North and East African mitochondrial genetic variation needs further characterization towards precision medicine

Anke Fähnrich[a,*], Isabel Stephan[a,*], Misa Hirose[b], Franziska Haarich[c], Mosab Ali Awadelkareem[d,e], Saleh Ibrahim[b,f], Hauke Busch[a,g,+], Inken Wohlers[a,+,§]

[a]Medical Systems Biology Division, Lübeck Institute of Experimental Dermatology and Institute for Cardiogenetics, University of Lübeck, Ratzeburger Allee 160, 23562, Lübeck, Germany

[b]Genetics Division, Lübeck Institute of Experimental Dermatology, University of Lübeck, Ratzeburger Allee 160, 23562 Lübeck, Germany

[c]Institute for Cardiogenetics, University of Lübeck, DZHK (German Research Centre for Cardiovascular Research), partner site Hamburg/Lübeck/Kiel, and University Heart Center Lübeck, Lübeck, Germany

[d]Faculty of Medical Laboratory Sciences, Al-Neelain University, Khartoum, Sudan

[e]Nuffield Department of Clinical Neurosciences, University of Oxford, Oxford OX3 9DU, United Kingdom

[f]Sharjah Institute for Medical Research, University of Sharjah, Sharjah, United Arab Emirates

[g]University Cancer Center Schleswig-Holstein, University Hospital of Schleswig-Holstein, Campus Lübeck, 23538 Lübeck, Germany

[*]Equal contributing first authors

[+] Equal contributing last authors

[§] Correspondence to Inken.Wohlers@uni-luebeck.de; Phone: +49 (0) 451 3101 8476; Fax: +49 (0) 451 3101 8404

Abstract

Introduction

Mitochondria are maternally inherited cell organelles with their own genome, and perform various functions in eukaryotic cells such as energy production and cellular homeostasis. Due to their inheritance and manifold biological roles in health and disease, mitochondrial genetics serves a dual purpose of tracing the history as well as disease susceptibility of human populations across the globe. This work requires a comprehensive catalogue of commonly observed genetic variations in the mitochondrial DNAs for all regions throughout the world. So far, however, certain regions, such as North and East Africa have been understudied.

Objectives

To address this shortcoming, we have created the most comprehensive quality-controlled North and East African mitochondrial dataset to date and use it for characterizing mitochondrial genetic variation in this region.

Methods

We compiled 11 published cohorts with novel data for mitochondrial genomes from 159 Sudanese individuals. We combined these 641 mitochondrial sequences with sequences from the 1000 Genomes (n=2,504) and the Human Genome Diversity Project (n=828) and used the tool haplocheck for extensive quality control and detection of in-sample contamination, as well as Nanopore long read sequencing for haplogroup validation of 18 samples.

Results

Using a subset of high-coverage mitochondrial sequences, we predict 15 potentially novel haplogroups in North and East African subjects and observe likely phylogenetic deviations from the established PhyloTree reference for haplogroups L0a1 and L2a1.

Conclusion

Our findings demonstrate common hitherto unexplored variants in mitochondrial genomes of North and East Africa that lead to novel phylogenetic relationships between haplogroups present in these regions. These observations call for further in-depth population genetic studies in that region to enable the prospective use of mitochondrial genetic variation for precision medicine.

Keywords: mtDNA, haplogroups, phylogenetics, energetics, next-generation sequencing, long-read sequencing

Introduction

Mitochondria are energy-producing, double-membrane-bound organelles that contain their own genome and that are central to many processes in eukaryotic cells (1). The mitochondrial (MT) genome is circular and typically 16,569 bases long in humans (2). Mitochondrial dysfunction is often linked to inefficient oxidative phosphorylation (OXPHOS) capacity with compromised ATP and increased mitochondrial reactive oxygen species (ROS) production that change cellular function and signaling (3). Dysfunction accompanies various pathologic conditions, such as metabolic diseases (4), neurodegenerative disorders (5), and autoimmune conditions as well as cancer (6) and is also linked to aging (7). Its root cause might stem from variations of the MT genome, which has thus moved into the center of research as a determining factor for complex diseases and precision medicine.

Since mitochondria are exclusively maternally inherited in humans, the mitochondrial DNA (mtDNA) phylogeny represents a global maternal genealogy, which has been summarized in an established and widely applied reference phylogenetic tree called PhyloTree (8,9). Its current version 17 is based on more than 24,000 mitochondrial sequences and constitutes more than 5,400 haplogroups, i.e., recurrent genetic variant profiles common in human mitochondrial DNA.

Recent studies assessed mitochondrial haplogroups in worldwide human diversity reference data sets from the 1000 Genome Project (1000G; n=2,504) (10,11), the Human Genome Diversity Project (HGDP; n=623 in (12); n=102 in (13)) and very recently gnomAD (14). Such mitochondrial phylogenetic studies have traced modern human settlement of the world (15) and support the hypothesis of Africa as origin of modern humans. The “Out-of-Africa” theory proposes the so-called “mitochondrial Eve”, which represents the mitochondrial Reconstructed Sapiens Reference Sequence (RSRS) mtDNA as the phylogenetic root and modern human origin (16).

Previous studies on mtDNA genetic variation in North and East African individuals largely focused on identifying and characterizing phylogenetic clades with respect to time of origin and geographic dispersal. However, with advances in next-generation sequencing

88 technologies and decreasing sequencing costs, the wide-spread use of mitochondrial genetic
89 variation for medical purposes becomes feasible. Many applications in this context can be
90 considered as precision medicine, i.e. they provide personalized diagnostics or treatment.
91 They fall into two areas: primary mitochondrial diseases and common diseases (17). Primary
92 mitochondrial diseases are caused by a genetic defect that impairs MT function, resulting into
93 a range of severe symptoms. They affect one of 4,300 people (18). To clinically diagnose these
94 genetic diseases, the underlying causal variant needs to be identified on an individual basis, a
95 process in which variants that occur in healthy individuals are ruled out. Thus, reference data
96 that is representative of human genetic mitochondrial variation will improve diagnosis of
97 primary mitochondrial diseases. The second area of application are common, polygenic
98 diseases; differences in mitochondrial DNA have been associated with a range of them.
99 Considering the central biological role of mitochondria, mtDNA variation is particularly
100 promising for disease stratification, identification of disease modifiers and inclusion into
101 personalized risk assessment, e.g. via polygenic risk scores. MtDNA for precision medicine in
102 common diseases is a comparably novel field with large potential (17). Owing to these recent
103 developments, we here view mitochondrial genetic variation of North and East Africa from
104 the point of its application in primary mitochondrial disease diagnostics and prospective
105 precision medicine.
106 In this work, we address the state of mtDNA reference data from the regions of North Africa
107 and East Africa. As of now, there are limited genetic data available from this world region, as
108 it is not included in large genome projects such as 1000G (19), gnomAD (20) or Topmed (21).
109 An exception is Egypt, for which an initial reference dataset has been compiled (22) and efforts
110 are ongoing to construct a much larger Egyptian genome reference towards genomics-based
111 precision medicine (23).
112 To address this reference data limitation, we overall compiled and subsequently quality
113 controlled data from overall 844 mitochondrial genomes (cf. Suppl. Table 1). These are from
114 a newly sequenced Sudanese cohort (n=159), an Egyptian cohort with targeted mitochondrial
115 sequencing (n=217) and an Egyptian cohort having whole genome sequencing data (n=110).
116 Both Egyptian cohorts have previously been investigated for haplogroup distribution (22,24)
117 and are here combined with mitochondrial sequencing data of ancient Egyptian mummies
118 (n=90) (25) as well as mtDNA sequences from Genbank from individuals with origins in North
119 or East African countries (n=268).

120 In addition to providing additional mitochondrial genomes from this understudied region, we
121 reanalyzed all available next generation sequencing data using a novel tool, called haplocheck
122 (26), to identify potential sample contamination and phantom variants (27). Haplocheck is a
123 recent tool which allows to detect sample contaminations from next-generation sequencing
124 data by assessing if multiallelic variant calls can be broken down into a mixture of two
125 haplogroups, one denoted as "major", the other as "minor". Presence of two haplogroups
126 indicates that genetic material of two individuals was jointly sequenced and thus DNA from
127 the individual relating to the major haplogroup contaminated with DNA from an individual
128 carrying the minor haplogroup. Our combined and new quality-controlled data results in 146
129 exceptionally high-quality and 641 high quality mitochondrial genome sequences after quality
130 control that we used to phylogenetically re-characterize genetic MT variation in North and
131 East Africa with respect to worldwide mtDNA diversity. We find that the haplogroups of the
132 region cover nearly all worldwide clades and identified 15 potential novel subhaplogroups,
133 among them two that descend from L0a1a1 and and two that are related to L2a1, for which
134 sequences from North and East Africa suggest a different phylogenetic relationship as
135 described by the reference PhyloTree.
136
137
138 **Results & Discussion**
139
140 **North and East African mitochondrial data collection and filtering**
141 To comprehensively cover the phylogenetic tree of the North and East African population we
142 sequenced a cohort of 159 Sudanese individuals which we combined with mitochondrial
143 sequencing data from 217 Egyptians. An additional 110 MT genomes were extracted from
144 whole genome sequencing data of Egyptians from Pagani et al. (24) and Wohlers et al. (22).
145 We further included mapped mitochondrial sequencing data from 90 ancient Egyptian
146 mummies from Schuenemann et al. (25); for three of these mummies, WGS data were
147 available (cf. Supplementary Table 1 for details).
148 As controls, we used two worldwide whole-genome references, 1000G with 2,504 samples
149 (19) and HGDP with 828 samples (28) from which we generated mitochondrial DNA
150 sequences. Further, we obtained quality-controlled and aligned Genbank sequences from
151 countries in North and East Africa from the supplement information of McInerny et al. (29),

152 which we manually traced to their eight respective publications provided in Suppl. Table 1.
153 Sampling location or ethnicity as well as number of sequences are provided in Figure 1.
154 Five of eight studies relating to Genbank entries included only specific haplogroups. These are
155 two studies on haplogroup U6 (Maca-Meyer et al. (30), n=11; Olivieri et al. (31), n=28) in
156 Berbers from Morocco; two studies on haplogroup L3 in Morocco (Harich et al. (32), n=8) and
157 Ethiopia (Soares et al. (33), n=70); and one study of both M1 and U6 in Moroccan (Pennarun
158 et al. (34), n=56). We also included 18 Genbank MT sequences from ancient Moroccans (Fregel
159 et al. (35)). Genbank-related studies that did not focus on a specific haplogroup covered 35
160 Egyptian (Kujanová et al. (36)) and 42 Tunisian mtDNA sequences (Costa et al. (37)).
161 To exclude study-specific batch effects and sequencing errors, we applied a two-step quality
162 control (QC) filter on all next-generation sequencing-based North and East African data sets.
163 This was important to correctly assess haplogroup distributions and identify putatively new
164 haplogroups. For the filter step, we kept all samples from Genbank as well as those (i) having
165 a haplocheck-reported major haplogroup equal to a minor haplogroup, and (ii) having
166 contamination assessed and not detected (i.e. “NO”). An overall of 641 samples from four
167 next-generation sequencing-based North and East African data sets and from eight Genbank
168 studies passed this lenient filter (cf. Suppl. Table 11 and Suppl. Table 18 for aligned
169 sequences). For the second QC filtering step, we excluded Genbank sequences, as
170 contamination checks for them were unfeasible, and additionally required a minimum
171 sequencing coverage greater than 1000x, which is considerably higher than the minimum 600x
172 coverage required by haplocheck to detect a 1% or greater contamination (26). The number
173 of total and filtered samples in each QC step are provided in Figure 1 for every cohort included.
174 For mitochondrial sequences obtained from Genbank, only the overall number is provided.
175

