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The Mitochondrial Origins of the Hellenistic Individuals of Ayasuluk Hill

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ABSTRACT

Objective: The present study aimed to extract ancient DNA from the remains of three individuals from the 4th century BC in order to determine the haplogroups through a mitochondrial DNA study, thus providing information about Anatolian migrations in ancient times.

Materials and Methods: For this purpose, the study examined the remains of three bodies found at the bottom of the city walls from the archeological excavations between 2007-2008 and dated to the 4th century BC. After taking anthropometric measurements, the study examined the mitochondrial *HVR1* and *HVR2* regions by using Sanger sequencing and then used online programs to evaluate the data from the sequencing.

Results: As a result of the study, death due to a possible injury from a sharp object was observed on the right femur of one of the three individuals. The maternal haplogroups of the individuals were determined to belong to the T2b group of European origin.

Conclusion: The present study obtained genetic information regarding three individuals found at the bottom of the ancient city walls on Ayasuluk Hill. These results will provide important information about the commander of the ruins found on the walls of the Ayasuluk Hill of the ancient city of Ephesus, which constantly changed hands during the Wars of the Diadochi.

Keywords: Mitochondria, genetics, ancient DNA

INTRODUCTION

The contributions of genetics to the field of archeology have increased over the past years and have influenced the re-examination of most of the information found in previous excavations or written texts (1-6).

During the Hellenistic period, Anatolia was a region where different societies reigned and fought each other. Those lands became a part of the empire with the conquests of Alexander the Great, but after his death in Babylon in 323 BC, their commanders divided them into kingdoms (7). The city of Ephesus occupies a very important place in Anatolia for hosting the Wars of the Diadochi during the Hellenistic period. The city of Ephesus was the first built independently under the control of Demetrius and with the assurance of Antigonus in 315 BC following Alexander the Great's death (7).

Due to Ayasuluk Hill constantly having changed hands, current archaeological data has yet to fully identify in which period or by which commander the walls of the city of Ephesus had been built. The pottery findings excavated in the area point to the late classical and early Hellenistic periods (4th-3rd centuries BC), but the exact date still remains uncertain.

As a result of the studies carried out using X-ray diffractometry (XRD) and scanning electron microscopy (SEM), the walls in the area on Ayasuluk Hill and the walls used in the reconstruction of Ephesus are thought to have been built in the 4th century BC. This leads to the idea about the skeletons that were found at the bottom of the city wall alongside the ceramic finds were soldiers who'd fought before Ephesus was moved. In order to prove this, the study examined the skeletons paleoanthropologically and genetically.

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The D-loop in the hypervariable region (HVR) is a 1.1 kb non-coding region in the human mitochondrial genome involved in the regulation of transcription and replication and has a highly variable sequence at the population level compared to the rest of the genome. It contains three short regions: *HVR1* (between nucleotides 16024-16400), *HVR2* (between nucleotides 44-340), and *HVR3* (between nucleotides 438-576). Among these, *HVR1* is the most frequently used one in population genetic studies (8-9). According to the literature, the mitochondrial genomes involved in this region were the first that used by the Cambridge group in 1981 and numbered in terms of the light strand defined as the Cambridge reference sequence (CRS) (10). However, this definition has since been updated due to an error in the used technique, with its final form being taken as the revised Cambridge reference sequence (rCRS) in 1999 and the reconstructed Sapiens reference sequence (RSRS) in 2012 as a result of the latest revisions (11, 12).

This study carried out mitochondrial DNA (mtDNA) analyses on samples of ancient DNA (aDNA) from the Seljuk Ayasuluk Castle on Ayasuluk Hill in southwest Anatolia. The study will contribute to the historical and archaeological findings regarding the makeup of the societies in the region and also identify the individuals who are presumed to have died in conflicts for the defense of Ephesus, one of the most active centers during the Wars of the Diadochi when regional turmoil occurred and the city frequently changed hands.

MATERIALS AND METHODS

The samples included in the study were based on the data obtained from archaeological studies and findings in the area and consisted of bones dated from the 4th-3rd century BC that were found alongside ceramic pieces at the bottom of the city wall during the excavation of 2007-2008. Evaluations of suitability of the sample for anthropometric measurement occurred prior to the analyses (13-15). The necessary permissions were obtained for the materials used in the study, which was approved for being conducted in the Altınbaş University Scientific Research Projects unit (Project code: PB2020-SHMYO-1).

