Short communication

Titanic's unknown child: The critical role of the mitochondrial DNA coding region in a re-identification effort☆

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ABSTRACT

This report describes a re-examination of the remains of a young male child recovered in the Northwest Atlantic following the loss of the Royal Mail Ship Titanic in 1912 and buried as an unknown in Halifax, Nova Scotia shortly thereafter. Following exhumation of the grave in 2001, mitochondrial DNA (mtDNA) hypervariable region 1 sequencing and odontological examination of the extremely limited skeletal remains resulted in the identification of the child as Eino Viljami Panula, a 13-month-old Finnish boy. This paper details recent and more extensive mitochondrial genome analyses that indicate the remains are instead most likely those of an English child, Sidney Leslie Goodwin. The case demonstrates the benefit of targeted mtDNA coding region typing in difficult forensic cases, and highlights the need for entire mtDNA sequence databases appropriate for forensic use.

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1. Introduction

One of history's greatest non-wartime maritime disasters was the sinking of the RMS Titanic on April 15, 1912. The accident resulted in the death of 1497 people; only 712 of the 2209 individuals aboard the Titanic survived [1]. Within a few days of the disaster the White Star Line dispatched a ship on a body-recovery mission [2] and on Sunday April 21, 1912 the crew of the Mackay-Bennett recovered the body of "a child of 2 or 3 years, a boy" (body No. 4) [2–4]. When the young boy went unidentified and unclaimed, the crew of the Mackay-Bennett arranged a funeral service and had a headstone dedicated "to the memory of an unknown child" placed on his grave in the Fairview Lawn Cemetery in Halifax, Nova Scotia [4].

The remains of the "Unknown Child" were long believed by some to be those of Gösta Leonard Pålsson, a 2-year-old Swedish boy. The association was made on the basis of several pieces of information: the age of the child as estimated by the crew of the Mackay-Bennett; a hand-written notation reading "Paulson child?" in the description of the child's body; eye witness accounts of the Pålsson child being washed overboard before the Titanic sank; and the recovery of the body of Alma Pålsson, Gösta's mother, with the tickets of all four of her children still in her pocket [2,4]. By request of the Pålsson family, and in coordination with the Titanic Ancient DNA Project, the remains of the unknown child were exhumed in May 2001 to investigate the child's identity [4].

The exhumation recovered only four small skeletal elements: a 6 cm shard of "poorly preserved" bone, and three teeth [5]. Presumed to be too degraded for nuclear DNA (nDNA) analysis, a mitochondrial DNA (mtDNA) hypervariable region 1 (HV1; base positions 16,024–16,365, numbered according to the revised Cambridge Reference Sequence (rCRS) [6,7]) profile was generated for the bone fragment and the only dental remains containing dentin. The concordant profile from the skeletal remains was compared to HV1 sequences from maternal relatives of the Pålsson child and did not match. Subsequently, maternal references were obtained for additional male children under the age of 3 who perished aboard the Titanic. The haplotype of the skeletal remains was consistent with the HV1 sequence of the references for two of these children: Eino Viljami Panula and Sidney Leslie Goodwin. In addition to the mtDNA analysis, an examination of the developmental features of the teeth recovered from the unknown child's grave estimated the age of the boy at 9–15 months. On the basis of the HV1 sequence data, the odontological evidence, and the ages of Eino Panula and Sidney Goodwin at the time of death (13 and 19 months, respectively), the child was identified as the Panula boy [5]. Despite publication of that conclusion in 2004, however, uncertainty about the identity of the unknown child lingered due to the HV1 match to the Goodwin references, an examination of Titanic artifacts at the Maritime Museum of the Atlantic (Halifax, Nova Scotia) which suggested that the shoes of the unknown child would have been too large for 13-month-old Eino Panula, and the identification decision having been based on an imprecise age determination.

This report details the genetic analyses performed on the remains following the earlier identification of the unknown child as Eino Panula, and includes mtDNA data from the maternal references of all six male children under the age of 3 years who were lost when the Titanic sank. Although minor contamination was evident in the dental remains, sequence data from additional portions of the mtDNA control region (CR) and coding region revealed two sequence differences in comparison to the Panula reference, and established that the rare mtDNA haplotype recovered from the remains was instead consistent with the Goodwin references. These new data therefore indicate that the unknown child is most likely Sidney Leslie Goodwin.

