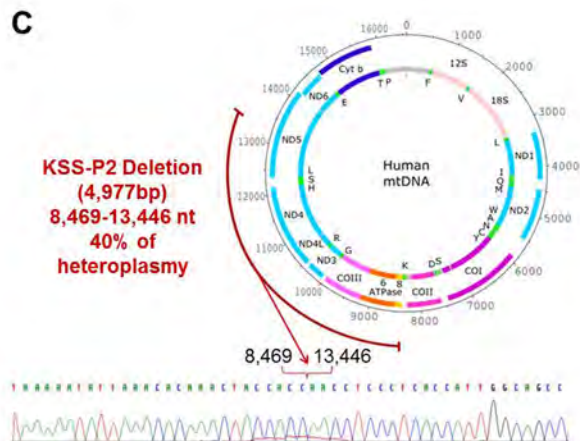
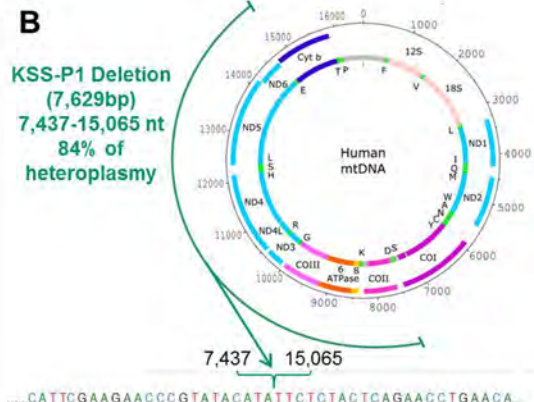
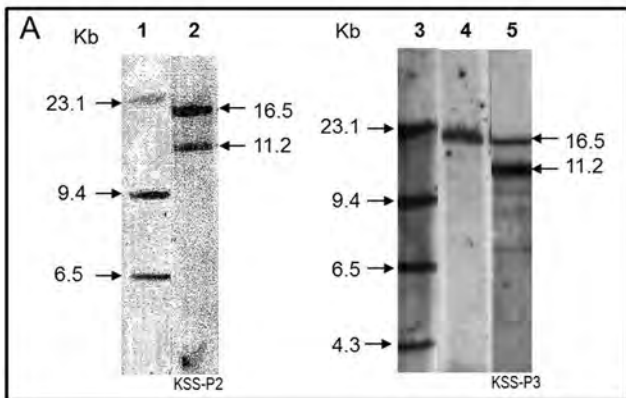