Isolation of the aDNA

The aDNA was isolated from the samples in the Altınbaş University Ancient DNA Laboratory. Sterile laminar flow cabinets (sections where DNA isolation, PCR, and Post PCR are performed) where the pre-study procedures would be performed were first sterilized with sodium hypochlorite (NaClO; Cat no: 7681-52-9, Sigma, Germany) and UV lights (15 WATT, 265 nm wavelength, duration 45 minutes) (16). Sterile pipette tips, disposable sterile gloves, and masks were also used in the study.

In order to minimize DNA contamination before the procedure, the upper portion of 1-2 mm of the bone fragment was removed from the target area using sterile Dremel tips (Cat no: 4000-1/45; Dremel, USA) around the area to be treated, with these tips being replaced after each procedure. The new UV-sterilized disposable Dremel tips were used on the powdered bone (0.04 to 0.5 g) that was to be taken for DNA sampling (17).

Isolation of the aDNA from the obtained bone powder (~50 mg) was carried out within a closed robotic system that had been sterilized before (and after) the study. This material was incubated in a 2 mL Eppendorf tube in 200 µL of incubation buffer and 10 µL proteinase K taken from the solutions in the isolation kit (TanBead Nucleic Acid Extraction Kit; Tissue Total DNA, Cat no: M6T2S46; TanBead; Taiwan) for 1 hour at room temperature before being processed.

The aDNA isolation was performed using the TanBead isolation robot and the tissue total DNA kits. Isolated samples were taken from the robot in 100 µL isolates and kept at 4°C until further use.

Following the isolation, the densities (ng/µL) and absorption measurements ($A_{260/280}$: 1.80 - 1.89) of the DNA samples were measured with a spectrophotometer and recorded (Cat No: ND-2000, ThermoFisher Scientific, USA; Table 1).

Table 1. Samples absorption measurements and concentrations.

Sample No	Conc. (ng/µL)	260/280	260/230
C320-1	135.1	1.43	0.63
C320-2	135.1	1.43	0.61
C320-3	14.8	1.37	0.76

Sanger Sequencing

AmpliTaq Gold DNA Polymerase (Cat No: N8080246, ThermoFisher Scientific, USA) and UDG (Uracil-DNA Glycosylase, Cat No: 78310100UN, ThermoFisher Scientific, USA) were used during the PCR stage due to its effectiveness on degraded regions with low copy numbers. For each PCR reaction, the study used 1 Unit of UDG, 1 µL of 360 GC Enhancer (Cat No: N8080246, ThermoFisher Scientific, USA), 1X buffer solution (AmpliTaq Gold® 360 Buffer, Cat No: N8080246, ThermoFisher Scientific, USA), 25 mM $MgCl_2$ (Cat No: N8080246, ThermoFisher Scientific, USA), 200 µM dNTP Mix (Cat No: N8080246, ThermoFisher Scientific, USA), 0.3 µM forward and reverse primers, 0.5 Unit Gold Taq polymerase enzyme, and 2 µL aDNA templates. The mixture was prepared by filling it with up to 25 µL of double distilled water (ddH_20). All studies were performed on ice. Each PCR cycle studied the region to be amplified alongside the control PCR (negative control) that contained no DNA. If bands were seen in the control PCRs, the PCR process was repeated for the problematic samples from the aDNA extraction step.

The PCR were performed in the thermal cycler following the protocol designed for the *HVR1* and *HVR2* regions of mtDNA (Table 2). Initial denaturation was performed over 30 min at 37°C followed by 10 min at 95°C. Then for 30 cycles, denaturation occurred over 30 sec at 95°C, primer binding over 30 sec at

Table 2. Primer list for PCR.

Primer Name	Primer Sequence
15876F	5´-TCAAATGGGCCTGTCCTTGTAG- 3´
132R	5´- GACAGATACTGCGACATAGG- 3´
15946F	5´- CAAGGACAAATCAGAGAAAA- 3´
639R	5´- GGGTGATGTGAGCCCGTCTA- 3´

60°C, elongation over 120 sec at 72°C (1 kb/c), and finally a 10 min extension time at 72°C. After the PCR, 5 µL of the products were taken and screened in the agarose gel electrophoresis containing 1.4% agarose (Cat No: 9012-36-6, Sigma, Germany) and 8 µg/mL ethidium bromide in the 1X TBE buffer, together with the 50 bp ladder marker (Cat No: D3812, Sigma, Germany) at 130 V for 20-25 min. The bands were visualized under UV light and recorded.