2. Materials and methods

An overview of the materials and methods is included in this section; experimental procedures are detailed more completely in the Supplementary Material.

A 6 cm fragment of poorly preserved ulna and three primary teeth: #55 (a maxillary right second primary molar), #73 (a mandibular left primary cuspid) and #84 (a mandibular right first primary molar) were used for analyses. Well-preserved dentin was only present in the pulp chamber of the first primary molar #84 [5]. DNA was extracted from either one or both sources of nucleic acids (bone and/or dentin) at multiple laboratories (Genesis Genomics, Binghamton University, Brigham Young University and the Paleo-DNA Laboratory at Lakehead University) between 2001 and 2004. DNA was extracted exclusively from dentin at the Armed Forces DNA Identification Laboratory (AFDIL) in 2007, as no bone material remained for re-extraction. Blood or buccal samples previously collected from maternal relatives of the six candidate children were extracted at both Genesis Genomics and at the AFDIL.

MtDNA CR amplification of the skeletal remains targeted HV1 and HV2 fragments ranging from 126 to 271 base pairs (bp) in length. The HV1 sequences obtained from the skeletal material prior to the initial identification publication in 2004 [5] were evaluated by re-testing in 2007–2008, and the HV2 data were generated in the years following the 2004 publication.

Amplification and sequencing of the reference samples targeted the entire CR.

Y-chromosome short tandem repeat (Y-STR) typing was performed for the dentin extract using the AmpFℓSTR® Yfiler™ PCR Amplification Kit (Applied Biosystems, Foster City, CA) according to the modified protocol described in Refs. [8–10]. Two amplifications were performed, using 1 and 3 μl of extract respectively, due to limited extract volume.

The first mtDNA coding region analyses consisted of SNaPshot® (Applied Biosystems) typing of 19 coding region single nucleotide polymorphisms (SNPs; multiplexes A and F; [11–13]). To identify additional sequence differences between the Panula and Goodwin references that could be targeted for further typing, the reference extracts were anonymized and sequenced for a portion of the mtDNA coding region (approximately 3500 bp, between positions 8164 and 11,600). Reference sample and dentin extracts were subsequently SNP-typed for nucleotide position 9923 using a 109 bp amplicon and the amplification products were sequenced to confirm the SNP typing results.

Likelihood ratios (LRs) were generated using haplotype frequencies, which were then included in the calculation of a posterior probability according to Bayes theorem as applied to alternate hypotheses [14]:

$$\frac{Pr(H_0)}{Pr(H_a)} \times \frac{Pr(E|H_0)}{Pr(E|H_a)} = \frac{Pr(H_0|E)}{Pr(H_a|E)}$$

where H_0 represents the maternal relation hypothesis and H_a represents the unrelated hypothesis. Calculations assumed 16.67% as the prior probability (on the basis of only six male children under the age of 3 years having been lost in the Titanic disaster).

3. Results and discussion

Entire CR sequences were obtained from maternal references for all six male children (Table 1). Where more than one maternal reference was tested for a single child, the data obtained from the maternal relatives matched. All of the references except for the Goodwin and Panula families were excluded on the basis of HV1 data, and the later HV2 sequencing confirmed these exclusions (Table 1). The Goodwin references differed from the rCRS at nucleotide positions 263 (G) and 315 (C insertion); the Panula references differed from the rCRS at the same positions and additionally at position 146 (excluding HV2 polycytosine length variants).

Sequence data obtained from the unknown child bone extract amplifications covered nucleotide positions 16,223–16,390 in HV1 and 35–267 in HV2. Sequence data obtained from the dentin extracts covered positions 16,009–16,390 in HV1 and 35–273 in HV2 (Table 1). In both cases, the sequence data from the unknown child matched the Goodwin child's references. The Y-STR typing did not produce any reliable results (data not shown); this was likely due to both a high degree of DNA degradation and the extremely limited quantity of dental material available (which severely restricted the number of amplifications and the input extract volume).