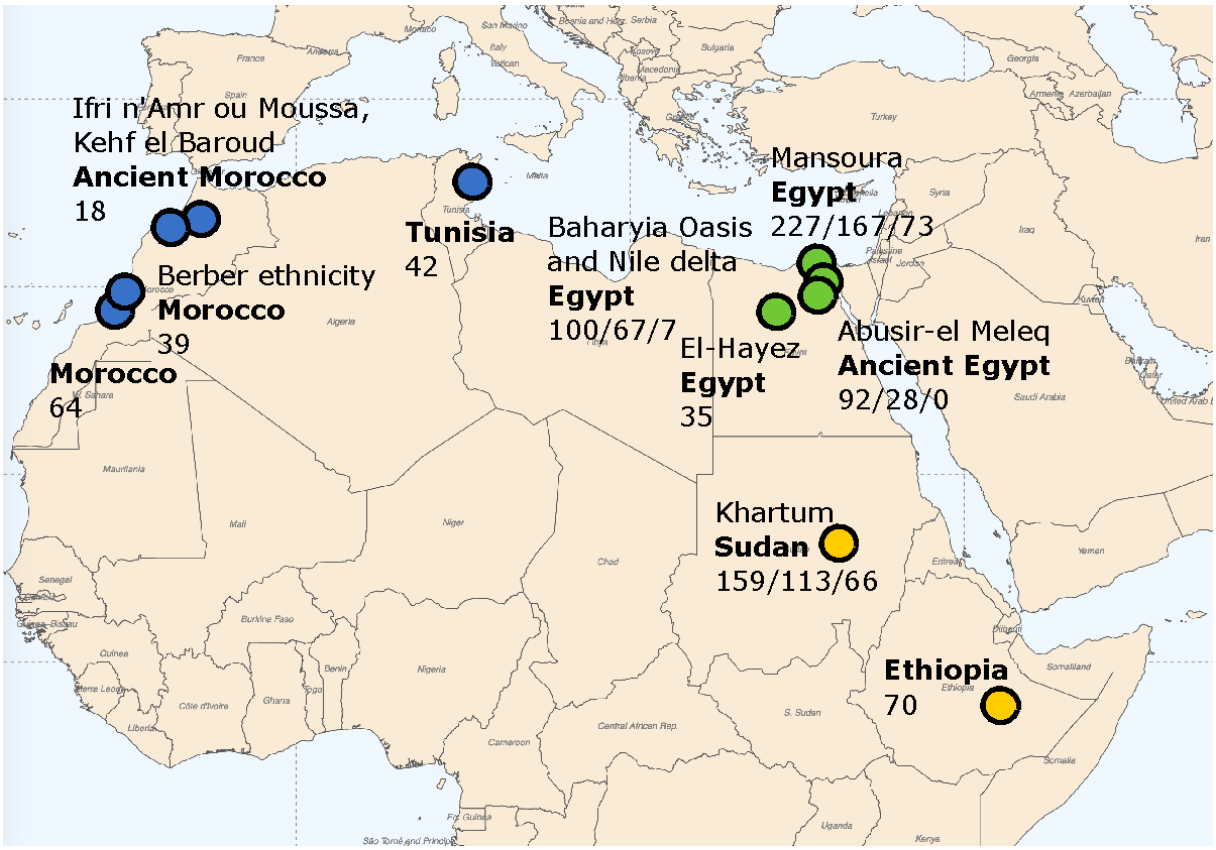

Figure 1: Mitochondrial DNA data compiled from North and East Africa. Blue color denotes cohorts referred to as North African, green color cohorts refer to Northeast African and yellow color cohorts refer to East African. Cohorts analyzed from raw next-generation sequencing data are denoted by three numbers: The overall number of samples; the number of samples remaining after lenient QC filtering; and the number of samples remaining after strict QC filtering. Locations with only one number refer to Genbank MT sequences obtained via McInerny et al. (29). Whenever Northeast Africa is not shown separately, its cohorts are included in North Africa and East Africa.

Haplogroup-based contamination assessment for diverse sequencing settings

We assessed potential contamination and related sequencing and variant calling metrics for all six sequencing-based datasets, including the worldwide references 1000G and HGDP (cf. Supplementary Tables 2-9 and 13-16 for details). Haplogroups assigned to 543 HGDP samples, for which Lippold *et al.* (12) determined haplogroups previously using targeted mitochondrial sequencing were largely consistent with the haplogroups assigned in this work (47% identical haplogroup; 86% a subclade of the previously assigned haplogroup; cf. Suppl. Table 17). Coverage of the 1000G and HGDP data sets is high with a median greater than 10,000X, while the other four cohorts have a significantly lower coverage of 840X (Egyptian MT sequencing), 670X (Egyptian WGS), 163X (ancient Egyptian mummies) and 992X for the Sudanese MT sequencing cohorts, respectively (see Figure 2 b).

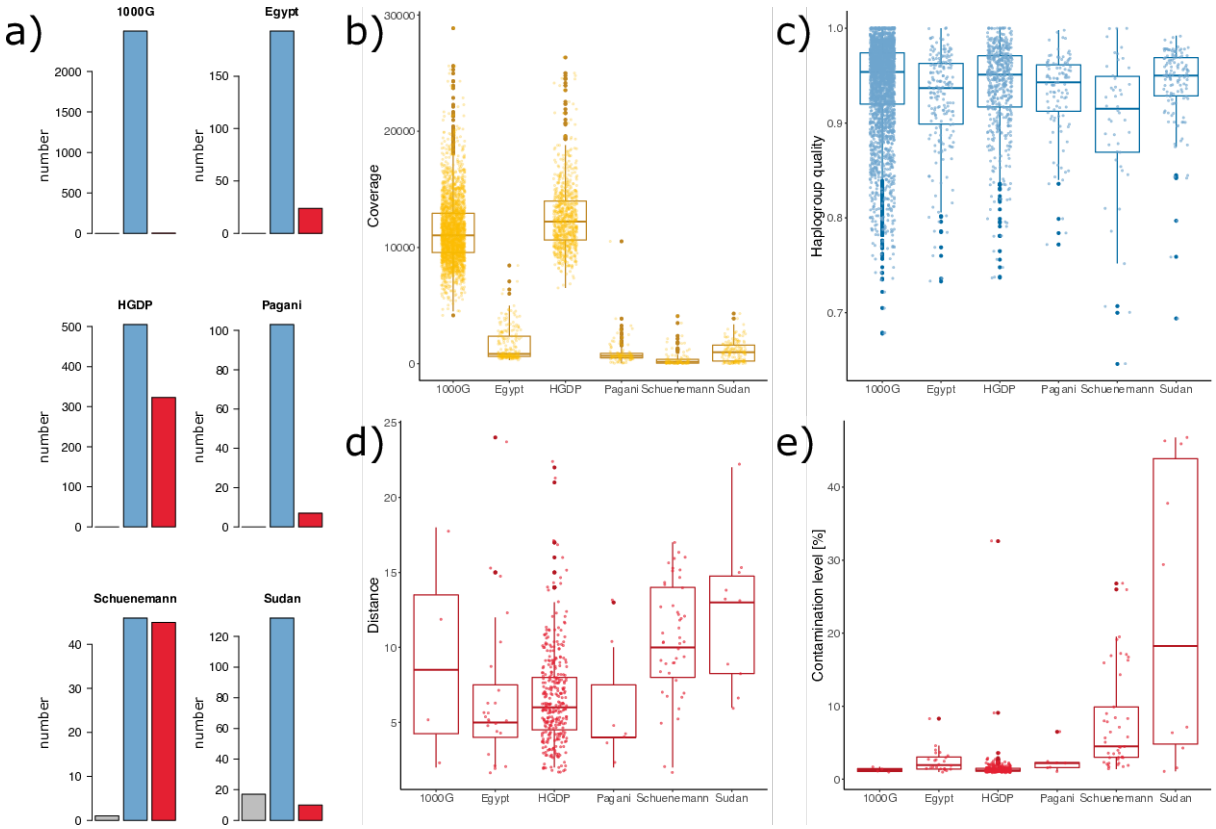

Figure 2: Characterization of the six sequencing-based data sets using numbers reported by haplocheck. (a) Histogram of number of samples with haplocheck contamination status not available ("NA") (gray), not contaminated ("NO") (blue) and predicted contaminated ("YES") (red); (b) Box plots for sequencing coverage; (c) Box plots for haplogroup quality of those samples not predicted to be contaminated; (d) For those samples predicted to be contaminated, box plots of the distance between major and minor haplogroup, i.e. the number of phylogenetic nodes between them within the phylogenetic tree; (e) Box plots of the contamination levels for samples predicted as contaminated. Box plots display median and lower/upper quartiles; whiskers denote the most extreme data point no more than 1.5 times the interquartile range; outliers are data points extending beyond whiskers.

Contamination is identified using the computational tool haplocheck, which identifies polymorphic sites within samples. It attempts to decompose the variants into a so-called major and another, minor, haplogroup. If such decomposition is possible and coherent, a mix of a sample of the major haplogroup with another sample of the minor haplogroup is predicted and the percentage of minor haplogroup allele frequency reported as contamination level. Interestingly, we found a large heterogeneity in contamination calls among the studies. Few of the WGS-based 1000G samples are predicted to be contaminated, yet more than 30% of the WGS-based HGDP samples (Figure 2a), albeit at a low contamination level of 2% (median 1.2%, Figure 2e). Comparable, low level high distance contamination is predicted for WGS samples of Wohlers *et al.* and Pagani *et al.* Possibly, these contamination calls are caused by NUclear MiTochondrial DNA sequences (NUMTs) causing mapping

artefacts, although a core genome coverage of 30x, a typically large haplogroup quality (Figure 2c) and a large phylogenetic distance between major and minor haplogroup detectable based on unusual mutation patterns (Figure 2d) indicate that this is rather not the case. High contamination levels (e.g. levels of 50% or 30%) are observed for very few samples largely from the Sudan cohort and may indicate partial swaps of sample material. For the ancient Egyptian mummy data set, haplocheck repeatedly identifies minor haplogroups related to H2a2a, which we consider an artifact caused by a lack of sequencing coverage that causes the revised Reference Sequence (rCRS) reference bases to be called, which refer to haplogroup H2a2a1. This is supported by the very low coverage for this cohort.