Enzymatic PCR purification (Exonuclease-I (Lot:00173016-ThermoFisher Scientific, USA) and rapid alkaline phosphatase (Cat No:04898133001, Roche, Switzerland) were performed on the products detected in the gel. After the purification step, the products were stored in the dark at 4°C until the sequence PCR reaction.

Sanger sequencing was performed on ABI3130 (Cat No: 3130XLR, ThermoFisher Scientific, USA). Electropherogram images were evaluated in the program Chromas, and the *HVR1* and *HVR2* regions of the mitochondria were identified according to RSRS based on the sequence results (18).

RESULTS

As a result of the paleopathological examination, the bones found in the area were determined to have belonged to 3 different adult males (Table 3) (15). The lesion seen on the anterior region of the right femur bone in one of these three individuals extends linearly from top to bottom and shows slight bends; it is thought to be a possible piercing injury (Figure 1) (19, 20). Upon examining the cut, the person is thought to have died shortly after receiving this blow as no signs of recovery were found.

Table 3. Samples anthropometric measurements.

Sample No	Sex	Age	Lenght	Sample Tissue
C320-1	Male	>20	1.74	Left Tibia
C320-2	Male	>20	1.73	Left Femur
C320-3	Male	N/A	N/A	Left Femur

N/A: not avaliable

Figure 1. C320-2 Right femur injury

As a result of investigation of the individuals included in the genetic evaluation, all the samples were identified to belong to the T2b2b haplogroup (Table 4). The same results were obtained in the independent PCR study performed for checking the results. Electropherogram images are given in

Table 4. Genetic results.

Sample No:	*HVR1*	*HVR2*	DNA Damage	Repeat	Haplogroup	*Defining Markers for Haplotypes*		Damaging Areas
						HVR1	HVR2	
C320-1	+	+	-	Sequencing	T (T2b2b)	T16126C C16294T C16296T (C16304T!)	73G	-
C320-2	+	+	+	Sequencing	T (T2b2b)	T16126C C16294T C16296T (C16304T!)		16126,16261,16294,16296, 16298, 16519
C320-3	+	+	+	Sequencing	T (T2b2b)	T16126C C16294T C16296T (C16304T!)	60C 73G	16189, 16289, 16400, 16519

!: back mutations

supplementary materials, with Table 4 providing the target regions in comparison for the haplogroup analysis.

DISCUSSION

Although developments in genetics and studies in the field of aDNA reveal new information about many historical events, consideration of these as pieces of auxiliary information rather than a definitive statement for questions to be asked is important. Recent decades have added the origin markers in mitochondria and Y chromosomes to the markers in the autosomal chromosomes. Without references to historical sources, such studies would have some difficulties for examining beyond the general screening tests, as the obtained data remains very weak without blending historical information with genetic information (3).

Although identifying DNA origin is based on mathematical proportions of the changes in the genomes of the living things under study, their sensitivity is directly proportional to an increase in the number of such studies. Although comprehensive methods exist for identifying origins in aDNA studies these days, one of the most practical ways for conducting a preliminary examination is to analyze easily examined mitochondrial *HVR* regions (21-23). The main reason for this and the accompanying problem is that the covered genomic region is relatively small (24).

Tracing the dominant alleles in gene pools in populations or the ancestry of important individuals is one of the important outcomes of the current study (6). Similarly, finding the place and time when a certain mutation first appeared (founding mutation) are also valuable in terms of genetics. Reflecting on the mutation rates in certain regions allows one to take into account the effects of not only geography but also time with regard to understanding the nature of variants in DNA (25, 26).

aDNA studies on excavations close to Ayasuluk Hill that cover similar historical periods have occurred with the Sagalassos and Kadıkalesi excavations (27-29). Both regions involve the European macrohaplogroups and, hence, the T haplogroup. The historical periods that followed observed the T group to start appearing prominently after the density of the region's H, HV, and RO groups.

The maternal T haplogroup is one of the common haplogroups seen in Europe during the middle and early Neolithic period (30). The T2b group is one of the subgroups of the T group and is thought to have come to Europe from Anatolia during the last Ice Age and then to have spread during the Neolithic period (31). In order to confirm this, the T2b group in the Bronze Age also appears in the Eastern European Pontic-Castian steppes, such as the Srubnaya, Yamnaya, and Tripoli cultures (32, 33). Modi et al. (34) identified the T2b group in their findings from the early Bronze Age in Bulgaria.

