AN ANALYSIS OF RODENT RESERVOIRS OF PLAGUE DURING THE SECOND PANDEMIC: SHIFTING FOCUS TOWARDS A MULTI-HOST AND MULTI-MECHANISM MODEL OF PLAGUE MAINTENANCE AND REINTRODUCTION IN THE PAST

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TITLE: An Analysis of Rodent Reservoirs of Plague During the Second Pandemic: Shifting Focus Towards a Multi-Host and Multi-Mechanism Model of Plague Maintenance and Reintroduction in the Past

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Abstract

The second pandemic persisted for 500 years throughout Europe and some regions of Africa and Asia and far surpasses the first and third pandemics in terms of temporal and spatial breadth. It is still unclear what mechanisms facilitated the long-term maintenance and continued disease reintroductions experienced during the second pandemic. Many researchers believe it was the spread of plague infected *Rattus* spp. via trade routes, while others are beginning to support a model that reflects modern plague ecology such that plague was maintained in local rodent reservoirs in the Mediterranean region or Europe following its 14th century introduction.

This study accumulated a list of rodent species known to host plague and analyzed host characteristics to determine what traits allow rodents to function as successful reservoir species. In addition, this study analyzed the ancient DNA within rodent remains in search of *Yersinia pestis* to add to the history of the pathogen.

The results of this study demonstrate that there are 45 non-*Rattus* rodent species known to host plague within a modern context. Although ancient *Y. pestis* DNA was not identified from zooarchaeological remains within this study, it is clear that rodent reservoirs were key players in past plague pandemics. When viewed through the lens of a single model (*i.e*. trade routes or human-ectoparasites), several questions about past plague maintenance remain unanswered. This study shows that a more complex composite model is best fit to describe the 500-year reign of the second pandemic and the quiescent periods that extended between the cyclical disease reintroductions.

This study presents the first comprehensive, interactive, and publicly available online database of rodent reservoirs to aid in future research and emphasizes the need to investigate a multi-mechanism model of plague maintenance and reintroduction in the past. With this research we facilitated a starting point for future studies, expanded the current knowledge on rodent reservoirs of plague, and contributed a valuable discussion to the field of plague studies on regions that have thus far gone underserved in plague studies, such as the Mediterranean littoral.

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List of Abbreviations

aDNA – Ancient DNA

BLAST – Basic Local Alignment Search Tool

BWA – Burrow-Wheeler Aligner (mapping software)

DNA – Deoxyribonucleic acid

GuHCL – Guanidinium hydrochloride

MQ – Mapping quality

NCBI – National Center for Biotechnology Information

PCR – Polymerase chain reaction

Pla – Plasminogen activator gene

qPCR – Quantitative polymerase chain reaction

spp. – Multiple species of the same genus

Declaration of Academic Achievement

I, Madeline Tapson, declare that this thesis titled "An Analysis of Rodent Reservoirs of Plague During the Second Pandemic: Shifting Focus Towards a Multi-Host and Multi-Mechanism Model of Plague Maintenance and Reintroduction in the Past" and the work presented in it are my own. I conducted all data collection for the database of this thesis and analysis of the data therein. Sample material was provided by Dr. Vedat Onar (Istanbul University) and Professor Guy Bar-Oz (University of Haifa, Israel). I participated in all lab work with assistance from Dr. Jennifer Klunk and performed bioinformatic analyses with assistance from Dr. Ana Duggan. The plan and structure for this thesis was discussed with my committee, Dr. Hendrik Poinar and Dr. Dan Salkeld. I wrote the initial draft for all chapters of this thesis with subsequent contributions from Dr. Hendrik Poinar, Dr. Dan Salkeld, Dr. Ann Carmichael, and Dr. Nukhet Varlık.

Chapter 1.0: Introduction and Background

1.1 Introduction

The path that the plague followed can be traced historically throughout Asia, Africa and Europe spanning from the 6th to the 20th century in three distinct pandemics. The first pandemic began with the Plague of Justinian between 541-544 AD that marked the spread of plague from Asia, through the Mediterranean, and into Europe, with rough estimates that it killed anywhere from 15-40% of the populations in its path (Perry & Fetherston 1997; Wagner *et al* 2014; Mordechai & Eisenberg 2019). Subsequent outbreaks of plague during the first pandemic continued in Africa, Asia, and Europe until the 8th century where the disease seemingly disappeared for several centuries before being reintroduced in the second pandemic (Bramanti *et al.* 2016; Wagner *et al.* 2014; Spyrou 2019).

The Black Death (A.D. 1347 to 1353) marks the onset of one of the most devastating disease pandemics known to date, the second pandemic of plague, which killed anywhere from 40-60% of the population where it travelled (Benedictow 2004; Green 2016). The second pandemic reigned over Africa, Asia and Europe for 500 years with cyclical outbreaks occurring approximately every 20 years into the 19th century (Perry and Fetherston 1997; Benedictow 2004). The second pandemic represents a very perplexing period of plague history as it is still unclear how it managed to persist for much longer and potentially have a more significant demographic impact than its predecessor, the first pandemic (Perry and Fetherston 1997; Wagner *et al* 2014).