Haplogroup validation with Nanopore long-read sequencing

We performed Nanopore long-read sequencing to validate haplogroups obtained from whole-genome sequencing data as well as from targeted mitochondrial sequencing data. We used 10 Egyptian samples whole genome-sequenced at 30x (covering subhaplogroups of H, L, M, and T). For all of them, the major haplogroup was confirmed. Further, we performed Nanopore sequencing for eight Sudanese samples for which mitochondrial sequencing data were available. The major haplogroup for all of them was confirmed, with the exception of L2a1+143, which according to Nanopore sequencing was assigned to the closely related haplogroup L2a1'2'3'4, with no contamination predicted (Suppl. Table 19). Manual inspection shows that all four variants that distinguish between L2a1'2'3'4 and L2a1+143 are clearly detectable from Nanopore sequencing, but have a large fraction of reads carrying the reference allele (chrM:143 16%; chrM:12693 16%; chrM:15784 24%; chrM:16309 7%). Interestingly, we observe a large number of predicted contaminations (n=13/18; 72%) with subhaplogroups of H2a2a, which, again, indicates a reference bias caused by a sequencing or variant calling artefact. Nanopore sequencing has a comparably low base accuracy and base calling errors affecting the reference base may be preferentially reported by MT DNA callers which have been designed for short-read data that has higher base accuracy. As technologies, protocols and tools for long-read data develop quickly, constant benchmarking is needed and has recently shown the validity for Nanopore-based somatic MT variant calling (38).

248 **Heteroplasmic variant calls**

249 Besides homoplastic variants, which are monoallelic, mtDNA variants can be heteroplasmic,
250 i.e. mtDNA of more than one allele is present in a sample or tissue. Highly accurate detection
251 of heteroplasmic variants, especially at low heteroplasmy levels, was not the scope of our
252 study and needs dedicated sequencing and analysis. Nevertheless, we investigated the overall
253 number of homoplasmic and heteroplasmic variant calls provided by haplocheck to
254 characterize the next-generation sequencing data sets (see Suppl. Tables 3, 5, 7, 9, 14 and 16).
255 For those samples not predicted to be contaminated (and thus not affected by artificial
256 heteroplasmic calls) the results are displayed in Figure 3 a) and b).

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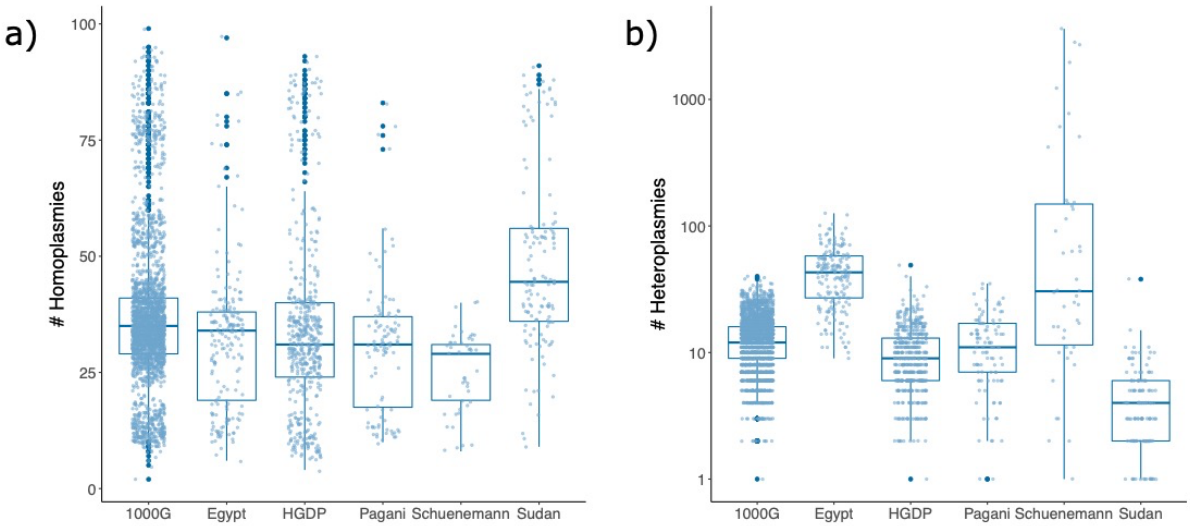

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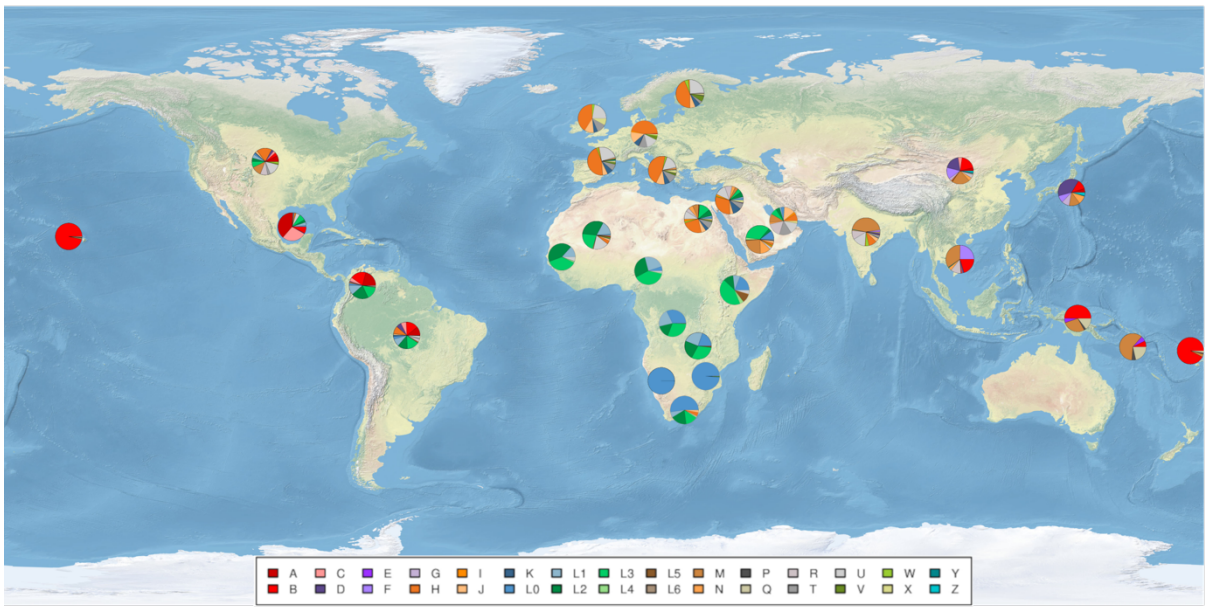
259 **Figure 3:** Boxplots of number of hompolasmic and heteroplasmic variant calls with respect to rCRS for all six next-generation
260 sequencing-based data sets. Box plots display median and lower/upper quartiles; whiskers denote the most extreme data
261 point no more than 1.5 times the interquartile range; outliers are data points extending beyond whiskers.

262

263 The number of homoplasmic variant calls reflects the haplogroups present in the respective
264 data set, because the larger the haplogroup's phylogenetic difference from the reference
265 sequence rCRS haplogroup H2a2a1, the more variants will be called with respect to rCRS. As
266 such, data sets containing for example many African haplogroups L will have more samples
267 with many homoplasmic variant calls. This also explains the comparably large number of
268 homoplasmic variant calls in the Sudanese data set. However, numbers of heteroplasmic
269 variant calls are expected to be comparable between data sets and independent of the
270 haplogroup content of the respective data set. This is however not the case and differences in
271 the distributions of heteroplasmic variant calls can be observed between datasets. These

272 differences indicate technical artefacts such as NUMTs, uneven or low coverage and base
273 errors. Interestingly, distributions of number of heteroplasmic variant calls are comparable
274 for all whole-genome sequencing data sets, i.e. 1000G, HGDP and Pagani *et al.* (24) with a
275 median number of heteroplasmic variant calls of 12, 14 and 12, respectively. Laricchia *et al.*
276 (14) recently report for the gnomAD WGS data set of 56,434 individuals that most
277 heteroplasmic variant calls are caused by NUMTs. This may also be the case here, because the
278 Sudan mitochondrial amplicon sequencing data set has a much lower number of
279 heteroplasmic variant calls (median 4), likely representing an upper bound of true
280 heteroplasmic variants expected in blood of healthy individuals. However, the Egyptian
281 mitochondrial amplicon sequencing data set has most heteroplasmic variant calls (median of
282 45). Finally, the Egyptian mummy data set has the widest range. Large numbers of
283 heteroplasmic variant calls in mitochondrial sequencing observed here are caused by low and
284 uneven coverage in conjunction with base errors. These base errors are possibly also reflecting
285 input DNA quality, which decreases with time and environmental exposure; an example for
286 this is the ancient DNA from Egyptian mummies. Thus, for accurate heteroplasmic variant
287 identification down to low heteroplasmy levels, deep sequencing at an even coverage will be
288 needed.
289
290 **Distribution of Western Asian and Northeast African mitochondrial haplogroup diversity**
291 The sequential accumulation of polymorphisms in the mtDNA sequence over time across
292 generations has led to the emergence of geographically isolable variation profiles and
293 haplogroups. As a result, geographic regions have characteristic distributions of mtDNA
294 haplogroups. The analysis of 9,264 worldwide sequences (see Methods) yielded haplogroup
295 distributions for distinct geographical regions as shown in Figure 4a.
296

a)



b)

