

6 PRELIMINARY ANALYSES OF DNA IN SKELETAL MATERIAL FROM THE HARBOR NECROPOLIS

The population structure and origin of Anatolians have been addressed in several studies, using genetic data from living people of present-day inhabitants of Turkey to extrapolate movements and origins of past populations²⁷². A mosaic of maternal lineages is found in Turkey, including traces of central Asian influence (Di Benedetto et al. 2001; Nasidze et al. 2004; Quintana-Murci et al. 2004; Schönberg et al. 2011). The admixture is more pronounced in paternal markers than in maternal markers (Nasidze et al. 2004), and recent analysis of unisexual markers shows that the Turkish population is rather distinct from European populations (Skoglund et al. 2012). However, the mode of introduction of the central Asian component to the people of Anatolia is not yet understood.

Ephesos, located on the coast of Ionia, was one of the major cities of the Roman Empire, and one of the largest in the eastern part of the Mediterranean. The rich and flourishing city promoted maritime trading, travelling and likely, intermarriages of people from different parts of the Roman Empire. The importance of the city as a commercial centre declined as the harbor was slowly silted up by the river, and Ephesos was a small village when the Seljuk Turks conquered it in 1090.

The Harbor Necropolis, located along the harbor channel of Ephesos, comprises five grave complexes containing skeletal remains of numerous humans of different coverage and preservation state (see chap. 3 and 5). The Harbor Necropolis was in use between the 2nd and the early 6th centuries; the burials of tomb 1/08 date from the 2nd to the early 5th century. In this study, preliminary results of the genetic constitution of the Roman population of coastal western Asia Minor is reported, and compared to the maternal genetic lineages found among the skeletal material from the Türbe in the Artemision in Ayasuluk/Selçuk, a grave complex from the Ottoman period of Ephesos (Bjørnstad 2015).

6.1 ANCIENT DNA ANALYSES

Ancient DNA (aDNA) is DNA isolated from organisms not preserved for the purpose of DNA studies, and includes skeletal material, feathers, coprolites, herbarium and sediment samples, among others. The history of the field goes back only four decades with the first reported DNA-study of an extinct quagga (Higuchi et al. 1984), followed by genetic analysis of Egyptian mummies (Pääbo 1985). Even though the field is young, and has seriously been set back several times, the development has been significant during the last years. Retrieval of DNA has even been possible for up to 700 000 years old specimens given exceptional preservation conditions in permafrost (Orlando et al 2013). However, there will be a maximal age of retrieval of aDNA; at some point all DNA will be in the form of single building blocks (Allentoft et al. 2011).

The question of authenticity has been a recurrent concern within the field. The DNA isolated from old remains is degraded and fragmented into smaller pieces. Thus, aDNA is vulnerable to contamination from high-quality modern DNA coming from people handling the bones or performing the genetic analyses, from laboratory consumables that often contain mammalian DNA, and from bacteria and fungi from the soil surrounding the skeletal remains. Some of this contamination will inhibit the amplification of aDNA, while other could create false sequences. Studies of aDNA therefore follow certain universal standards to minimize the risk of contamination (Cooper – Poinar 2000; Hofreiter et al. 2001).

Recent developments of sequencing technologies, and implementation of the so-called next generation sequencing (NGS), have nearly revolutionised the field of genetics, making it possible

²⁷² Since the co-author of this volume and main author of this chapter, Gro Bjørnstad, prefers writing in English chap. 6 was left in its original version. See also Steskal – Bjørnstad 2018.

to sequence whole genomes in a fraction of time compared to conventional sequencing. A major advantage with this technology is that through analysing the high number of DNA fragments preserved in each aliquot of amplified DNA, the degradation of the samples could be evaluated, giving statistical support to the most likely authentic sequence (e.g. c-statistics; Helgason et al. 2009). Thus, NGS has almost circumvented the always impending danger of contamination from surrounding material, and has rather introduced computational limitations by the huge amount of data produced.