A question that many plague researchers have asked, and one that will be addressed throughout this study, is through what mechanisms was the plague able to be reintroduced into human populations and subsequently maintained over such long periods of time during the second pandemic? There is much speculation surrounding this question, with the most prominent theories revolving around the repeated reintroduction of plague infected rodents into Europe along trade routes from regions to the east (Schmid *et al*. 2015; Yue *et al.* 2016; Yue *et al.* 2017). However, there is no reason to believe that the second pandemic operated under different mechanisms than its successor, the third pandemic, where plague was established in rodent reservoirs after its introduction in the 19th century and persists into the present within natural foci (WHO 2016). This study will investigate potential rodent species as candidate reservoir hosts for *Yersinia pestis*, the etiologic agent of plague (Perry & Fetherston 1997), during the second pandemic. From this, we can infer patterns of plague persistence and reintroduction into human populations to elucidate how the disease could be maintained over its 500-year time span.

1.2 Research Questions and Objectives

The primary objective of this study was to build an extensive database of potential rodent plague reservoirs in the Mediterranean littoral – specifically in the regions of northern Africa, southeastern Europe, and western Asia (Fig. 1.1) In doing so, the current research presents a theory of plague maintenance and reintroduction – supported by various scholars (Bos *et al.* 2016; Carmichael 2016; Green 2016; Seifert *et al.* 2016) – that features local rodent reservoirs as long-term hosts of disease that periodically reintroduce the plague into human populations. This research used multiple sources

stemming from historical, ecological, and anthropological accounts to study rodent species that have the ability to host plague today and assess their potential role in long term plague maintenance in the past. I then compared this rodent based model of plague maintenance against the other leading theories for plague persistence or re-occurrence in Europe – including the 'trade route' model of plague dissemination and the 'human-ectoparasite' model of plague transmission. This accumulation of data and analysis has been presented and discussed in Chapter Two.

The second objective of this study was to locate, acquire, and screen the remains of potential animal reservoirs from the Byzantine period (4th-15th century) for the presence of *Y. pestis*. In total, 72 rodent samples were obtained from Istanbul, Turkey (42) and Haifa, Israel (30). In order to assess these samples for the presence of *Y. pestis* I employed a protocol adapted by the McMaster Ancient DNA Centre for use on aDNA and utilized various bioinformatic tools to analyze the sequenced data, which will be further discussed in the Laboratory Methods and Results section in Chapter Three.

1.3 Sylvatic Plague

Plague has shaped human history and devastated human populations in several world regions and continues to have serious ramifications for humans today, particularly in Madagascar (Richard *et al.* 2015; Vogler *et al.* 2017; Andrianaivoarimanana *et al.* 2019). It is, however, primarily a disease of rodents and their associated fleas that can – under the right conditions – jump to humans (Morelli *et al.* 2010). Understanding the plague cycle within rodent populations can therefore provide valuable insight into how

the disease was able to continually spill over into human populations during each cyclical outbreak of the second pandemic.

In addition to simply being infected with plague, a number of modern studies have demonstrated the ability of certain rodent species to maintain a level of resistance to the infection, thus allowing them to harbor the disease over long periods of time. For example, a study carried out by Smith *et al*. (2010) in the United States involving 28 years of rodent surveillance showed high seroprevalence and prolonged persistence of *Y. pestis* antibodies in several species of chipmunks. These results demonstrate that certain rodents infected with plague are able to survive over at least a two-year period (Smith *et al.* 2010).

Furthermore, studies such as Salkeld *et al.* (2010) and Stapp *et al.* (2009) have shown that interactions between certain species, such as prairie dogs (*Cynomys* spp.) and grasshopper mice (*Onychomys leucogaster*) in the United States, results in the increased spread of plague thus demonstrating the complex role of rodent reservoirs in maintaining and redistributing the bacterium. Studies such as these demonstrate the ability of rodents to act as both short-term hosts and long-term reservoirs of plague and indicate the need for further research into the possibility of such species existing during the second pandemic.

1.4 Mechanisms of Plague Maintenance and Reintroduction

As we know that plague is a rodent- and flea-borne disease and that certain rodents – particularly rats – were commonly found on ships, it is easily extrapolated that infected rodents effectively “hitch-hiked” their way from Asia into Europe and Africa on

trade ships. This model of plague transmission is consistent with the worldwide distribution pattern of the black rat (*Rattus rattus*), the rodent most notoriously associated with the plague (Shiels 2018). However, it is certain that *Rattus* spp. are not the only rodents associated with plague as it has been documented in several studies that many other rodent species are able to act as plague reservoirs today (Salkeld *et al.* 2010; Smith *et al.* 2010; Malek *et al.* 2015).