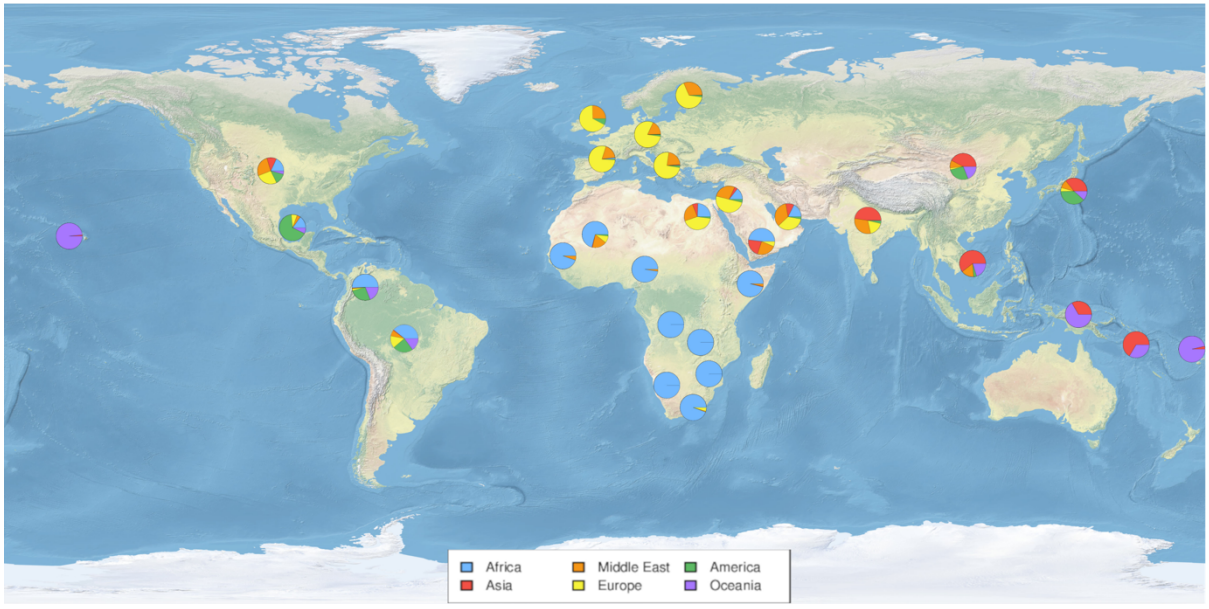


Figure 4: Worldwide haplogroup distributions by geographic region using top-level alphabetic clade denominators as well as first level subclades of haplogroup L. Please note that for some haplogroups geographic incidence of individual subclades can differ substantially (e.g. different B subclades occurring in America and Asia). a) Haplogroup level. b) Haplogroups have been assigned to continents of highest prevalence, representing their hypothetical regions of origin. We decided on the following assignment: Africa: L haplogroups; Asia: E, F, G, M, Y, Z; Middle East: N, R, U, I, W; Europe: H, J, K, T, V; America: A, C, D, X; Oceania: B, P, Q. Please note that tracing geographic origins of haplogroups is complex, e.g. due to autochthonous subclades, and the haplogroup levels and groupings we chose here are not representing all these relations, e.g. for haplogroup B.

As described previously, multiple haplogroups are predominant in different continental region. Here, we decided on the following assignment: Africa: L haplogroups; Asia: E, F, G, M, Y, Z; Middle East: N, R, U, I, W; Europe: H, J, K, T, V; Americas: A, C, D, X; Oceania: B, P, Q. Of note, we are aware that haplogroups overlap between continents, e.g., especially in Europe

310 and the Middle East. The grouping above therefore does not entail exclusivity with respect to
311 the listed haplogroups. Based on our assignment, we determined the relative proportion of
312 haplogroups associated with different continents for each clustered group (see Figure 4b).
313 Regions in Asia, North America, Northeast Africa, and the Middle East are particularly diverse.
314 Historically, this can be attributed to events such as colonization and slave trade in the
315 Americas as well as early human migration in the Middle East (39). In addition to its unique
316 transcontinental location between Africa and Asia, Northeast Africa is adjacent to the center
317 of modern human settlement in the world (15) and has been affected by complex events of
318 prehistoric back migrations (40).
319 The relative frequency of mitochondrial haplogroups of the 641 QC filtered North and East
320 African samples are depicted in Figure 5a and the individual haplogroup assignment per
321 sample is given in Supplementary Table 11.
322

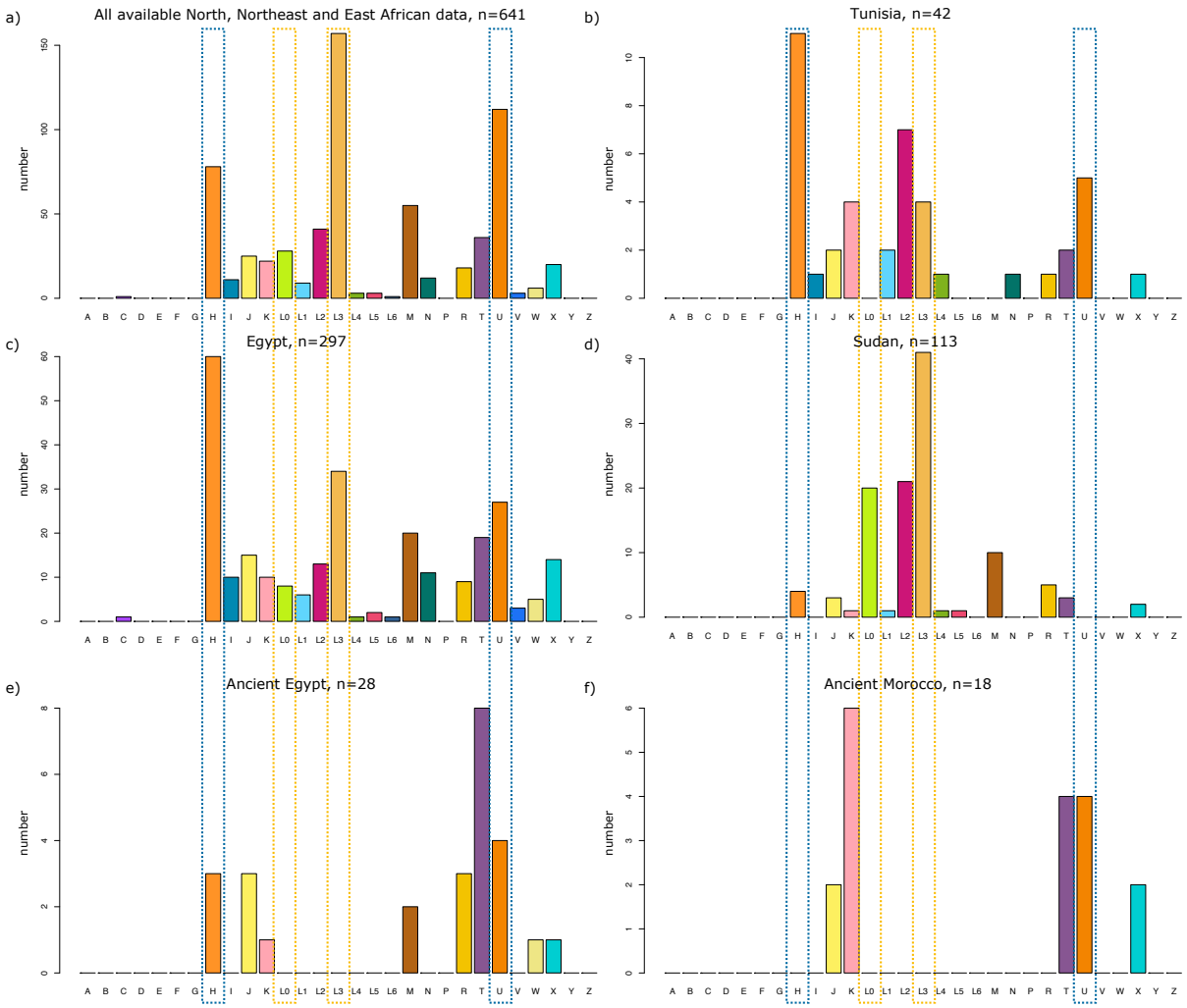


Figure 5: Histograms of haplogroup frequencies. Blue dashed line boxes indicate predominantly North African and yellow dashed line boxes predominantly East African haplogroups within this region. a) For all n=641 North, Northeast and East African samples passing the lenient QC filter and including Genbank sequences. This includes also Genbank sequences of studies addressing particular haplogroups (e.g. L3, M1 and U6) and thus is not a geographically representative distribution. b) Tunisian haplogroup numbers out of overall 42. c) Egyptian haplogroup numbers out of overall 297. d) Sudanese haplogroup numbers out of overall 113. e) Haplogroup numbers in ancient Egyptian mummies. f) Haplogroup numbers in ancient Moroccan individuals.

Country-specific haplogroup frequencies for Tunisia, Egypt, Sudan, ancient Morocco and ancient Egypt are shown in Figures 5b-f. Geographic neighborhoods are clearly reflected in the haplogroup distributions and indicate a geographic gradient from North Africa via Northeast Africa towards East Africa. This can be noted particularly for haplogroups H and U, that are most prevalent in Tunisia, less abundant in Egypt, and rare in Sudan. Conversely, L3 and L0 are prevalent in Sudan, and occur to a lesser extent in Egypt but are rarely observed in Tunisia. Interestingly, L2 is commonly observed in both Tunisia and Sudan, but rarer in Egypt.

Overall, most haplogroup clades are observed in the Egyptian cohort (compiled from three data sets). This finding might be explained by the larger sample sizes and compilation of various data sets available for Egypt, compared to only one data set each for Tunisia and Sudan. Yet, more haplogroup clades reach high fractions compared to Tunisia and Sudan, supporting the hypothesis that Egypt is the region of highest mitochondrial DNA diversity within North- and East Africa.

Northeast African mitochondrial lineages cover nearly all major worldwide clades

In order to elucidate the haplogroup abundance in North and East Africa relative to the rest of the world, we computed a phylogenetic tree from the 641 sequences in combination with the 1000G and HGDP reference data. The resulting trees in Figures 6 and 7 demonstrate that nearly all worldwide mitochondrial clades are represented in North and East Africa and, apart from the haplogroup M that is mainly represented by M1, a variety of subclades occurs. This is a haplogroup known to be restricted to North and East Africa that is absent from the 1000G and present in HGDP data only once.