6.2 MITOCHONDRIAL DNA

Mitochondrial DNA (mtDNA) is to date the most frequently applied genetic marker system for investigating the history and origin of human populations. MtDNA, a small, extra-nuclear circular organelle, has a maternal inheritance mode, meaning that it is transmitted directly, and only, from mothers to childrens. Unlike genes on the chromosomes, mtDNA has no recombination or major rearrangements, and mutational events occur relatively often over generations, making it informative even in a short evolutionary scale. While autosomal DNA is found in only two copies (on the chromosomes) in each cell, every body cell contains high numbers of mtDNA copies, a major advantage for investigation of old and degraded biological samples of low DNA quantity and quality. MtDNA diversity has been thoroughly documented worldwide, making a large reference material available, both from present and past human populations. However, mtDNA reflects the female history only, not necessarily the population as a whole. Autosomal markers (i.e. unisexual genetic markers) reflect the complex mosaic of the genetic history of all past relatives, while Y-chromosome markers are the male counterpart to mtDNA, providing information on the paternal history.

Each mtDNA variant, called haplotype, can be classified according to the particular set of mutations or variable positions found within a sequence stretch of the mtDNA. Sequences with similar mutation motifs are grouped together in haplogroups, denoted with capital letters. Ancient human expansions and migrations are reflected in the geographical distributions of haplogroups. The mtDNA lineages of the world are presented in figure 83. In Africa, the ancestral L-hap-

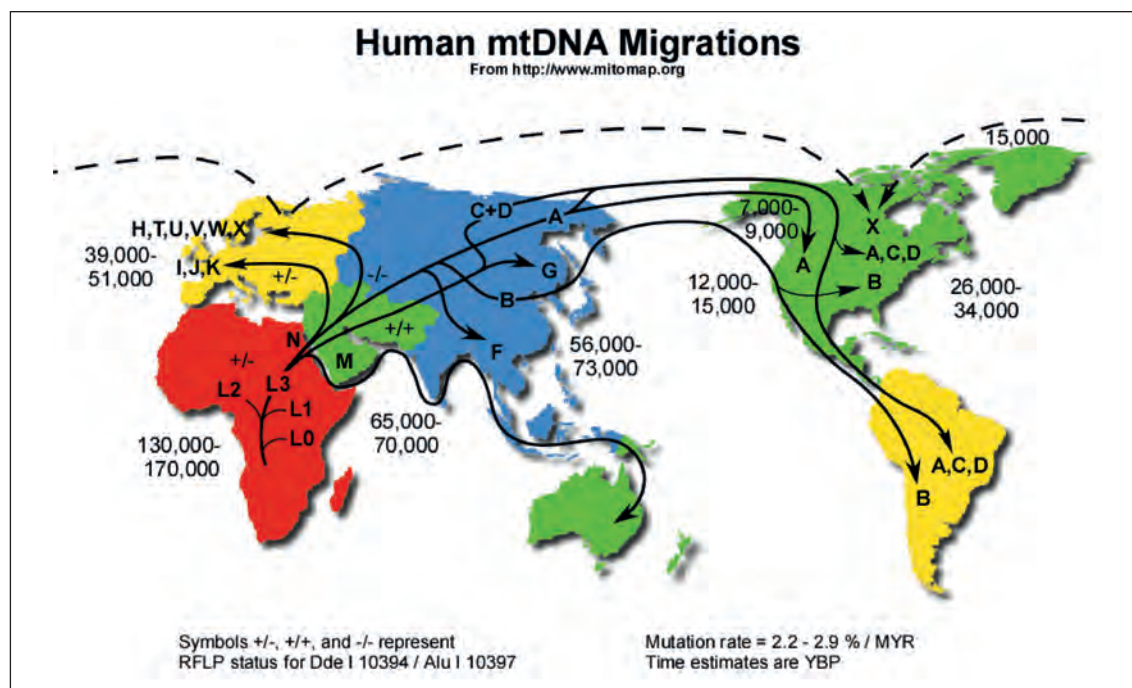


Fig. 83 Worldwide human migrations estimated by mtDNA haplogroups. Time estimates for continent expansions are given as years before present (YBP) (Foto © <http://www.mitomap.org> [accessed on July 05, 2016])

logroups are found. When the anatomically modern human left Africa, two haplogroups derived from L, haplogroups M and N, migrating respectively eastwards to eastern Eurasia and north- and westwards to western Eurasia. In Europe and western Eurasia the haplogroups H, I, J, K, T, U, V, W and X, all deriving from the ancestral N-haplogroup, are found in a complex distribution with relatively little geographical structuring. Haplogroup H is by far the most common with a prevalence of 40–50 % across Europe (Richards et al. 2000). In eastern Eurasia other haplogroups are more prevalent (C, D and G), deriving from the other major ancestral haplogroup M.