Studies such as Schmid *et al.* (2015) and Yue *et al.* (2016 & 2017) have taken historical and ecological approaches to negate the involvement of long-term rodent reservoirs in the Mediterranean region and Europe in the past. To do this, they evaluate climate patterns that could account for repeated waves of plague out of Asia and study the proportion of plague in rural versus urban settings during the second pandemic, respectively. Similarly, Hufthammer & Walløe (2013) have taken a historical approach to demonstrate the lack of rodent reservoirs in the archaeological record, while Dean *et al.* (2018) have used statistical modeling to argue that human ectoparasites, instead of rodent fleas, better account for second pandemic mortality curves. However, as discussed further in Chapter Two, these theories do not prove conclusively that rodent reservoirs were unable to persist in the past and as such this study calls for deeper investigation into a multi-mechanism model of plague reintroduction that includes a rodent reservoir component.

1.4.1 Ancient DNA and Plague Reservoirs

As *Y. pestis* is a bloodborne pathogen, upon infection it enters the bloodstream of the host and leaves behind traces of its DNA that can be located using specific techniques

adapted to extract and analyze DNA from ancient bones, teeth, and tissue of infected individuals. It is through the analysis of ancient skeletal material of plague victims that recent genomic studies have been able to evaluate the evolutionary relationships between strains of *Y. pestis* from the 18th century plague outbreak in Marseille, France to demonstrate that after the introduction of the bacterium into Europe, distinct strains diverged and evolved independently in different regions (Bos *et al*. 2016; Seifert *et al.* 2016; Spyrou 2016; Spyrou 2019). This data demonstrates that there must have been local persistence of the bacteria during the second pandemic, which supports the theory that it was circulating within wildlife reservoirs (*i.e.* rodent populations) until spillover events into human populations occurred (Bos *et al*. 2016).

Expanding aDNA plague research to include the hunt for such rodent reservoirs would add a tremendous amount of answers to how plague cycles functioned in the past. As of yet, there have been no published studies searching for or identifying *Y. pestis* in ancient rodents. This gap in plague scholarship is likely due, in part, to the limited understanding of what rodent species could have been acting as reservoirs in the past. It is in this vein that I explore rodent reservoirs in greater depth in this thesis by presenting both the first known attempt to identify plague in ancient rodent remains via aDNA techniques, discussed in Chapter Three, as well as the first comprehensive and interactive database of rodent reservoirs to aid in future aDNA investigations.

1.4 Second Pandemic Plague in the Mediterranean Littoral

Although *Y. pestis* spread throughout Europe and several regions of Asia and Africa, the majority of plague research has been Eurocentrically focused (*i.e.* England,

France, and Italy), thus reinforcing colonialist "othering" by neglecting regions of perceived less importance (Varlık 2016). Evaluating the plague experience in these underserved regions would fill a substantial gap in plague scholarship as it would allow us to increase our understanding of the plague experience outside of western Europe and give a voice to the supposed 'origin locations' of plague reintroduction.