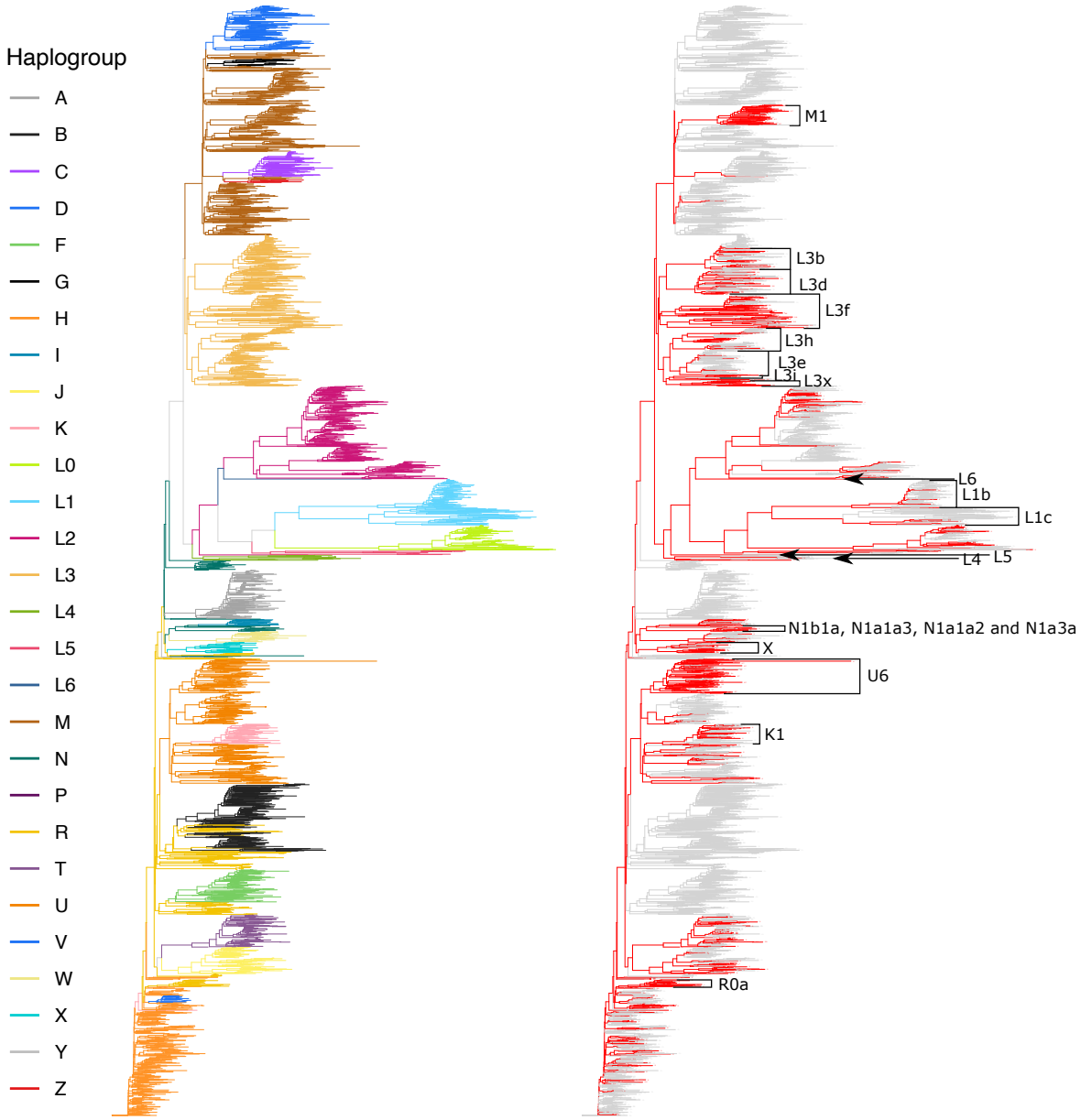


Figure 6: Phylogenetic tree of 641 North and East African mitochondrial sequences together with sequences from 1000G as worldwide reference data sets. This tree was computed by a Neighbor Joining-based tree and is colored according to haplogroup assigned by haplocheck (left) and colored according to data source, with North, Northeast and East African mitochondrial sequences compiled in this study shown in red and reference data shown in gray (right).

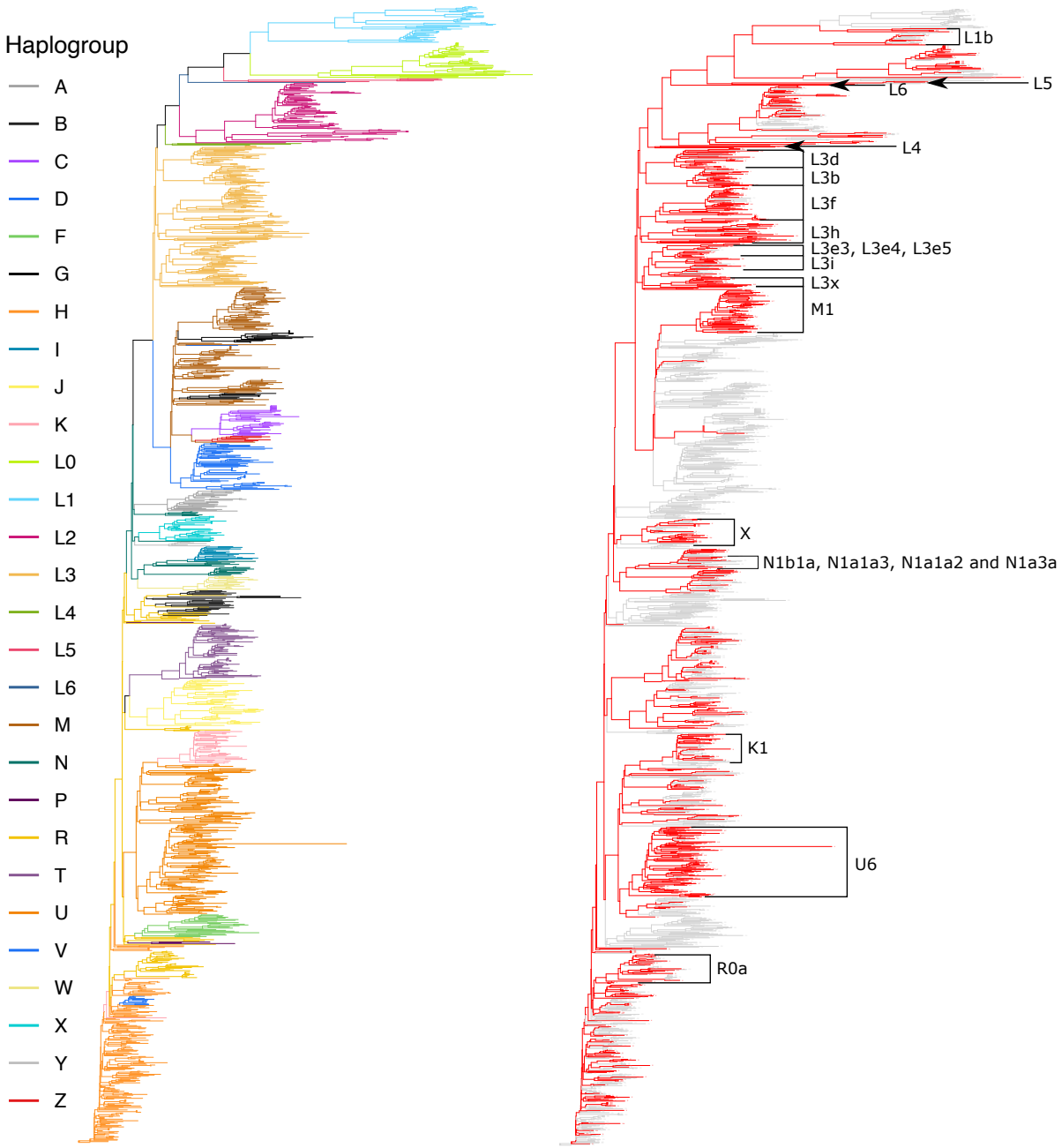


Figure 7: Phylogenetic tree of 641 North and East African mitochondrial sequences together with sequences from HGDP as worldwide reference data sets. This tree was computed by a Neighbor Joining-based tree and is colored according to haplogroup assigned by haplocheck (left) and colored according to data source, with North, Northeast and East African mitochondrial sequences compiled in this study shown in red and reference data shown in gray (right).

368 The worldwide haplogroups not observed in North and East Africa are A, B, D, E, F, G, P, Y and
369 Z, which are predominantly North Asian, Northeast Asian, East Asian, Southeast Asian,
370 American or Oceanian.
371 The predominantly European haplogroup H is more common in Tunisia and Egypt and less
372 common in Sudan. The same applies to the haplogroups I, J, K, N and U.
373 Haplogroup I is a sister clade of N1a1b, as described earlier (41). Haplogroup J is also observed
374 in both ancient data sets. North African sequences belong to J1 as well as J2 and are with
375 respect to RSRS more deep-rooting than 1000G and HGDP sequences.
376 Haplogroup K represents a subgroup of U8, as expected (42). In the North and East African
377 data, only K1 was observed, whereas 1000G and HGDP samples also covered K2.
378 African haplogroups L0, L1, L2 and L3 show a high abundance in Egypt, Sudan and Tunisia with
379 the exceptions of L0 in Tunisia and L1 in Sudan being absent and lowly abundant, respectively.
380 L3 is the founder of haplogroups N and M, which have given rise to all worldwide haplogroups
381 outside of Africa, and thus L3 has been studied extensively and exclusively (33,40). Haplogroup
382 L3 is observed in all data sets, but predominantly in Sudan with a share of 40%. This is in line
383 with previous studies that suggest East Africa as the origin of L3 (33).
384 Besides pan-African haplogroups, we observe haplogroups L4, L5 and L6. All these
385 haplogroups have previously been observed mainly in East Africa (43,44). Due to low
386 prevalence and due to being restricted to this region, they have not been studied by explicitly
387 sequencing mtDNA sequences of these haplogroups .
388 All but one sample relating to North and East African M haplogroups belong to the M1 clade,
389 which is the only M subclade in Africa (45). The data sets compiled here cover a large diversity
390 of M1. Haplogroup M1 has neither been observed in the comparably small Tunisian nor in the
391 ancient Moroccan data set, but was present in the ancient Egyptian mummy data.
392 Sequences belonging to the N subhaplogroups build subclades that are located at different
393 positions in the phylogenetic trees. All of them occur in the same phylogenetic branch, the
394 same holds true for the haplogroup R. In the R subclade, we only observe haplogroups R0a
395 (n=17) in North and East Africa, which previously has been described to be worldwide most
396 frequent in the Middle East and North and East Africa (46), and encounter haplogroup R
397 among the filtered sequences of an ancient Egyptian mummy once.
398 Nearly all T subclades are detected in North or East Africa, and their proportion is elevated in
399 both Moroccan and Egyptian ancient data sets. The presence of haplogroup T, as well as its

400 sister clade J, has previously been observed and considered to be of Near Eastern origin
401 (25,36).
402 North and East African haplogroup U mtDNAs cover all subclades (U1 to U8) except the rare
403 U9 clade. Haplogroup U6 has been studied in a targeted way (30,31,34) previously. Together
404 with M1, it is the only clade of a worldwide haplogroup that is autochthonous in Africa, i.e.,
405 predominantly occurring there. Corresponding U6 sequences from (30) and (31) integrated
406 into our data compilation as well as Egyptian and worldwide reference data support this
407 notion.
408 Finally, haplogroup X is present in data from all North and East African regions, including data
409 from three ancient individuals. The North and East African dataset contains 12 mtDNA
410 sequences belonging to the phylogenetically old subclade X1, which is largely restricted to
411 North Africa (47). The coalescence of X in the phylogenetic tree with 1000G and with HGDP,
412 which both do not contain X1 mtDNA sequences, differs between both references and from
413 the PhyloTree reference, involving N9b and R11 as sister clades, both of which are
414 haplogroups that are poorly represented in the 1000G and HGDP reference data. In order to
415 accurately resolve relationships between haplogroup X and other clades, more mtDNA
416 sequences will be needed.
417
418 **Clades occurring predominantly in North and East Africa are often poorly characterized**
419 We next investigated North and East African haplogroup profiles within the geographic
420 context using selected 1000G and HGDP populations from neighboring regions (Figure 8). A
421 corresponding histogram is shown in Supplementary Figure 1.