6.3 MTDNA OF PRESENT-DAY TURKEY, OTTOMAN AND BYZANTINE ASIA MINOR

A mosaic of maternal lineages is found in Turkey today (fig. 84 a), including traces of central Asian influence (Di Benedetto et al. 2001; Nasidze et al. 2004; Quintana-Murci et al. 2004; Schönberg et al. 2011). Haplogroup H is most prevalent in Turkey, as in Europe, but some of the H-lineages are ancestral in the region, while others have back-migrated from Europe (Richards et al. 2000). The common haplogroups of Europe is found here (e.g. U, T, J, K, I), in co-existence with Asian lineages (A, D, F). Currently, our knowledge about the genetic constitution of ancient Asia Minor is limited to a study of late Byzantine Sagalassos (11th–13th c.; Ottoni et al. 2011) and Ottoman Ephesos (16th–17th c.; Bjørnstad 2015). European profiles (fig. 84 b) and maternal affiliation between Sagalassos and the neighbouring areas like Balkan, Greece, Persia and Italy was documented. However, the central Asian component present in the Turkish population today

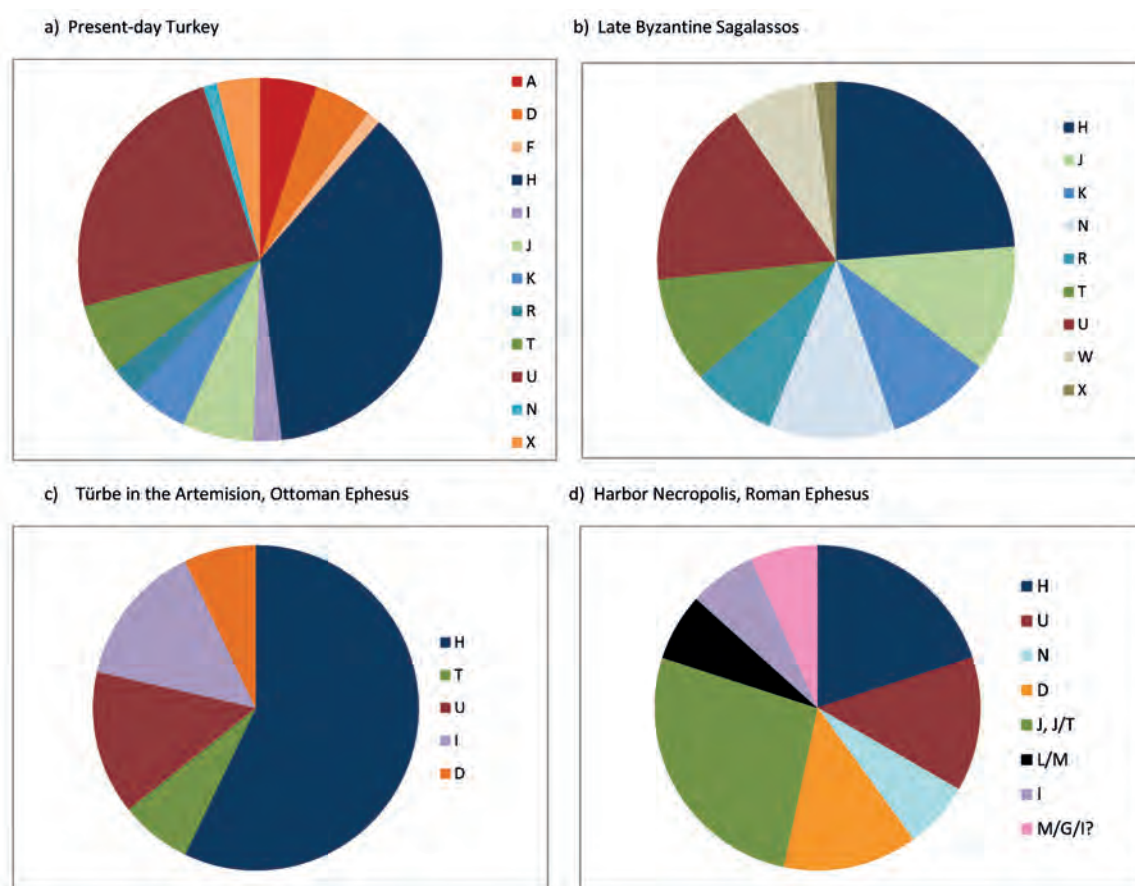


Fig. 84 Pie charts of frequency distribution of mitochondrial haplogroups of (a) present day Turkey ($n = 79$, Quintana-Murci et al. 2004, Schönberg et al. 2011), (b) late Byzantine Sagalassos ($n = 53$, Ottoni et al. 2011), (c) Türbe in the Artemision of Ottoman Ephesus ($n = 13$, Bjørnstad 2015) and (d) Harbor Necropolis of Roman Ephesus ($n = 15$) (Graphics: G. Bjørnstad)

was not observed in this inland Byzantine population. A similar genetic constitution was observed among the 13 skeletons from Ottoman Ephesos (fig. 84 c), but here, in this later population, one haplotype of East Asian origin (D) was also found. This haplotype could have been introduced once as of the invasion of the Seljuk Turks or with successive introgressions over many centuries as to continuous interaction with central Asians belonging to similar language groups as the present-day Turkish population. Alternatively, it might be just as likely that the lineage is older in Anatolia and could have been brought here by multiple human migrations over the past millennia, given the central geographical and cultural position of Asia Minor.

6.4 BONE SAMPLES AND GENETIC ANALYSES OF THE HARBOR NECROPOLIS

The excavations of the Harbor Necropolis started in 2005, several years before the samples for the DNA-analyses were taken in 2011 and 2013. Thus, the skeletal material had already been handled by numerous persons, probably leaving modern DNA on the surface of the bones. Since DNA is easily transferred between objects we touch it is important to decontaminate the bones as best as possible and carry out proper controls in the lab, to try to identify if contamination occur.