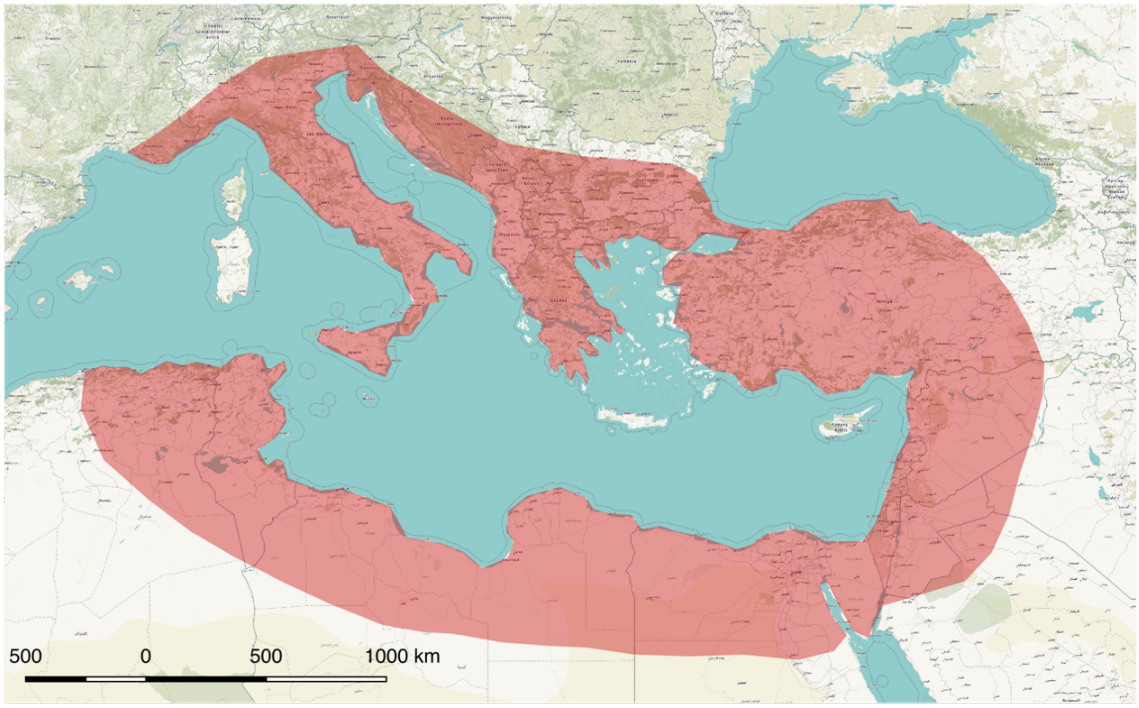

Figure 1.1: Map showing an approximated representation of the Mediterranean littoral (*i.e.* regions in north Africa, western Asia, and southeast Europe) shaded in red.

Specifically, the Mediterranean littoral (Fig. 1.1) represents an under researched yet key intermediary region in the pathway of past plague where rodent reservoirs capable of hosting plague still persist today in natural foci following plague's third pandemic introduction (WHO 2016).

During the 500-year time span of the second pandemic, several regions in the Mediterranean – such as Constantinople (Istanbul), Salonica, Cairo, Damascus, etc. –

experienced repeated outbreaks of plague (Varlık 2016). Additionally, the Mediterranean Sea connected trade routes throughout Afro-Eurasia, which could have facilitated the spread of plague-infected rodents and people between geographic regions according to the 'trade route' model of plague reintroduction (Benedictow 2013; Green 2018). Although such trade routes have led to suggestions that the continued reintroduction of *Y. pestis* originated from port cities, such as those in the Mediterranean (*e.g.*, Constantinople (Istanbul), Smyrna (Izmir), and Alexandria), little research has focused on the plague experience there (Varlık 2016).

The limited historical attention to plague outside of Europe has thus led to the exclusion of several Mediterranean and southwest Asian regions (*i.e*. North Africa, Syria, Iran, etc.) in modern aDNA analysis in terms of the level and quality of research devoted to plague as opposed to that conducted on western Europe. Focusing only on certain areas of plague outbreak at a time when the disease had spread throughout Asia, Africa, and the Middle East in addition to Europe severely limits the scope of plague studies and skews the history of the pathogen. Without sufficient historical analysis on plague in the Mediterranean we will not be able to accurately infer the spatial and temporal path followed by plague or the complete evolutionary history of *Y. pestis*. Future research on the history and effects of plague in the Mediterranean during the second pandemic will elucidate the mechanisms of plague transmission and maintenance that allowed for this pandemic to persist over such a prolonged time period.

1.5 Sandwich Thesis Structure

The following thesis is broken down into three chapters. Chapter Two lists the paper prepared for submission, entitled: *A rodent reservoir-based model as the main driving force behind long-term persistence and reintroduction of plague during the second pandemic*. This paper will discuss mechanisms of plague maintenance and reintroduction during the second pandemic by analyzing what we know today about rodent reservoirs and extrapolating this data into the past. In addition, Chapter Two will include supplementary information submitted along with the paper such as a comprehensive excel database of the rodent reservoirs discussed as well as an interactive online database, titles *Rodent Reservoirs of Plague: Modern Distributions and Host Characteristics,* detailing the geographic spread of these reservoirs and their associated key traits. Chapter Three provides a discussion of aDNA laboratory methods and bioinformatic analyses employed to assess relevant samples for *Y. pestis* DNA, the results obtained, as well as a greater discussion on the reality and significance of negative results in the realm of aDNA research. Chapter Four summarizes the key points of the thesis and provides areas for future research.

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Chapter 2.0

A rodent reservoir-based model as the main driving force behind long-term persistence and reintroduction of plague during the second pandemic

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2.1 Abstract

The second pandemic devastated human populations in Europe and regions of Africa and Asia from the 14th-19th century. As of yet, there is no clear consensus on how this pandemic was able to persist for approximately 500 years. While many researchers argue for a 'trade route' model of continued reintroduction, recent genomic studies strongly suggest that plague was maintained after being introduced during the Black Death. As such, rodent reservoirs were almost certainly involved, acting to maintain and locally reintroduce the disease to nearby humans. We present a database of potential rodent reservoirs known to be capable of hosting plague today and evaluate their host characteristics. In addition, we demonstrate that the current 'trade route' or 'human-ectoparasite' models of plague maintenance and transmission do not conclusively eliminate the role of rodent reservoirs in the past and do not align with recent genomic evidence supporting a local persistence theory. Our analyses ultimately demonstrate that a composite model including rodent reservoirs is more likely to account for the repeated outbreaks experienced during the second pandemic and the intermittent quiescent periods that extended in between.