Currently in maternal haplogroup studies, the T group is observed mostly in Europe and rarely in India (35, 36). This strengthen the argument that this haplogroup is of Indo-European origin. Recent studies have also reported the T2 group to be one of the highest observed maternal haplogroups in the Macedonian population at 6.2% (37).

The soldiers provided by the city of Ephesus to the regions on Ayasuluk Hill during the struggle of Demetrius against Lysimakhos and the other Wars of the Diadochi in the region are said to have been sent from Kingdom of Macedon. The same source also states that Lysimakhos had left the slain soldiers where they were (7). The actual aim of this study was to obtain preliminary information about the possible origins of these soldiers. Evidence of at least the maternal origins of the samples was obtained by isolation of the aDNA and subsequently sequencing the HVR1 and HVR2 regions.

As a result of the study, the mitochondrial (maternal) haplogroups were found to be of European origin (T2b), and the skeletons found in the paleoanthropological examination are thought to have been soldiers probably from the army of Antigonus' son Demetrius or from Lysimachus, who came to Ayasuluk Hill to fight. The fact that the soldiers were not buried in a proper grave might show that they had lost the war, and this supports the view that Lysimachus was not a militaristic settlement.

In ancient DNA studies, while mitochondrial studies on their own give very general population data, they are very suitable for preliminary studies. However, the supplementation of Y chromosomal and autosomal markers when necessary, as well as using C14 carbon dating to date the materials, will lead to more detailed information being obtained about the samples in question. Examining the data from such individual or regional genetic identification studies using population genetics leads to larger data pools being obtained (16). In this way, all these data will not only be important in their own right but will also become important as the vast data they allow access to once the data regarding modern human genome sets accumulate. Although the information obtained as a result of such studies provides less information than broader sets, it does provide more rapid and cheaper preliminary results for excavation areas.

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Ethics Committee Approval: There is no need for ethics committee approval, since the study was carried out on samples taken from ancient individuals belonging to the year 4th century BC.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study – F.T., M.G.; Data Acquisition – F.T., M.G.; Data Analysis/Interpretation – F.T., M.G.; Drafting Manuscript – F.T., M.G.; Critical Revision of Manuscript - F.T., M.G.; Final Approval and Accountability – F.T., M.G.;