After the skeletons had been examined anthropologically by Kristina Scheelen and Jan Nováček, bone samples were taken by cutting a slice of the femur with a hacksaw using protective gloves and cleaning the equipment with ethanol between consecutive samples, or when available, by removing a tooth from the jaw. Samples were exported to the University of Oslo for further processing and DNA analyses. In total, samples from 68 skeletons were collected from four of the five graves of the Harbor Necropolis (grave 1: $n = 24$, grave 2: $n = 2$, grave 3: $n = 33$, grave 4: $n = 9$).

In the aDNA laboratory, standards were followed to avoid contamination including protective clothing. Thorough bone treatment was undertaken to remove contamination from people handling the skeletal material (fig. 85, 1. 1). For each bone piece, the outer surface layer was removed, followed by UV irradiation. After the bones were powdered, the bone powder was bleached. These processes are all implemented to remove potential contamination of bacterial, fungal and modern human origin. In addition, frequent use of negative controls and physical separation of the facilities for handling original and amplified DNA, to minimize the risk of contamination from amplified aDNA copies that could outnumber original aDNA, followed the standards for working with aDNA.

DNA was extracted and amplified as described by Malmström et al. (2009). In short, a 343-base pair long sequence stretch of the mtDNA control region corresponding to the positions 16 050–16 392 in the mitochondrial reference sequence was targeted (Anderson et al. 1981), using five overlapping fragments (fig. 85, 1. 2). All targeted fragments were short, between 120–150 base pairs long, to maximize the possibility to amplify authentic DNA.

Next generation sequencing (NGS) was implemented on the skeletal material from the Harbor Necropolis, performed at the Norwegian Sequencing Centre along with traditional sequencing of mitochondrial DNA performed at the ABI facilities at the UiO and Macrogen (Netherlands) for 20 samples. Several of the samples were sequenced using both methods. A major advantage with NGS is that a high number – typically hundreds or thousands – of DNA fragments present in an aliquot of amplified DNA are reported from each sample. The degradation pattern of the samples could be examined, as shown in figure 85, 1. 3. In ancient samples, DNA degradation is expected, as decomposition also of the DNA strands start at time of death. For poorly preserved samples, potentially no original DNA strands could be retrieved, and only degraded DNA-strands are observed. Thus, based on the degradation pattern the most statistically supported authentic sequence could be predicted even if it is not present (e.g. c-statistics; Helgason et al. 2009).

Haplotype assignment was based on Vincent Macaulay, mtDNAMANAGER and Genographic databases²⁷³.