2.2 The Second Pandemic

The plague, caused by *Yersinia pestis*, is responsible for several disease outbreaks that devastated human populations both in terms of spatial and temporal breadth as well as overall impact on human population health. The history of plague is characterized by three distinct pandemics, the first of which began with the Plague of Justinian (541-543 AD) that marked its spread across north Africa and southwest Asia and into Europe and continued for some 200 years until the 8th century (Bramanti *et al.* 2016; Wagner *et al.* 2014). The Black Death, the most notorious plague epidemic in history, represents the beginning of the second pandemic with its early arrival at Constantinople and in Italy in 1347 (Perry and Fetherston 1997; Benedictow 2004). This epidemic spread throughout Europe, the Middle East and North Africa from 1347 to 1353 AD killing an estimated 40-60% of the populations (Benedictow 2004; Green 2016).

Following plague's 14th century introduction to regions afflicted during the second pandemic, cyclical outbreaks occurred approximately every 20 years (though intervals varied in different areas and centuries) throughout Afro-Eurasia until the 19th century making the second pandemic by far the longest and most devastating of the three (Perry and Fetherston 1997; Benedictow 2004; Varlık 2015). Although many historic sources of past plague pandemics illuminate the scale of the mortality, geography, and diffusion of *Y. pestis*, they do not as of yet explain why it persisted for nearly 500 years. Current hypotheses tend to lean towards the repeated reintroduction of plague from regions outside of Western Europe (Schmid *et al.* 2015; Namouchi *et al.* 2018), thereby reinforcing colonialist othering of locations that have already gone underserved in plague

studies, such as the Mediterranean and Middle East. Alternatively, the cyclical outbreaks of plague experienced during the second pandemic could reflect modern plague ecology, such that local reservoirs facilitated the maintenance and eventual spillover of the disease into human populations. A consensus amongst researchers has yet to be reached as no single model has been able to fully explain the peculiar experience of the second pandemic's lengthy reign and as such it is clear that further investigation into a more complex multi-mechanism model is required.

2.3 Models of Plague Maintenance

Many researchers have tried to answer the long-standing question of how plague was able to be maintained during the second pandemic through the use of historic and archaeological records or statistical modeling. The resulting studies have thus far discounted the role of rodent reservoirs in the past – although they are known to be active in modern plague ecology (Stapp *et al.* 2008; Salkeld *et al.* 2010) – in favour of two predominant hypotheses: 1) the 'trade route' model of repeated plague transmission between regions (*e.g.* Schmid *et al.* 2015; Yue *et al.* 2016; Yue *et al.* 2017); and more recently, 2) the 'human ectoparasite' model that relies on human-to-human plague transmission via vectors such as lice (*e.g*. Dean *et al*. 2018, Hufthammer & Walløe 2019).

2.3.1 'Trade Route' Model

For decades, the repeated re-introduction of plague-carrying rats along interregional trade routes from various regions to the east was the most prominent model used to explain the reoccurring nature of plague in Europe (Devaux 2013; Schmid *et al*.

2015; Yue *et al.* 2016; Yue *et al.* 2017; Namouchi *et al.* 2018). Various researchers have relied solely on this model while too easily dismissing - or outright neglecting - other possibilities, namely the potential involvement of rodent reservoirs.

Climate patterns have been used to demonstrate the inability of rodent reservoirs to have existed in Europe in the past, thus leaving trade routes as the only possible mechanism of plague reintroduction (Schmid *et al.* 2015). This theory argues that the climate in western Europe was not well suited for the peak activity of black rats (*i.e. Rattus rattus*) and their associated fleas, making a local reservoir unlikely (Schmid *et al.* 2015). It was instead climate fluctuations in Asia that resulted in the spread of infected rodents and/or their infected fleas towards Europe via human trade and travel, spreading plague to different populations along the way (Schmid *et al.* 2015). According to this school of thought, plague would slowly spread out of Asia in successive waves taking approximately 15 years to reach Europe, which would explain the 20-year cycling of plague during the second pandemic (Schmid *et al.* 2015).

However, this theory does not account for the possibility that other non-rat rodents – operating under varying climatic conditions – could have been acting as plague reservoirs in Europe and the surrounding regions during the second pandemic. The present study will clearly demonstrate that there are many other modern rodents in Africa, Asia, and Europe capable of hosting plague that rely on vastly different climates. For example, this diverse range can be seen in species such as *M. persicus* - which thrives in arid and rocky high-altitude mountainous regions in the Middle East - *Otomys angoniensis* - which prefers wet grasslands in Africa - and *Spermophilus pallidicauda* -

which persists primarily in temperate grassland regions in China and Mongolia. Thus, the results of the current study suggest that if we move beyond *R. rattus*, these climate based arguments in support of the 'trade route' model become problematic as climate fluctuations may not be the only indicator for the presence or absence of active plague reservoirs as there are other species and interactions at play.