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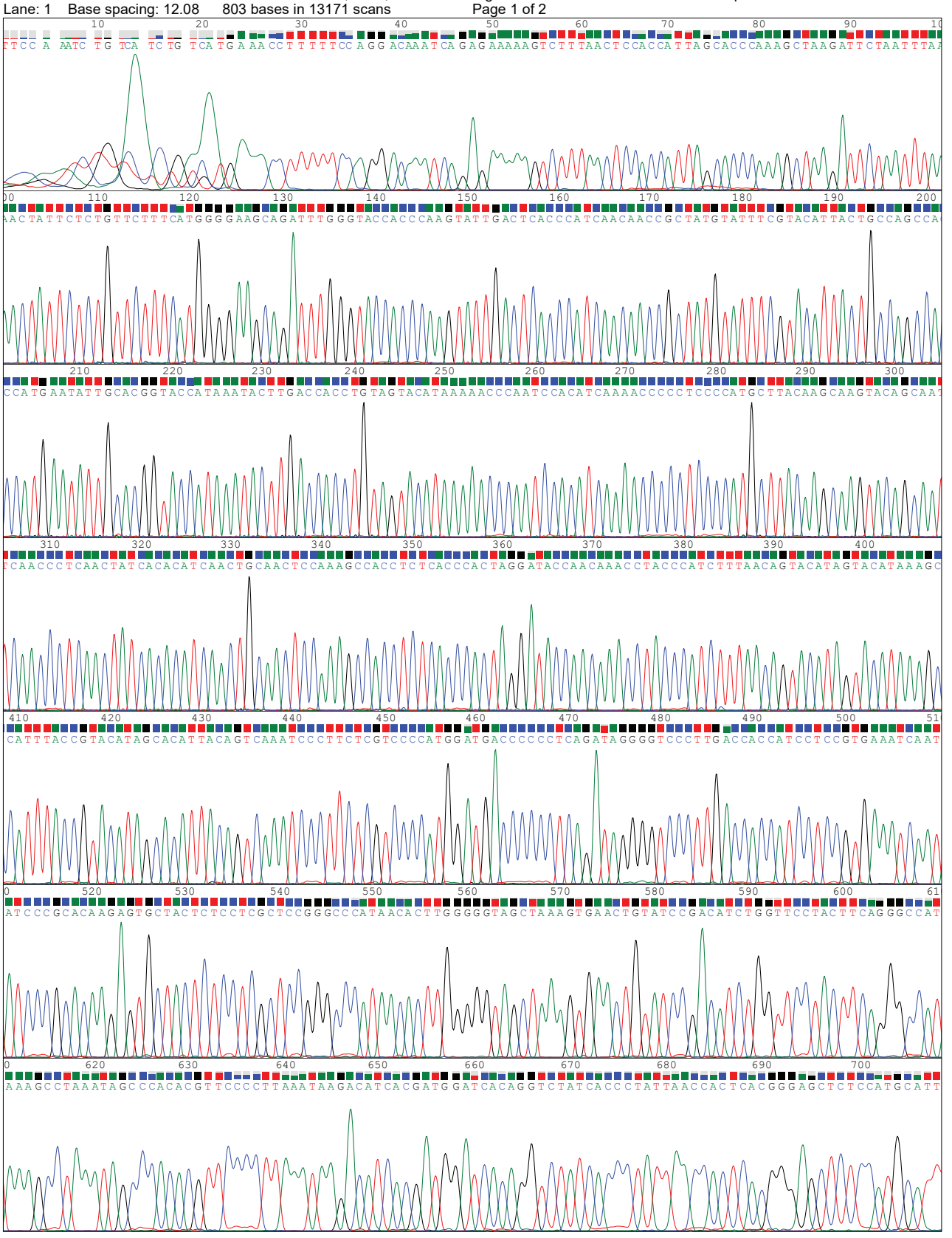