²⁷³ <<http://www.stats.gla.ac.uk/~vincent/founder2000/motif.html>> (accessed 05/07/2016); <<http://mtmanager.yonsei.ac.kr/>> (accessed 05/07/2016).

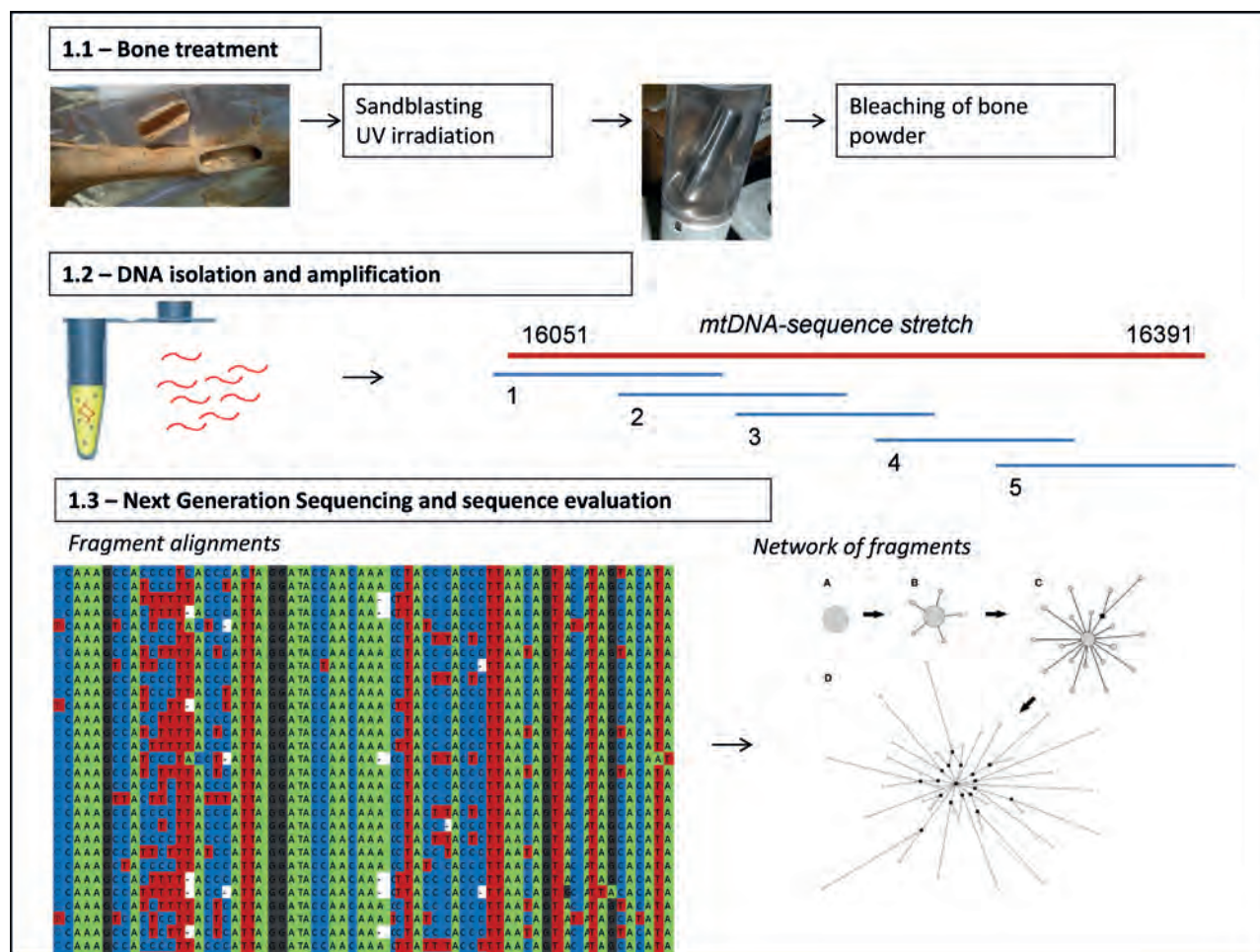


Fig. 85 The procedure from bone to DNA sequence is illustrated as sequential steps. Since DNA is on everything we touch with our bare hands, thorough bone treatment is undertaken to remove contamination from people handling the skeletal material or from bacteria or fungi from the soil surrounding the material (1.1). For each bone piece, the outer surface layer is physically removed using a sand blaster, followed by UV irradiation of the bone piece in a crosslinker cabinet. The bone pieces are then powdered in a Freezer Mill in the presence of liquid nitrogen, and the bone powder is bleached to further remove contaminants and amplification inhibitors.