Researchers have also attempted to support a 'trade route' model by demonstrating a positive correlation between an increase in past plague outbreak and close proximity to major cities (Yue *et al.* 2016; Yue *et al.* 2017). Based on this positive correlation, it is suggested that no inland plague reservoirs could exist due to the steep reduction of plague outbreaks away from major trade routes or navigable rivers (Yue *et al.* 2016; Yue *et al.* 2017). These findings have been based largely on the only currently available digitized database of plague outbreaks during the second pandemic created by Jean Noel Biraben (1975) (Fig. 2.1), which is biased to western Europe, France in particular, as well as major cities (Roosen & Curtis 2018), leaving rural areas - where records would have been less likely to survive over time - largely underrepresented in the digitized history of plague. The bias of this database to urban centres combined with generally little investigation into the history of plague in rural areas results in misleading trends that show higher plague occurrences in larger cities along common throughways and again places the focus of plague transmission on ship rats, *i.e. R. rattus*, at the expense of other inland rodent species that could have been contributing to plague at the time.

2.3.2 'Human Ectoparasite' Model

This study also highlights potential issues with a sustained human-human transmission model which suggests that human fleas and body lice were the true drivers behind past pandemics (Drancourt and Raoult 2006; Houhamdi *et al*. 2006; Hufthammer & Walløe 2013; Dean *et al*. 2018). Studies in support of this model have suggested that rodents and their associated fleas could not have been acting as plague vectors during medieval plague in Nordic regions due to the scarcity of *R. rattus* remains in the medieval and early modern archaeological record (*e.g.* Hufthammer & Walløe 2013). These arguments have been largely based upon the fact that when rodent remains were found they were concentrated close to trade routes with little to no evidence of them moving inland, whereas human dwellings throughout the region were supposedly teeming with body lice and human fleas allowing for the rapid spread of plague observed in medieval epidemics (Hufthammer & Walløe 2013). The scarcity of rat remains in the archaeological record, however, does little to prove conclusively that trade routes were the sole mechanism of plague reintroduction as it does not account for the potential involvement of other species or flea transmission via animal furs. Moreover, this argument is problematic as small animal bones are often missed during excavation, lost entirely due to poor preservation, misidentified at the genus/species level, or not recorded due to perceived lack of importance (Morlan 1994; Lyman 2012).

Recently, Dean *et al.* (2018) also demonstrated that a model of sustained human-human transmission via human ectoparasites fits mortality curves from plague outbreaks better than a model based solely on rodent or pneumonic transmission. However, these

models are shown to be poorly supported as a result of assumptions that lead to underestimations of uncertainty and overinflated confidence in a model based on human ectoparasites as opposed to a rodent-flea model (Park *et al.* 2019). Furthermore, a model based solely on human-human transmission would not account for the intermittent plague free periods and sporadic re-emergences experienced during the second pandemic. Such plague free periods would require some sort of spatially removed reservoir, which would maintain the disease outside of human populations until sporadic reintroductions allowed for the rapid human-human transmission experienced during outbreaks.

2.3.3 Problems That Arise in a Single Model System

Although popular in some circles, the 'trade route' and 'human ectoparasite' models are at odds with the evidence garnered from third pandemic studies showing plague's ability to establish itself in natural foci where newly introduced, as witnessed from the Andes Mountains to Central Africa to the Asian Steppe (Echenberg 2002; Schneider *et al.* 2014; Abedi *et al.* 2018). Plague persists in well-described enzootic cycles following its third pandemic introduction and has been documented extensively in wild North American rodents (Salkeld *et al.* 2008; Tripp *et al.* 2009; Matchett *et al.* 2010). In these reservoirs, the disease can lay dormant for years before re-emerging as human epidemics as witnessed most recently in East Africa (Lofty 2015; Rahelinirina *et al.* 2017; Andrianaivoarimanana *et al.* 2019). The 'trade route' model is also at odds with the recent explosion of genomic evidence from skeletal material such as those from the infamous 18th century Peste de Provence, that show late second pandemic strains of *Y. pestis* to be derivatives of earlier second pandemic strains (Bos *et al.* 2016; Seifert *et al.*

2016). Together these studies indicate that plague persisted in the Mediterranean region and Europe following a single introduction of the disease (Bos *et al.* 2016; Spyrou *et al.* 2016; Spyrou *et al.* 2019).

Although these alternative models of plague maintenance and transmission remain the preferred models for the second pandemic by several researchers, they ultimately fail to conclusively exclude rodent reservoirs as important drivers of plague re-emergence. Nor do they explain how the pathogen could have been locally maintained for long periods of time between human outbreaks. This goes to show that these "opposing" models of plague reintroduction and long-term persistence could not have been acting alone and thus are not mutually exclusive. Instead, it was likely a combination of large-scale plague diaspora via human mediated travel and trade followed by the development of local reservoirs where the disease was able to percolate during "plague free" periods. Recurring spillovers could potentially have been enhanced by human ectoparasite transmission until die off events led to a quiescent period where the disease once again remained percolating within rodent reservoirs until subsequent spillover events. By emphasizing the importance of rodents in this complex multi-mechanism system, this study seeks to refocus plague research towards a model of past plague persistence and reintroduction that incorporates a local reservoir component.