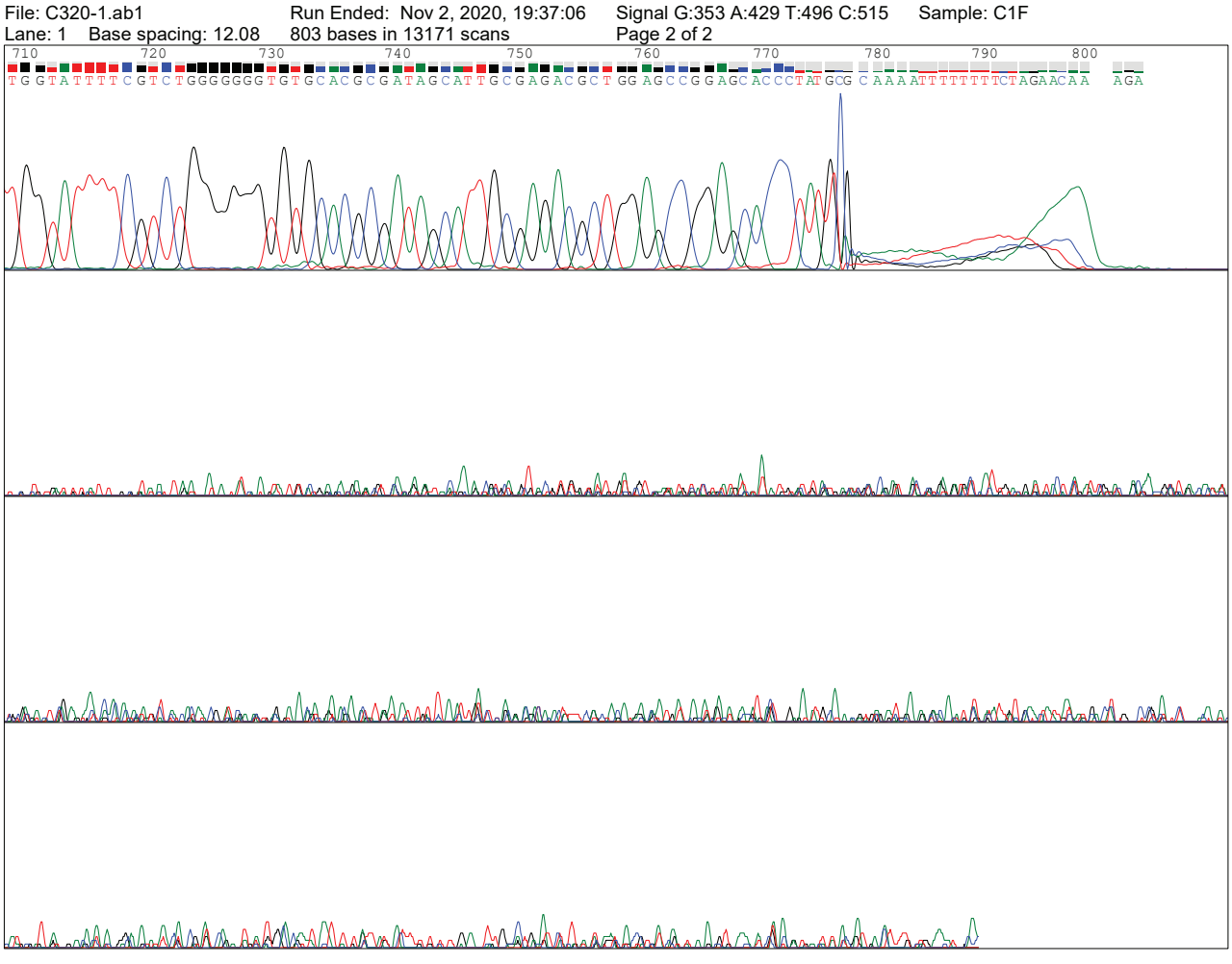
SUPPLEMENTARY FILE