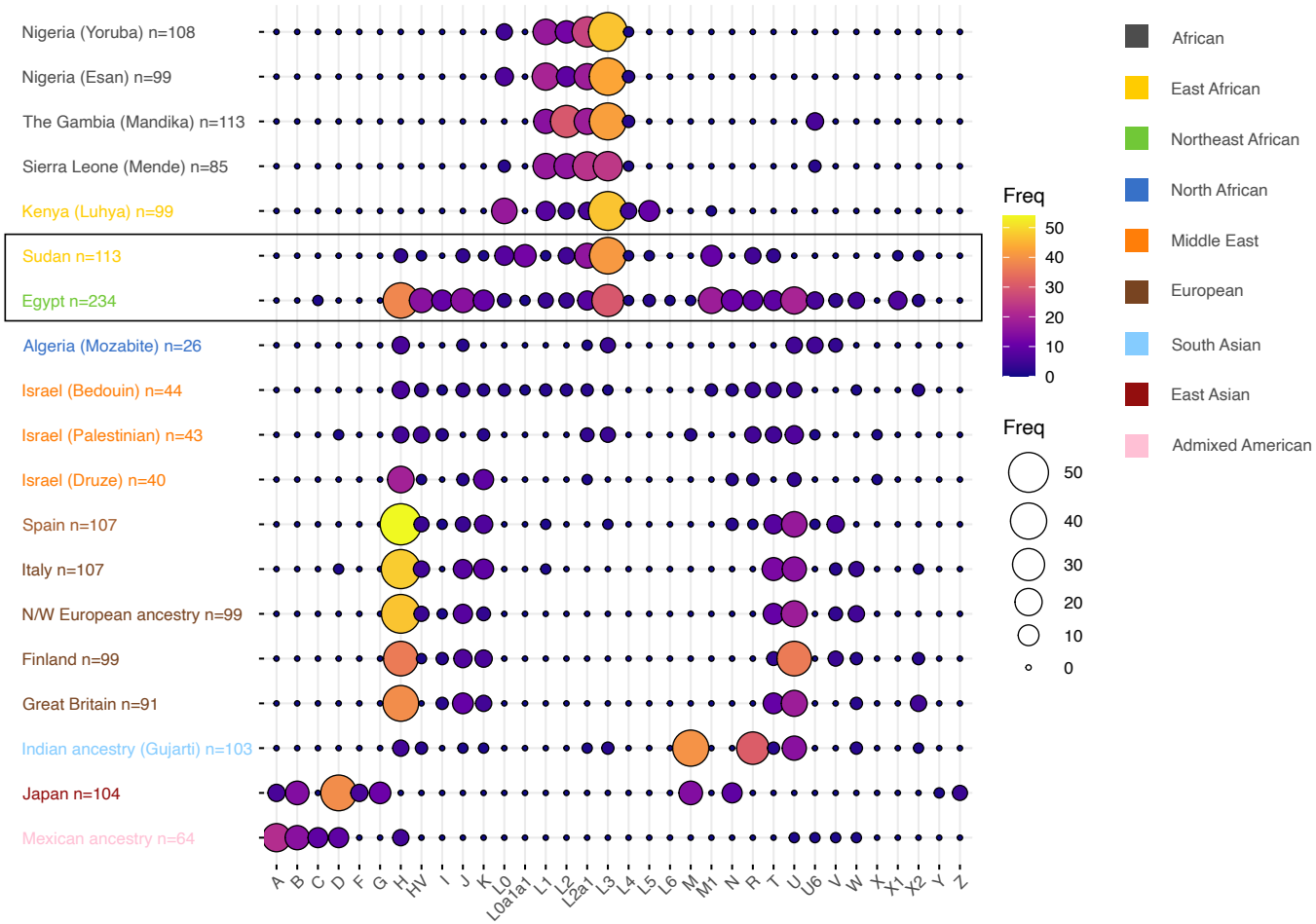


Figure 8: Haplogroup frequencies of selected 1000G and HGDP populations together with the Sudanese and Egyptian haplogroups of this work (highlighted in box). The populations are colored according to geographic region. Shown in the histogram are clades that are particularly relevant and/or prevalent in North or East Africa and discussed in this work., e.g. L0a1a1 and L2a1. Note that a sequence is counted only once, i.e., a sequence of haplogroup M1 is not counted towards haplogroup M.

425
426 This diagram shows that both Egyptian and Sudanese mitochondrial profiles are mediating
427 between the profiles of populations assessed by these public references, constituting a
428 genetic continuum between them. For example, 1000G covers African, European, South Asian,
429 East Asian and Admixed American populations, none of which represents mitochondrial
430 haplogroup occurrences observed in Sudan or Egypt according to data generated and
431 compiled in this work (cf. Figure 8). Middle Eastern and North African populations of the HGDP
432 reference show a combination of predominantly European, African and Asian haplogroups
433 comparable to what is seen in Sudan and Egypt. However, HGDP population sample sizes are
434 low and thus corresponding haplogroup distributions may not be representative.
435 Further, some haplogroups from the North and East African region are absent from both major
436 global references 1000G and HGDP. This applies especially to specific haplogroups that
437 exclusively or predominantly occur in North and/or East Africa, e.g. subclades of L0a1a1, L3,
438 L4, L6, M1, U6 and X1. Some of them, with a particular interest to worldwide human
439 prehistoric migration routes, have explicitly been addressed by studies and selectively been
440 sequenced before, e.g., L3, M1 and U6 and are included in our study. However, local clades
441 possibly not related to archaeogenetic hypothesis are more poorly characterized, e.g., L0a1a1.
442 Finally, there is little information on rare haplogroups such as L6. They differ a lot from all
443 other, well characterized haplogroups and as such may convey different mitochondrial
444 function and disease susceptibility. Thus, also and especially such rare haplogroups will need
445 better characterization in the context of mitochondrial genetics-based precision medicine.
446
447 **Variation suggesting novel haplogroups**
448 To obtain a dataset of exceptional high-quality, we next selected (n=146) samples with high
449 sequencing coverage and without predicted contamination (provided in Suppl. Table 12). The
450 phylogenetic tree of these sequences with corresponding haplogroups is depicted in Figure 9.

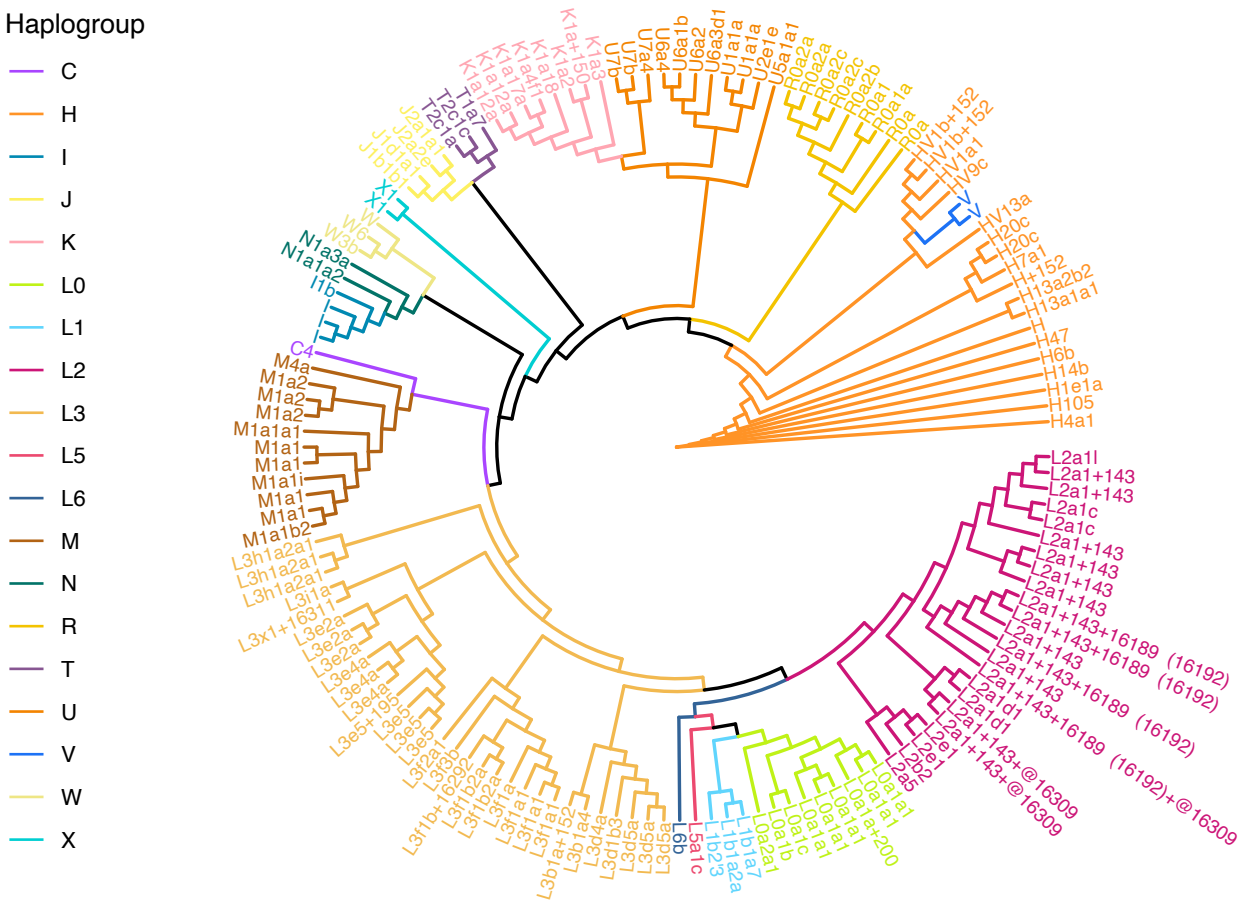


Figure 9: Phylogenetic tree of the 146 exceptional high-quality sequences used for detection of novel haplogroups. This tree was computed by a Neighbor Joining-based tree. The circular tree depicts the topological information, while branch lengths are not preserved. An alternative visualization preserving branch lengths is shown in Supplementary Figure 2.

In the search for novel and previously undefined subclades, we investigated this data set using the phantom mutation module of the online haplogrep2 tool. In this module, sequences are checked for recurrent variants that are not haplogroup-defining and that have a 'Soares score' of less than or equal to 2, i.e., having been detected no more than twice in a large collection of mitochondrial sequences (according to Table S3 of Soares et al. (48)). We checked for such variants occurring in samples of the same haplogroup to ensure that they were likely specific to haplogroup carriers and defined a novel, previously undefined subclade. A summary is provided in Table 1.