2.3.4 Reintroducing a 'Local Reservoir' Model

A model that includes the perpetuation of plague amongst local rodent reservoirs is more in line with plague's recent history and new genomic evidence (Seifert *et al*. 2016; Bos *et al.* 2016; Spyrou *et al.* 2016; Vogler *et al.* 2017; Spyrou *et al.* 2019), which

argues against the old theory of repeated reintroduction based solely on trade ships. Here we hope to revive the theory long understood by various plague scholars (Carmichael 2016; Green 2016; Prybil 2017) that rodent reservoirs were key components of past plague.

This model is rooted in analyses of rodent species capable of maintaining plague today in areas long afflicted and the creation of the first comprehensive database, *Rodent Reservoirs of Plague: Modern Distributions and Host Characteristics* (available at ArcGIS Online on an interactive platform), which highlights life history characteristics and geographic distributions of such species. Using modern examples and case studies from regions in the Mediterranean littoral, this research both expands the focus of plague scholarship beyond western Europe and demonstrates that plague was likely not solely re-introduced from "outside" sources following the Black Death via the importation of plague-carrying rats. Rather, we provide more weight to the argument that plague perpetuated in enzootic cycles within affected areas and repeatedly spilled over into human populations from local rodent reservoirs. With this research we hope to facilitate a starting point to spur further investigation into these rodents in the archaeological record to conclusively identify which species could have been acting as long-term reservoirs during past plague pandemics.

2.4 The Black Rat

Rattus rattus, commonly known as the black rat, is most often mentioned as the main culprit behind the transmission of plague. This is likely a result of its commensal nature as it was the rodent most commonly observed and most widely known to the

general public. The focus many researchers have placed on the black rat when working to support either a solely trade route or human ectoparasite based model of plague maintenance is ultimately an oversimplification and has the potential to produce overconfidence in the models presented when no alternative reservoir species are considered.

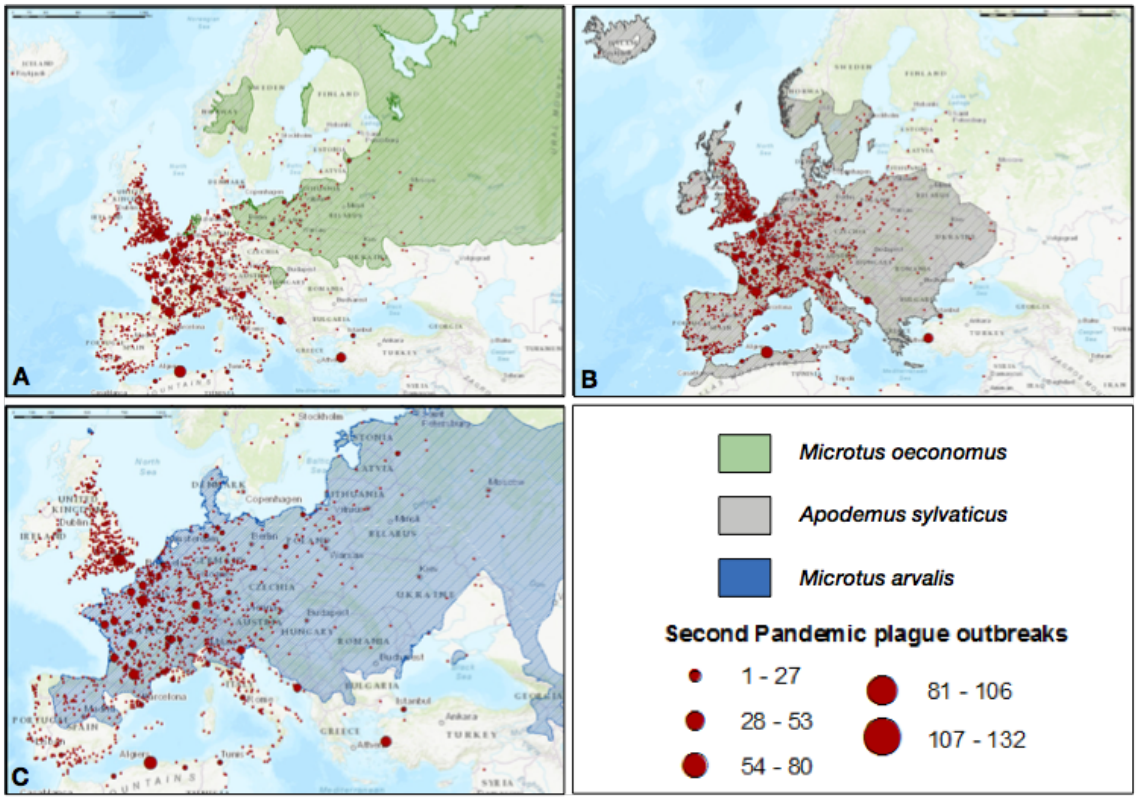

Figure 2.1: Geographic distributions of rodent reservoirs extending into Europe demonstrating the presence of non-Rattus spp. in the region capable of hosting plague. Rodent species shown overlapping with second pandemic plague epidemics included in Biraben (1975) dataset, whose work does not account for every second pandemic plague epidemic (Roosen & Curtis 2018). Rodent species presented here, *M. oeconomus* (A), *A. sylvaticus* (B), and *M. arvalis* (C), could indicate the presence of natural rodent plague foci yet to be discovered in the region. Maps created with ArcMap and all data available in interactive database, *Rodent Reservoirs of Plague: Modern Distributions and Host Characteristics* available at ArcGIS Online.