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Base spacing: 12.08
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Page 2 of 2
710
720
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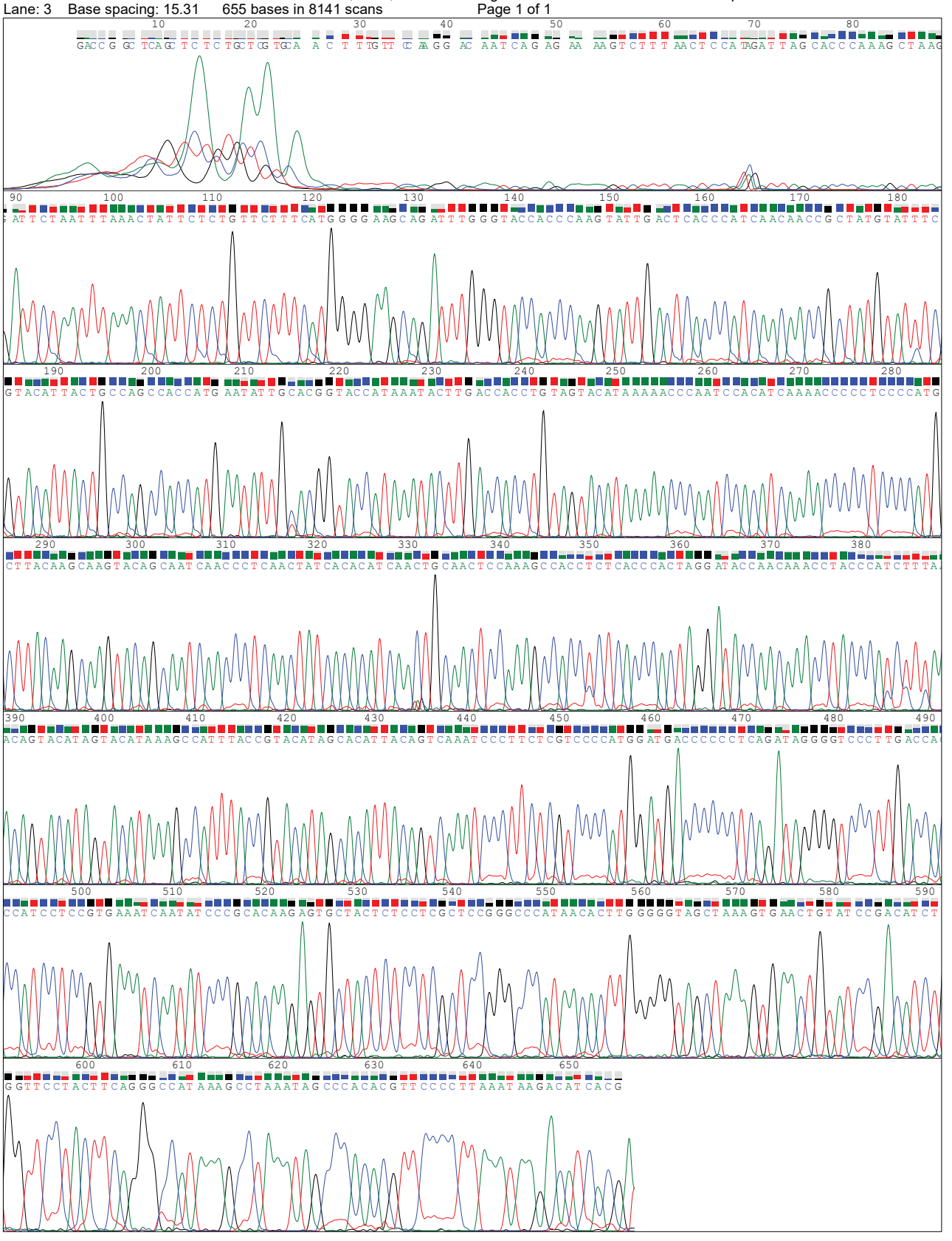
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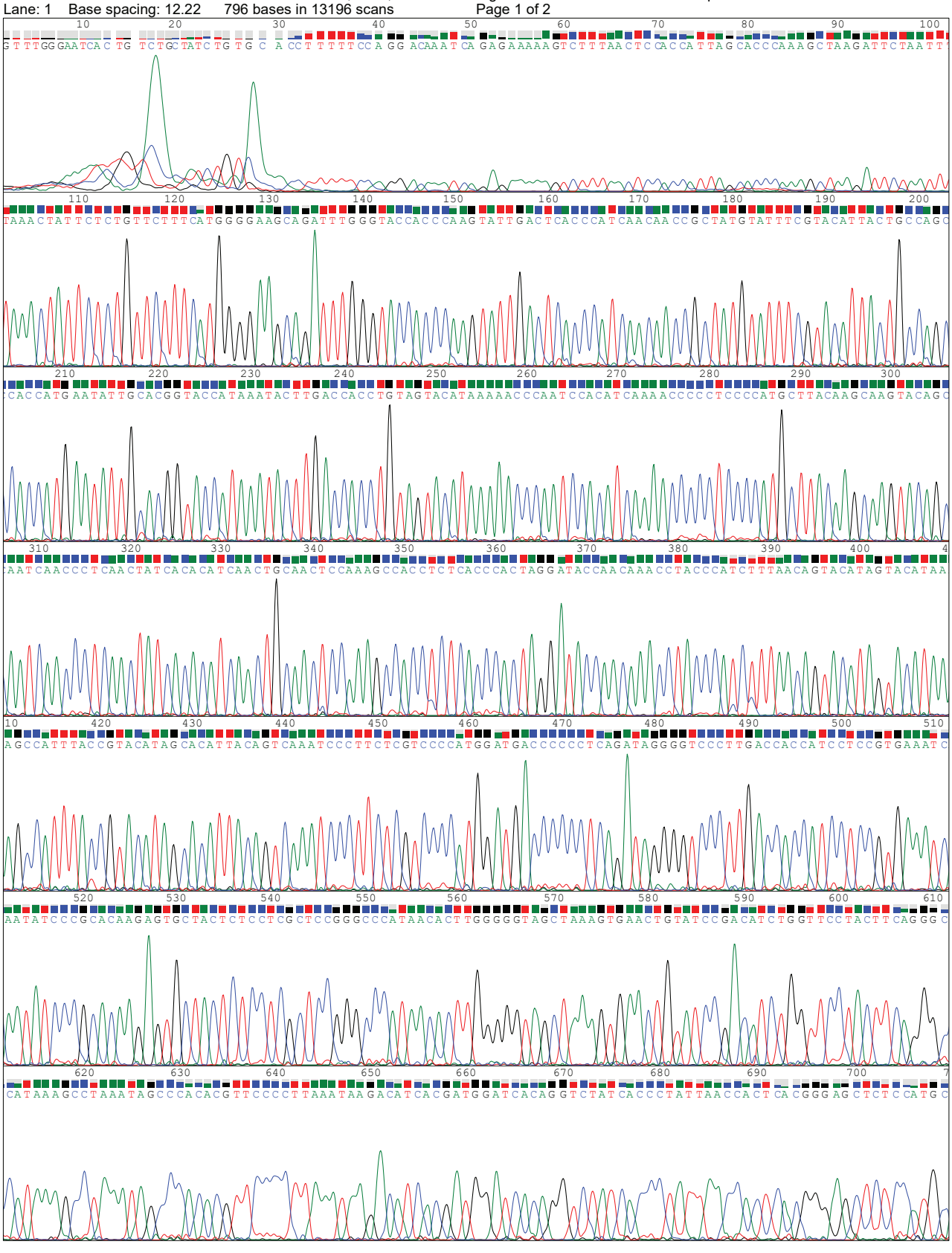