465

Haplogroup	Subhaplogroup variant	# HQ	# LQ	Soares score
HV1b+152	8640T; 4590G	2	1	0; 0
I	567C	3	0	0
I	567C; 4772C	2	3	0; 2
L0a1a1	8869G	2	1	1
L0a1a1	8017T	3	9	0
L2a1c	5186G; 9116C	2	0	2; 2
L2a1+143	7897A; 398C; 9389G	2	0	1; 2; 2
L2e1	13356C	2	0	0
L3d5a	15457A; 14410A	2	1	0; 1
L3e4a	470G; 9455G	2	2	0; 1
L3e5	711C	2	2	0
L3f1a1	12441C; 736T; 328G;15151G; 13967T; 14554G	3	3	0; 0; 0; 1; 2; 2
M1a2	16182G; 2858G; 15401G; 1282A	2	0	1; 1; 1; 1
R0a2c	5981C	2	1	0
V	9126C	2	1	0

466 **Table 1**: Haplogroups having potentially novel and previously undescribed sub-haplogroups. The table columns denote, in
467 this order, haplogroup, recurrent novel variants, the number of samples after strict filtering (# HQ; out of n=146) and after
468 lenient filtering (#LQ; out of n=641-146=495) and the Soares score (48), which represents the previous occurrence of the
469 variant in worldwide data sets.

470

471 For ten putatively novel haplogroups, we find further support among samples with lower
472 sequencing coverage. In seven cases, we find one novel, recurrent variant in at least two
473 individuals of the same haplogroup, for example variant 8017T in overall 12 carriers of L0a1a1.
474 In eight cases, more than one recurrent variant was detected within carriers of the same
475 haplogroup, e.g. six carriers of L3f1a1 share six novel variants (12441C; 736T; 328G;15151G;
476 13967T; 14554G). Thereby, 14 of 30 potential novel haplogroup variants have a Soares score
477 of 0 (47%), 9 have a score of 1 (30%) and the remaining 7 have a score of 2 (23%). The
478 haplogroups of which potential novel subhaplogroups have been identified are either
479 haplogroups that are largely restricted to North and/or East Africa, e.g., L2a1, M1, and
480 different L3 clades (d, e and f), or are potential local clades diverging from basic haplogroups,
481 e.g., I and V.

482

483 **Variation suggesting revision of phylogenetic relationships**

484 For the 146 exceptional high-quality sequences, we examined the phylogenetic tree according
485 to the PhyloTree reference to search for potential new haplogroups using a function from the
486 web-based haplogrep2 tool (see Supplementary Figure 3). We manually investigated all
487 variants present in the mtDNA sequence as well as all variants expected for the haplogroup
488 assigned, but found to be missing in the sequence. We found two clades, L0a1a1 and L2a1,
489 which were better represented, when revising or extending the phylogenetic tree provided by
490 PhyloTree (Figure 10).

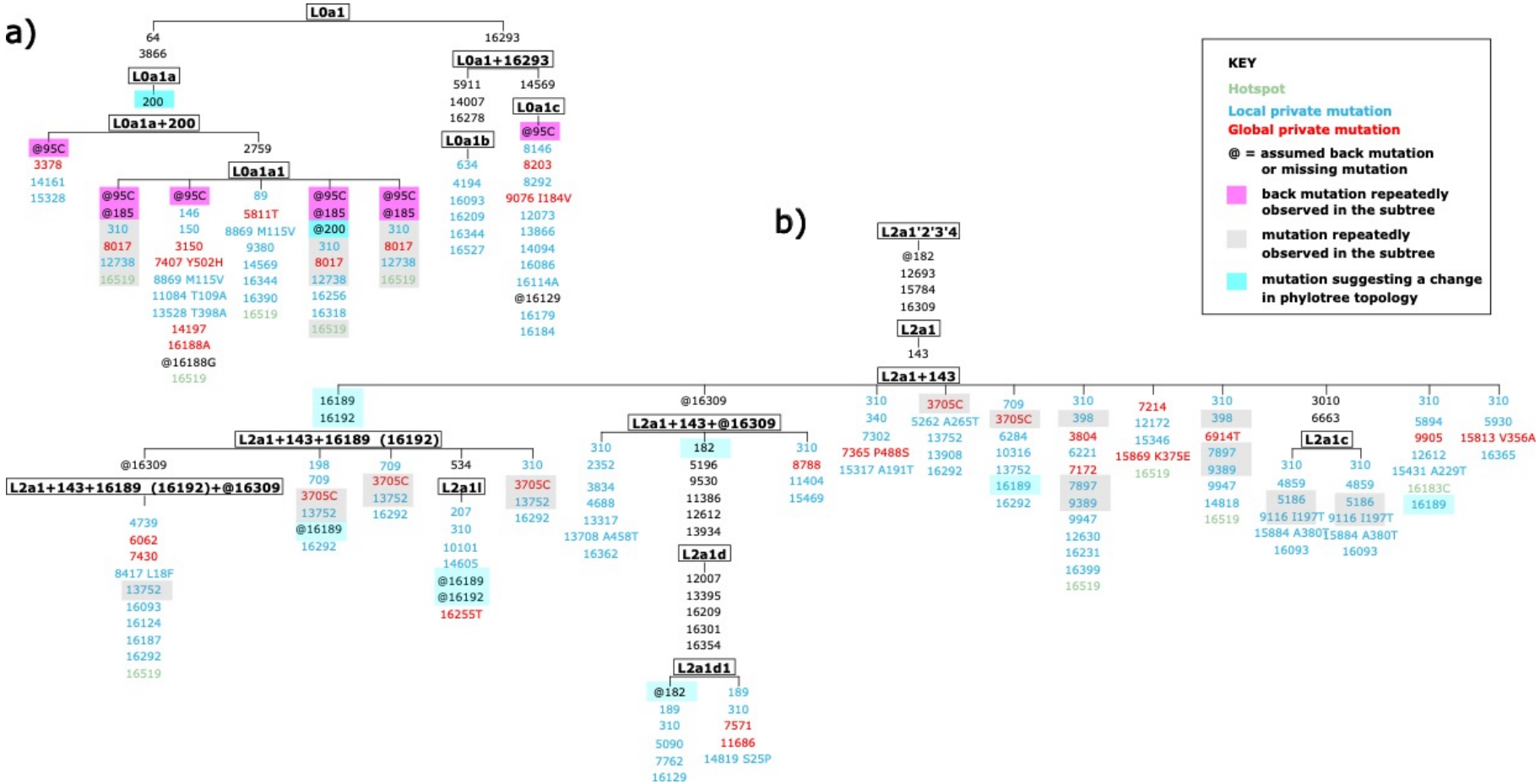


Figure 10: Phylogenetic tree according to the PhyloTree reference for those samples that were assigned to subclades a) L0a1 and b) L2a1. We highlight with magenta, gray and turquoise boxes those variants that indicate that a different phylogenetic tree may better explain the samples from North and East Africa, see legend in Figure a). Variants denoted for the leaves of the tree, i.e. samples, denote variant differences w.r.t. the haplogroup assigned. Shared back mutations (magenta) denote that parental haplotypes may be missing in PhyloTree. Mutations repeatedly observed in a subtree (gray) suggest that child haplogroups are missing. Variants that occur in multiple samples and differ from variants defining a parental haplogroup (turquoise) suggest that different variant combinations and haplogroup specifications may better explain North and East African mtDNA sequences.

There are eight samples assigned to L0a1 sub-haplogroups. In most of them, the haplogroup-defining variant 95C is not observed, which is already noted within the PhyloTree reference. Further, three samples show a back mutation at position 185. Such a back mutation is expected for haplogroup L0a2 according to PhyloTree, so meaning the samples we sequenced may link clades L0a1 and L0a2. In one sample, an expected mutation at position 200 was not observed, showing the possibility that 95C and 185 together with 310, 8017, 12738 and possibly 16519 are a hitherto undescribed subclade of L0a1.

The second branch of interest is L2a1. Out of 19 samples to which sub-haplogroups of L2a1 have been assigned, most showed extensive, recurrent deviations from PhyloTree (Figure 10 b). For example, resolving the haplogroup variants 16189 and 16192 within the PhyloTree reference seems not representative for North and East African samples. Further, variants 3705C, 13752, 7897, 9389, 5186 and 9116, which are recurrent in the L2a1 clade, are not represented in sequences appearing in PhyloTree, yet. However, if included, they would implicate changes in phylogeny.

Conclusion

We investigated whether current global mitochondrial genetic data adequately represents the genetic mitochondrial variation of the North and East African regions. Genetic data from this region are sparse until now, despite an apparent interest due to autochthonous ancestry components being identified (22,49) and despite the importance of the region as origin or transit of modern humans outside of Africa. Besides an in-depth analysis of mitochondrial sequence data underlying Egyptian haplogroup distributions reported previously (22), we have generated novel sequence data of mitochondrial genomes from Sudan, another region with few genetic data available. We characterized North and East African mitochondrial DNA variation by combining these data with publicly available sequence data as well as with mitochondrial DNA sequences from Genbank relating to 10 other studies. This work has resulted, to the best of our knowledge, in the largest mitochondrial data collection from this region to date.

529 With a stringent quality control via the novel tool haplocheck, we related North and East
530 African phylogenetics to the worldwide context by restricting the analysis to mitochondrial
531 sequences unaffected by potential contamination or other shortcomings such as insufficient
532 sequencing coverage. An additional filter for a minimal 1000x sequencing coverage results in
533 exceptionally high-quality mitochondrial sequences, which makes us very confident that novel
534 mitochondrial variations, novel haplogroups and observed PhyloTree inconsistencies are
535 indeed caused by a hitherto insufficient genetic representation of this geographic region. Thus
536 this work complements recent efforts to improve mitochondrial reference data (50,51).
537 We find that North and East African mitochondrial genomes are phylogenetically diverse and
538 cover many of the major haplogroups seen in global populations. Likewise, specific subclades
539 are prevalent in the region, supporting that North and East Africa, and particularly Northeast
540 Africa harbors ancestry components typically attributed to Africa, Europe and Asia (22) , all of
541 which are likely present in the region since prehistoric times (52). Although our study focused
542 on assessing the state of mtDNA reference data for precision medicine, the combined data
543 allowed to rediscover geographic differences in genetic ancestry that many earlier studies
544 related to prehistorical and historical conditions and events (53), e.g. migration between Egypt
545 and Sudan along the Nile river (54). Further, several North or East African clade
546 representatives highlighted by our work and not studied in-depth so far may still add further
547 information to worldwide maternal human genetic history (e.g., the haplogroups L4, L6, N, I
548 and X).
549 In conclusion, the initial North and East African mitochondrial reference dataset compiled as
550 part of our work calls for further extension in order to support genetics-based precision
551 medicine.
552
553
554 **Material and Methods**
555
556 **Haplogroup data compilation for worldwide mitochondrial haplogroup distribution**
557 Based on a comprehensive literature research, n=7,414 publicly available mitochondrial
558 (mt)DNA sequences with available haplogroup and geographic information in the form of the
559 country of origin were collected. Additionally, data from two reference datasets, the 1000
560 Genomes dataset (19) (n=2,504) and the Human Genome Diversity Project (28) (n=828) were