DNA is extracted and amplified with frequent use of negative controls (i.e. empty samples including only water and chemical consumables). The negative controls are included to detect contamination if present. A 343 base pair long sequence stretch of the mtDNA control region, corresponding to the positions 16 050–16 392 of the mitochondrial reference sequence, was amplified in five different, but overlapping fragments using PCR (1.2). PCR – *Polymerase Chain Reactions* – is a method to make millions of copies of each requested target DNA sequence. All targeted fragments were short, between 120–150 base pairs, to maximize the possibility to amplify fragmented authentic DNA.

Next generation sequencing (NGS) technology reports a high number (typically hundreds or thousands) of DNA fragments present in an aliquot of amplified DNA from each sample (1.3). By aligning the sequences underneath each other, the degradation pattern (like *deamination*, an incident occurring post-mortem where biochemical changes in the DNA make the instrument read an alternative building block of the DNA strand) of the samples could be examined and evaluated using networks. When building the networks, identical fragments are grouped together, presented in a circle (A) and the size of the circle reflects the number of times a variant is observed. In the next step of network building, variants with one observed difference compared to the main type are added (B), followed by variants with two observed differences (C), and so on. For poorly preserved samples, potentially no original DNA strands could be retrieved, and only degraded DNA-strands are observed (D). The degradation pattern of the network could be statistically evaluated and the most likely authentic sequence predicted (Graphics: G. Bjørnstad)

6.5 PRELIMINARY MTDNA RESULTS AND GENETIC CONSTITUTION OF THE HARBOR NECROPOLIS

The presented results are a combination of conventional sequencing and NGS (tab. 93). No sample yielded DNA information in all five DNA-stretches, many of the samples gave DNA sequences in only three or four of the five fragments. Statistical evaluation of the authenticity of the sequences has not been performed, the observed mutations remain to be confirmed, and missing sequence information must be added before confirming haplogroup assignments. The suggested haplogroup assignments for 15 samples are presented as pie charts in figure 84 d. The high number of haplotypes and diversity of haplogroups indicate a broad and complex origin of this population of Roman Ephesos. The presence of the probable haplogroups H, U, N, J/T and I, all of western Eurasian origin, is in accordance with a European profile. The structure of the Roman population, interpreted from the Harbor Necropolis is putatively more complex than the later Ottoman population, presenting eight haplogroups among the 15 skeletons, compared to the Ottoman population described through the Türbe in the Artemision-study, presenting five haplogroups in 13 skeletons (fig. 84 c). However, both studies are based on a low number of skeletons and exposed to stochastic effects.

Table 93 Preliminary haplogroups assignments of 15 individuals representing four graves of the Harbor Necropolis

Sample no.	Grave	Individual	Bone/Tooth	Haplogroup	Radiocarbon dating
276	1	VIII	M2	U	
267	1	XV	M2	H	
61	2	XVII	Femur	N	
62	2	XVIII	Femur	D	321 ±50 A.D.
41	3	II	Femur	U	
54	3	IX	Femur	M/G/I	
57	3	V	Femur	H	
55	3	VIII	Femur	J/T	
43	3	XI	Femur	H	
38	3	XIV	Femur	J/T	
47	3	XVII	Femur	J/T	
1	4	I	Femur	D	179 ±34 A.D.
6	4	II	Femur	L/M	171 ±35 A.D.
58	4	IV	Femur	I	
14	4	VI	Femur	J/T	

Three samples had mutations indicative of haplogroups D/M (2) and M/L2 (1). The mutations of these samples are not conclusive for haplogroup assignment, and further analyses have to be performed to confirm the haplogroup. These haplogroups are uncommon in western Eurasia. Haplogroup M is one of the two main ancestral lineages leaving Africa in an eastward direction (fig. 83). Haplogroup D derived from haplogroup M and has an eastern Eurasian origin. The presence of three samples representing an eastern Eurasian or African origin could further be interpreted to reflect the throbbing life of this harbor city, attracting people from near and distant. It should be emphasized that it is not necessarily the examined individuals themselves that have an exotic origin, but rather their maternal ancestors.