Despite independent extraction in multiple laboratories accustomed to handling highly degraded human remains, some of the unknown child dentin extracts exhibited evidence of contamination. The HV1 data was mixed at nucleotide positions 16,126, 16,294, 16,296, and 16,304 (Fig. S1 and Table S1, Supplementary Material); no mixtures were observed in the HV2 data. All but one of the HV1 amplicons in which a mixture was observed resulted in similar minor component signals (estimated at 5–15%) with peak height ratios reproduced in duplicate amplifications. These data provide some support for linkage of the major and (separately) the

Table 1
Differences from the rCRS as identified by mtDNA control region sequencing and coding region SNP typing.

Missing child (country of birth)	Age at death (months)	# of references tested	Differences from the revised Cambridge Reference Sequence (rCRS)[a]				
			HV1	HV2	Additional CR	Multiplexes A and F SNPs	9923
Gilbert Danbom (Sweden)	5	2	16,186, 16,189	263, 315.1C	16,519	n/a	–
Alfred Peacock (England)	7	2	16,126, 16,294, 16,311	73, 152, 263, 315.1C	16,519	n/a	–
Eino Panula (Finland)	13	3	–	**146**, 263, *309.1C*, **309.2C**, 315.1C	16,519	3010	–
Sidney Goodwin (England)	19	2	–	263, *309.1C*, 315.1C	16,519	3010	**9923**
Gösta Pålsson (Sweden)	28	1	16,153, 16,291, 16,298	72, 93, 263, *309.1C*, 315.1C	-	n/a	–
Eugene Rice (United States)	30	2	16,126, 16,153, 16,294, 16,296	73, 150, 263, *309.1C*, 315.1C	16,519	n/a	–
Unknown child consensus profile			–	263, *309.1C*, 315.1C	n/a	n/a	**9923**

Reference samples were sequenced for the entire CR, while the skeletal remains were sequenced for only HV1/HV2. HV1 sequence data was sufficient to exclude all but the Panula and Goodwin families. Multiplexes A and F SNP typing was performed to distinguish the Panula and Goodwin families; no sequence differences were detected and thus the skeletal remains were not typed using the SNP multiplexes. 9923 SNP typing was performed on all reference samples as well as the skeletal remains; the mutation was observed for only the Goodwin references and the skeletal remains. The sequence differences at positions 146 and 9923 (in addition to the HV2 poly-C length polymorphism), shown in bold type, exclude the Panula family as a match to the unknown child.

[a] Nucleotides are only included in the case of insertions; all observed substitutions were transitions. Regions typed in which no sequence differences were observed are marked with a dash. Regions which were not typed are marked as n/a.

minor components across multiple amplicons [15]. The minor sequence (T16126C, C16294T, C16296T, T16304C) in these HV1 amplifications did not match any of the six reference families or any of the laboratory scientists involved with the case, but does match a base sequence motif generally attributed to sub-haplogroup T2b [16]. This haplogroup-specific motif further supports linkage, and the observation of a mixture at only these haplogroup-specific positions strongly suggests contamination by a T2b individual rather than either postmortem DNA damage or base misincorporation during PCR.

In combination, the bone extract results and the majority sequences from the dentin extracts for the mtDNA CR matched only the Goodwin references, and differed at a single base position (146) from the Panula references (Table 1). Forensic mtDNA interpretation guidelines recommend that exclusions be made on the basis of two sequence differences, due to the high mutation rate of mtDNA [17,18]. The need for more than a single sequence difference to exclude the Panula family as a match to the unknown child remains was particularly necessary in this case, given the contamination observed in the dentin sequence data, the high frequency of the Goodwin and unknown child HV haplotype among West Eurasians and the especially high rate of substitution and heteroplasmy at nucleotide position 146 [19–21].

In previous studies, analysis of portions of the mtDNA coding region have successfully identified variable positions that can be used to distinguish lineages which match, or nearly match, in the CR [11,22,23]. In this case, the Goodwin references and the unknown child possessed the most common West Eurasian HV haplotype, present in nearly 7% of that population (based on a search of 3830 unrelated West Eurasians in the European DNA Profiling Group (EDNAP) MtDNA Population Database (EMPOP) [24]). Numerous publications have demonstrated the utility of coding region data for resolving this and other common HV haplotypes, and various assays have been developed for the purpose [11–13,23,25–28]. Although the two coding region SNP assays applied to this case have previously proven useful for the resolution of lineages with the same HV haplotype as the Goodwin family and unknown child [29,30], they unfortunately did not identify any additional sequence differences between the Panula and Goodwin families.