561 added, resulting in a total number of n=10,746 entries. As quality assurance, for all database
562 entries haplogroups were reassigned using for mitochondrial sequences haplogrep2 (10) and
563 for next generation sequencing-based cohorts haplocheck (26). Subsequently, entries were
564 filtered such that only those remained that were representative for the population of a region.
565 Towards this, studies analyzing only specific haplogroups as well as data from archaeological
566 studies was removed. Eventually, 9,264 entries were retained for further analysis.
567
568 **Objective geographic grouping for worldwide mitochondrial haplogroup distributions**
569 For determining the relative amount of haplogroups per geographic region, we grouped the
570 database entries into clusters of minimal geographic distance and having a size of at least 100
571 entries. At the beginning, only countries with less than 100 entries were considered, i.e., those
572 that could not form a group on their own. Countries with an initial number less than 100 were
573 assigned to group 1, all other countries to group 2. In order to classify a country from group
574 1, an attempt was made to combine it in a first step with other countries or already connected
575 groups of countries in group 1. A continent-dependent maximum distance was set as a
576 termination criterion. For Europe, this was set to 1,000 km, for Africa to 1,750 km, for the
577 Middle East to 1,600 km, for Asia to 1,900 km, for the Americas to 2,600 km and for Oceania
578 to 1,600 km. This distance was determined as a function of the average area of a country on
579 the continent and the existing sample density. If after this first step, the sample number of a
580 country or a group was still less than 100, the countries with an initial sample number greater
581 than or equal to 100 were included as possible connection partners. In this second step, the
582 country or group to be classified was in any case merged with the country from group 1 or
583 group 2 with minimal distance.
584
585 **Mitochondrial DNA short-read sequencing**
586 Sudanese samples were acquired under approval of the Central Institution Review Board, Al-
587 Neelain University, Sudan, IRB Serial Number NU-IRB-17-7-7-91. All subjects gave written
588 informed consent in accordance with the Declaration of Helsinki. Genomic DNA samples were
589 processed for library preparation, using the Human mtDNA Genome protocol for Illumina
590 Sequencing Platform (https://emea.support.illumina.com/content/dam/illumina-
591 support/documents/documentation/chemistry_documentation/samplepreps_legacy/huma
592 n-mtdna-genome-guide-15037958-01.pdf) as described previously (22,55) for Egyptian

593 samples and 63 Sudanese samples. In brief, a primer set [MTL-F1
594 (AAAGCACATACCAAGGCCAC) and MTL-R1 (TTGGCTCTCCTTGCAAAGTT); MTL-F2
595 (TATCCGCCATCCCATACATT) and MTL-R2 (AATGTTGAGCCGTAGATGCC)] was used to amplify
596 the mtDNA by long-range PCR (LR-PCR). Library preparation of the LR-PCR products was
597 performed using a Nextera XT DNA Library Preparation Kit (Illumina Inc., CA, USA). For 96
598 Sudanese samples, we applied a slightly modified protocol to improve the evenness of the
599 coverage. More specifically, the LR-PCR was conducted using another primer set [hMT-1_F
600 (AAATCTTACCCCGCCTGTTT) and hMT-1_R (AATTAGGCTGTGGGTGGTTG); hMT-2_F
601 (GCCATACTAGTCTTTGCCGC) and hMT-2_R (GGCAGGTCAATTTCACTGGT)] and the library
602 preparation of the LR-PCR products of hMT1 and hMT2 primers was conducted using DNA
603 Prep Tagmentation (Illumina Inc.). This approach allows the fragment size of the tagmented
604 LR-PCR products to be evenly 300 bp, as the transposome reaction occurs every 300 bp on the
605 beads, in contrast to the Nextera XT DNA library preparation Kit, by which transposome
606 reaction occurs randomly, thus, results in various size of the tagmented LR-PCR products (i.e.,
607 larger fragments were removed by beads purification steps). The libraries prepared by both
608 methods were further purified, concentration quantified using Qubit Fluorometer (Thermo
609 Fisher GmbH, Dreieich, Germany) and the library size was determined by 2100 Bioanalyzer
610 Instrument (Agilent, Santa Clara, CA, USA). The final library was sequenced on the Illumina
611 MiSeq sequencing platform, using v2 chemistry (2 × 150 bp paired-end reads) (Illumina Inc.).
612

613 **Mitochondrial DNA long-read sequencing**

614 Genomic DNA (gDNA) from peripheral blood for the long-read sequencing was prepared using
615 Qiagen MagAttract HMW DNA kit (Qiagen, Hilden, Germany). Two GridION sequencing runs
616 were conducted using one GridION Flow Cell version R10.3 (FLO-MIN111; Oxford Nanopore
617 Technologies, Oxford, UK). Full-length mitochondrial DNA (16,595 bp) was amplified from
618 gDNA by long-range PCR using a set of primers tagged with universal sequences (show in
619 Italic): [US-hmt_F-2120
620 (*TTTCTGTTGGTGCTGATATTGC*GGACACTAGGAAAAAACCTTGTAGAGAGAG and US-hmt_R-2110
621 (*ACTTGCCTGTCGCTCTATCTTC*AAAGAGCTGTTCCTCTTTGGACTAACA)]. Fifty µl PCR reaction was
622 prepared with 200 ng template gDNA, 50 nM of each forward and reverse primers, 25 µl
623 LongAmp Hot Start Taq 2´ Master Mix (New England BioLabs, Frankfurt am Main, Germany).
624 The PCR program was 94°C for 1 min; 30 cycles of 98°C for 10 s, and 68°C for 16 min; 72°C for

625 10 min; hold at 4°C. The PCR products were purified using GeneJET PCR purification Kit
626 (Thermo Fisher Scientific GmbH, Dreireich, Germany). The purified PCR product were
627 barcoded by PCR using the primer sets, which were the same sequences as Nanopore PCR
628 Barcoding Expansion 1-96 (EXP-PBC096; Oxford Nanopore Technologies, UK), but were
629 commercially synthesized (Biomers.net, Germany). The barcoding PCR condition was 95°C for
630 3 min; 15 cycles of 95°C for 15 sec, 62°C for 15 sec, and 65°C for 16 min; 65°C for 16 min; hold
631 at 4°C.
632 The barcoded PCR products were further prepared for the DNA library using the Ligation
633 Sequencing Kit (SQK-LSK110; Oxford Nanopore, UK) according to the manufacturer's
634 instructions. In brief, the end-repair, dA-tailing, sequencing adapter ligation, and final
635 purification of DNA libraries were performed according to the manufacturer's instructions
636 using NEBNext Companion Module for Oxford Nanopore Technologies Ligation Sequencing
637 (New England BioLabs GmbH, Germany). The GridION Flow Cell was primed and the DNA
638 library was loaded according to the manufacturer's instructions. Both sequencing durations
639 were approximately 24 hours.
640
641 **Analysis of long-read MT sequencing data**
642 Basecalling was performed with guppy version 6.0.1+652ffd1 and flowcell option FLO-
643 MIN111, sequencing kit option SQK-LSK110 and barcode kit option EXP-PBC096, which
644 defaults to guppy config file dna_r10.3_450bps_hac.cfg and model file
645 template_r10.3_450bps_hac.jsn, i.e. using high accuracy base calling. Reads with a Q-score
646 larger 10 were considered pass reads and kept for further analysis. PycoQC (56) was run for
647 quality control and mapping performed with minimap2 against GRCh38, which includes rCRS.
648 Haplogroups were assigned based on this BAM file using the haplocheck tool within mitoVerse
649 (https://mitoverse.i-med.ac.at).
650
651 **Sudanese and Egyptian mitochondrial sequencing characteristics and QC**
652 Running FastQC on all Sudanese and Egyptian mitochondrial sequencing FASTQ files resulted
653 in no systematic, data quality compromising warnings or errors. Sudanese sequencing data
654 has an average length of 140 bases, Egyptian sequencing comprises samples sequenced with
655 150 and samples sequenced with 300 bp target read length, resulting in an average length of
656 205 bases across the cohort. Average bases mapped per sample are about 18 Mb for the

657 Sudanese cohort and about 30 Mb for the Egyptian cohort, amounting to an approximate
658 average coverage of more than 1000X. In the Sudanese cohort, five samples have less than
659 1000 reads and are considered dropouts. The Egyptian cohort does not contain samples with
660 less than 1000 reads.
661
662 **Variant calling, haplogroup assignment and contamination detection**
663 Raw reads from FASTQ files of Sudanese and Egyptian sequencing data were mapped to
664 mitochondrial sequence MT of GRCh38, which resembles the historic revised Cambridge
665 Reference Sequence (rCRS), a sequence of haplogroup H2a2a1. For samples with available
666 BAM files, we used these directly. Our analysis is largely based on haplocheck (version 1.1.2)
667 (26) and its internal accompanying tools haplogrep2 (10) and mutserve (version 1.3.4) as
668 implemented via the mtDNA-server (57). Variant caller mutserv was run with default settings,
669 which amounts to reporting heteroplasmic variants at a level larger than 1% (--level 0.01),
670 calling deletions (--deletions), base alignment quality turned off (--noBaq), considering a
671 mapping quality of 20 (--mapQ 20) and a base quality of 20 (--basQ).
672
673 **Phylogenetic analyses**
674 In order to obtain a FASTA format mitochondrial sequence that relates to the haplogrep2
675 output reported by haplocheck, we perform variant calling with mutserv using the default
676 parameters also used within haplocheck. Then we select from the VCF those variants for which
677 the major base is the non-reference base. Using bcftools consensus, we then convert the VCF
678 file into FASTA. Note that mutserv does not call insertions, only deletions which are denoted
679 with a gap symbol (within FASTA, it is "-"), thus ensuring that the sequence length is always
680 exactly the length of rCRS, i.e. 16,569 bases, and that sequences align correctly without re-
681 alignment. The same holds for the Genbank files processed by McInerny et al. (29). Thus, we
682 can simply combine FASTA files of cohorts by pasting them into one combined FASTA file. The
683 combined FASTA file was then loaded into Jalview (58) where a Neighbour Joining-based tree
684 was computed and exported into Newick format. This tree was then visualized with ggtree
685 (59) using various graphical settings.
686
687 **Ethics statement**

688 This study was approved by the Central Institution Review Board, Al-Neelain University,
689 Sudan, IRB Serial Number NU-IRB-17-7-7-91. All subjects gave written informed consent in
690 accordance with the Declaration of Helsinki.
691
692 **Data availability**
693 The public data compiled as well as the corresponding publications and repository IDs are
694 provided in Supplementary Table 1. Sudanese raw sequencing data generated as part of this
695 study is available from the European Genome Phenome Archive (EGA) under study ID
696 EGAD00001008315. The analysis workflow used to run haplocheck, perform variant calling
697 and generate and compile mitochondrial sequences is available at
698 https://github.com/iwohlers/2020_mt_analyses.
699
700
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708
709
710 **Conflict of interest**
711 The authors have declared no conflicting interests.
712
713
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