Two samples had a sequence that could correspond to the eastern Eurasian haplogroup D or M. A variant of this haplogroup, D4, was found in Ottoman Ephesos (Bjørnstad 2015). D4 is the most frequently occurring haplotype of northeast Asia, and spread all over Southeast Asia, Central Asia and the Americas. The frequency of D in Turkmen is around 30 % (Comas et al. 2004). Haplogroup D is found in the modern Turkish population at around 7 % (Schönberg et al.

2011) but was not observed in the late-Byzantine population of Sagalassos (Ottoni et al. 2011). The lineage could have been brought into the Anatolian population by the Seljuk Turks or during later contact with central Asia. However, the skeletal material from the Harbor Necropolis suggests that these lineages are also more ancient in Anatolia.

One individual had mutations that could suggest it belongs to haplogroup L2 (or M). Haplogroup L2 is African. It is likely that the maritime trading networks of eastern Mediterranean promoted contacts between coastal Ephesos and northern Africa, contacts that could have left permanent genetic traces in the Ephesian population. Interactions between Sagalassos, an inland city, and Egypt/Levant, have been documented through finds of ancient fish bones originating from Northern Africa/Middle East in Sagalassos (Arndt et al. 2003).

The lack of information of partial DNA-sequences is challenging, particularly for detecting H-haplotypes. Absence or minor differences compared to the reference sequence (Cambridge Reference Sequence, CRS) indicate that a haplotype belong to haplogroup H, the most prevalent haplogroup of present-day Turkey as well as Europe. Thus, many of the samples without detected variation and not reported in this study most likely belong to haplogroup H but are not assigned haplogroup since we do not possess the complete sequence information of these samples without (conclusive) mutations.

6.6 FUTURE ANALYSES OF THE GRAVES OF THE HARBOR NECROPOLIS

Grave 3 and 4 appear to have best biological preservation of the five Harbor Necropolis graves. Grave 1, 2 and 5 have been limited tested, but the physical appearance of the bones suggests that amplification of aDNA could be more challenging from these graves. The biological preservation of the skeletal material from grave 3 and 4 could warrant analyses also of nuclear markers like SNPs (Single Nuclear Polymorphisms). SNPs include the complex genetic information of all past direct ancestors and could elucidate complex cultural and historical trends undetectable by maternally inherited markers (mtDNA). Further, reliable sex determination of skeletal remains based on biometrical measurements is challenging for incomplete skeletons and children, but analyses of sex-linked markers, detectable with SNPs, could reveal valuable information regarding gender composition, population structure, health aspects, burial traditions and so on.

Multiple samples were taken from many of the skeletons from the Harbor Necropolis for isotope analyses. Stable isotopes of carbon and nitrogen provide information on diet composition while isotopes of strontium provide information on geographical origin. Values of strontium isotopes are imprinted by the geology of the landscape, giving an area a unique strontium pattern. The strontium values of teeth are identical with the geographical landscape values of where the individual grew up, as the teeth were formed. By comparing the strontium values of the human teeth with reference values of the landscape (e.g. soil, plants, molluscs, domestic animals) indicate if the individual have lived the whole life in the region or if it was a migrant, growing up in another region with a different strontium profile. Food consumption is reflected in the isotopic composition of human tissue. Stable isotopes of carbon and nitrogen reflect the protein intake and indicate if a person's diet was mainly terrestrial or marine, vegetarian or meat based, or if special plants (C4-plants like millet, sorghum) were part of the diet. The isotope analyses of the skeletal remains from the Harbor Necropolis are initiated in cooperation with Michael P. Richards (Simon Fraser University in Burnaby, Canada) and will provide valuable information about diet and origin of people buried in the Harbor Necropolis of Roman Ephesos.

In conclusion, preliminary analyses of the skeletal material from the Harbor Necropolis suggest a complex distribution of maternal lineages among the Roman Ephesians. Both European signatures, along with more exotic maternal lineages potentially of Asian and/or African origin were detected within this coastal site. This suggests that Roman Ephesos was a meeting point for people from near and far, from ›all corners of the world‹. Further investigations using nuclear markers and analyses of isotopes could elucidate significant cultural and historical issues of this Roman population.