However, given that this type of targeted approach – in which SNPs variable for *particular* HV or CR haplotypes are analyzed – has been shown to be an effective method for identifying discriminatory information in the mtDNA coding region [31], a similar strategy was employed in this case to identify additional polymorphic sites that were not already targeted in the two

aforementioned SNP assays. Based on a synthesis of 92 published mtDNA genomes with the observed HV type, a 3500 bp region was identified that harbored high levels of inter-individual variation. Targeted sequencing of the anonymized Panula and Goodwin references between nucleotide positions 8164 and 11,160 success-fully identified a single sequence difference between the two families at third codon position 9923. Subsequent SNP typing of the reference samples for position 9923 identified a C–T transition in the Goodwin references, while the Panula references matched the rCRS (Table 1).

As with the CR sequence data, the SNP typing of the dentin extracts for position 9923 revealed the presence of two mtDNA types; the SNP data included a large T (green) peak and a small C (blue) peak, comprising approximately 83% and 17% of the total fluorescent signal, respectively (Fig. 1). The 17% contaminant signal in the SNP profile is likely an over-estimation of the actual contamination, due to a substantial signal strength disparity between the dR110 (blue) and dR6G (green) dyes (ddG and ddA incorporation, respectively) used with the SNaPshot chemistry [12,32]. Indeed, subsequent sequencing of both dentin extracts for the 109 base pair amplicon encompassing position 9923 (to confirm the SNP typing results) demonstrated that the majority of the molecules present in the samples had a T at 9923, while the contaminant signal was so low as to be nearly undetectable (Fig. S1, Supplementary Material).

Though the HV haplotype observed in the Goodwin references and the unknown child is common among West Eurasians (frequency is 0.06710; the 95% upper confidence limit is 0.07503), the shared T–C transition at nucleotide position 9923 is rare, with only three occurrences among the more than 6000 human coding region sequences available in GenBank. A recent analysis recognized the 9923 T–C transition as the molecular definition for mtDNA sub-haplogroup H1u [33]; however, only two haplogroup H sequences with the 9923 mutation have been published in GenBank. Among the 92 entire mtGenomes in GenBank with HV haplotypes matching the Goodwin reference and the unknown child, only one possessed the 9923 T–C transition (a frequency of 0.01087; the 95% upper confidence limit is 0.03206). In combination these data result in a likelihood ratio (LR) of 416 in support of the hypothesis that the tooth is from an individual related to the maternal reference for Sidney Goodwin, rather than from an unrelated individual. Using a prior probability of 16.67%, the posterior probability is thus 98.81%.