Page 1 of 1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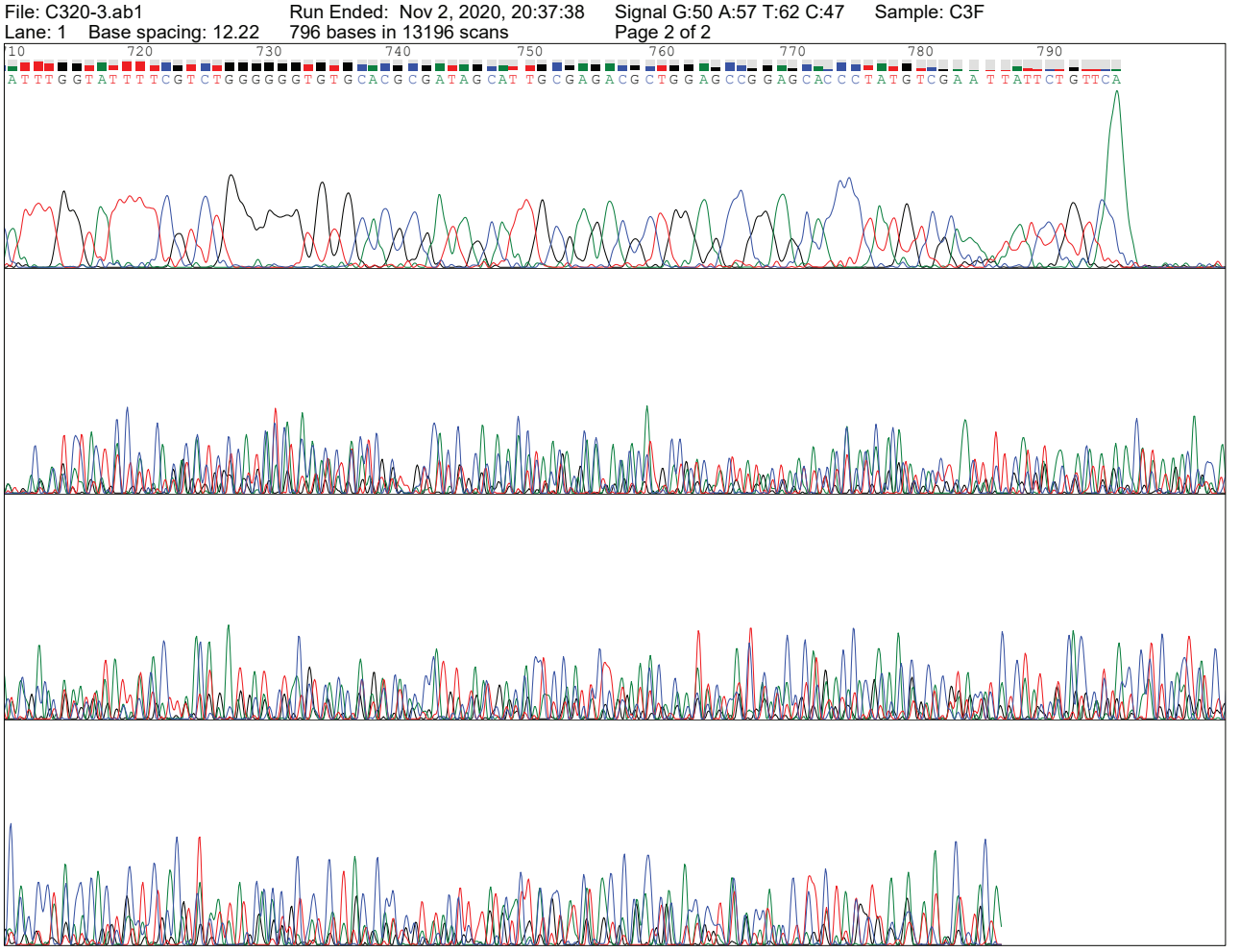
Lane: 1 Base spacing: 12.22
796 bases in 13196 scans
Page 1 of 2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Signal G:50 A:57 T:62 C:47
Sample: C3F
Lane: 1
Base spacing: 12.22
796 bases in 13196 scans
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