Figure 1



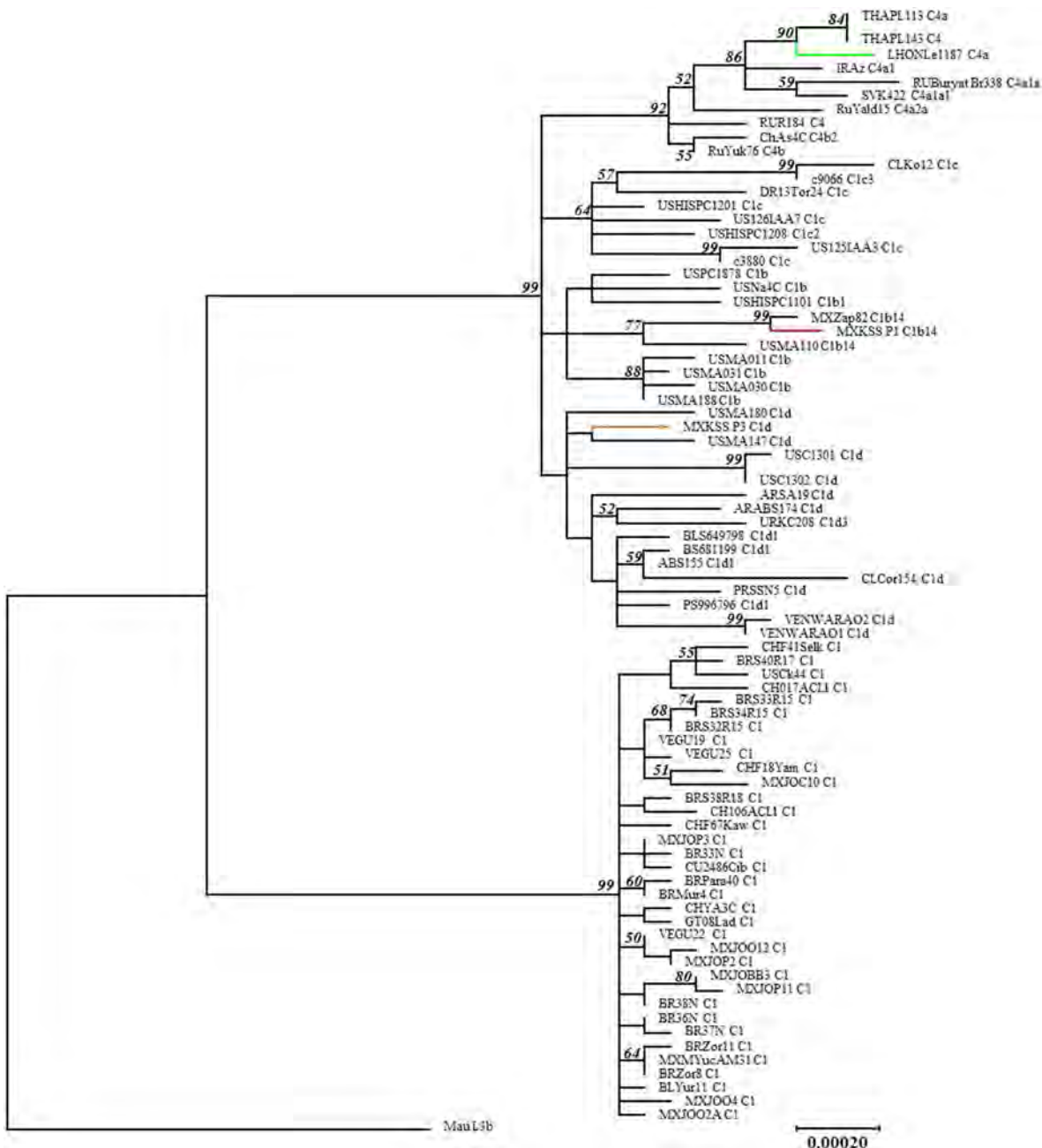


Figure 2

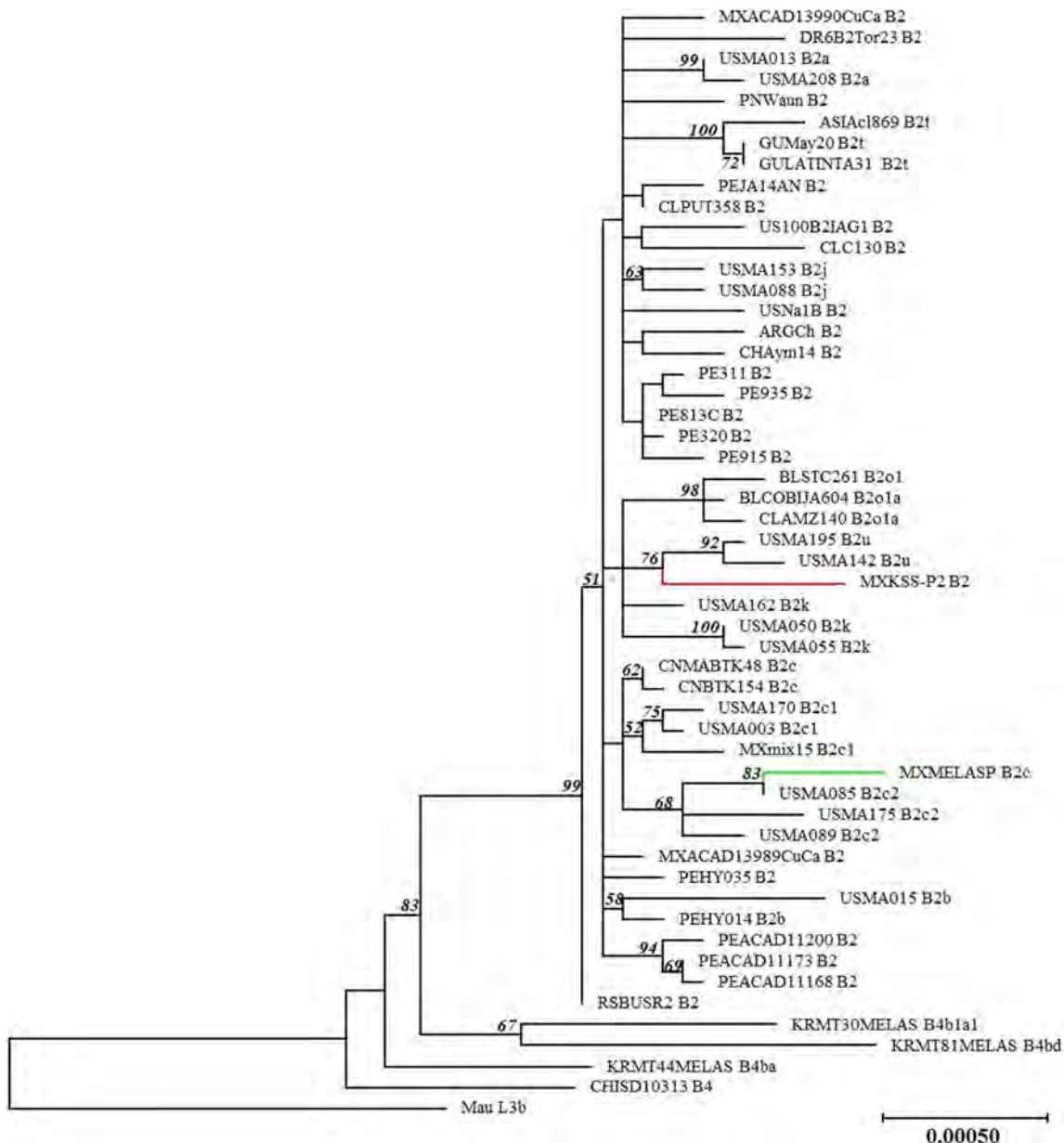


Figure 3