The statistical support for the maternal relation hypothesis is strong, especially given that only mtDNA profiles could be obtained from the skeletal remains; however, it is likely that
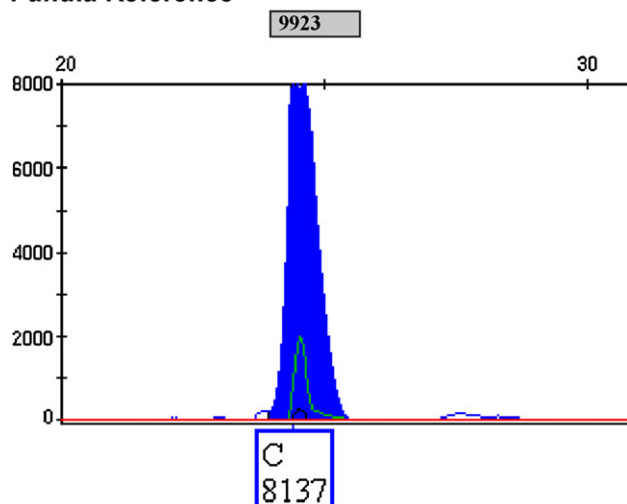
Unknown Child
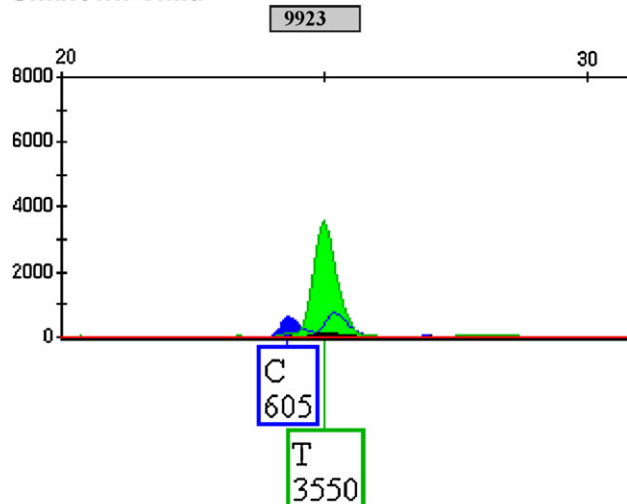
Goodwin Reference
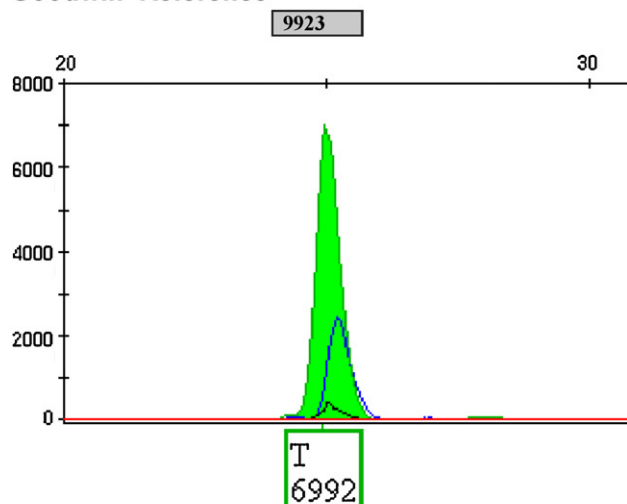
Panula Reference

Fig. 1. 9923 SNP typing results for the unknown child, Goodwin and Panula references. Utilizing a reverse primer for the singleplex SNP typing using SNaPshot chemistry (Applied Biosystems), dR110-labeled ddG (blue) and dR6G-labeled ddA (green) incorporation represent C and T, respectively, at position 9923. Relative fluorescent unit values for each peak are included beneath the nucleotide designation. (For interpretation of the references to color in the citation of this figure, the reader is referred to the web version of the article.)

comparison of the matching HV profile to an entire mtDNA genome database suitable for forensics would have resulted in a higher posterior probability. The entire mtDNA genomes available in GenBank that were used for our frequency estimation are an imperfect substitute for a forensic database, but the GenBank collection is nearly the only up-to-date resource available for investigation of the frequency of a rare mtDNA haplotype that includes coding region data. While we used this repository of published mtDNA genomes to generate a haplotype frequency in support of a non-exclusion in a century-old, closed population case, the use of mtDNA coding region profiles in other types of forensic case work would likely require a more extensive and/or representative database. The need for such a database will increase as new technologies which enable access to mtDNA coding region data with relative ease (e.g. linear arrays, mass spectrometry, and next-generation sequencing) become more widely applied in forensics [34].

4. Conclusion

The work reported here confirms the previous mtDNA HV1 data that eliminated four of the six male casualties under the age of 3 as sources of the unknown child remains. More extensive CR sequencing, to include portions of HV2, identified a single sequence difference between the Panula family and the skeletal remains. Two coding region SNP assays failed to identify any additional discriminatory information in this case, and the extremely limited and highly degraded skeletal material prevented generation of a Y-STR profile. However, targeted mtDNA coding region sequencing resulted in the identification of a second sequence difference between the Panula references and the skeletal remains, resulting in a high posterior probability for the match to the Goodwin family. Though evidence of minor contamination was apparent in the dentin extracts, the consistent mtDNA HV profiles and especially the rare mtDNA coding region polymorphism shared with the Goodwin maternal references indicate that the unknown child remains are most likely those of Sidney Leslie Goodwin. While this closed population case could be resolved to a reasonable degree of certainty by the exclusion of all other candidate children, the need for a forensic database that includes mtDNA coding region data is evident and should be a focus of future mtDNA databasing efforts.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fsigen.2010.01.012.

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