Although it is suggested that black rats were the primary reservoir of plague in Europe in the past and that no modern plague reservoirs exist there currently (Benedictow 2004; Schmid *et al.* 2015), other rodents known to persist in Europe - such as *Apodemus*

sylvaticus (Wood mouse), *Microtus arvalis* (Common vole), and *Microtus oeconomus* (Tundra vole) - were identified in this study as having the potential to host plague though are scarcely investigated and discussed (Fig. 2.1). This indicates that there could be other reservoirs that have gone unnoticed in Europe due to the preferential focus consistently placed on *Rattus* spp. in plague scholarship.

This is not to say that *Rattus* spp. cannot act as plague reservoirs in any capacity, as can be seen most recently in Madagascar where rats have been documented as the primary reservoir for the recurring plague outbreaks (Andrianaivoarimanana *et al.* 2012; Brouat *et al.* 2013). Rats are in fact the most commonly found rodents in plague surveillance studies in Madagascar (Rahelinirina *et al.* 2010; Richard *et al.* 2015; Miarinjara *et al.* 2016), however, Asian house shrews (*Suncus murinus*) have also been noted for their involvement as reservoir species in past plague outbreaks in the region (1991-1999) and could potentially be involved in the recent plague outbreaks as well (Boisier *et al.* 2002; Rahelinirina *et al.* 2017).

A multi-host system is most likely to account for the complexities of reservoir-based plague reintroduction, especially when considering the dynamics between humans and highly commensal species. *R. rattus* and *R. norvegicus* live primarily in or around human dwellings (most commonly in roof or floor structures, respectively) (Feng and Himsworth 2014), which would result in very high contact with human populations and the direct transfer of plague if such populations were harbouring the disease. This high contact with human populations makes *Rattus* spp. better suited to be intermediary species between a long-term wild reservoir and human populations. Interactions between

these different species could result in the spread of plague and inevitable spillover from rat populations into their closely associated human populations and thus account for the intermittent plague free periods followed by sudden reappearances of plague during the second pandemic.

2.5 The Plague Cycle

The plague cycle is well defined in modern rodent reservoirs, where researchers have studied the dynamics of different non-*Rattus* rodent species to understand the inner workings of sporadic disease flare ups and the more perplexing quiescent periods that extend in between (Stapp *et al.* 2008; Foley and Foley 2010; Salkeld *et al.* 2010). Several studies in particular have focused on enzootic plague cycles in prairie dog (*Cynomys* spp.) colonies throughout the United States, where plague persists as a very prominent natural focus (Stapp *et al.* 2008; Stapp *et al.* 2009; Salkeld *et al.* 2010; Hoogland *et al.* 2018; Russell *et al.* 2019).

Prairie dogs are typically highly susceptible to death from *Y. pestis* infection and as a result they are typically unable to maintain plague over long periods of time. However, it has been shown that prairie dog coteries (*i.e.* family groups) are in fact capable of locally maintaining the pathogen in low levels due to social constraints inhibiting the flow of the pathogen between different coteries and rapidly spreading throughout the entire colony (Salkeld *et al.* 2010). The driving force behind epizootic outbreaks of plague in these populations can in fact be attributed to the movement of another less susceptible rodent species, grasshopper mice (*Onychomys leucogaster*), which transfer infected fleas between colonies (Salkeld *et al.* 2010). This demonstrates

the varying roles of non-*Rattus* plague reservoirs and their ability to interact with their surrounding environment and other reservoir species to either locally maintain the disease over long periods of time or spread the disease between different populations.

The complex roles of different plague reservoirs calls into question the classic dichotomy of *enzootic* versus *epizootic* host classifications - where the former relates to maintenance hosts able to resist death from infection over long periods of time and the latter indicates species better suited to rapidly amplify the disease and facilitate spillover events (Gratz 1999). These distinctions imply that a species will always either be resistant or susceptible to the infection and that all rodents within each species will have the same response to infection. Instead, there is likely a biological gradient to reservoir species in terms of their ability to host plague and their susceptibility to infection as well as social and environmental constraints that may affect their ability to maintain the infection within their population, as seen in certain prairie dog colonies (Gratz 1999; Salkeld *et al.* 2010). Thus, the reservoir database included in this study represents certain populations of these species where plague is known to persist and does not indicate that all species included are long-term reservoirs or that all rodents within a particular species will act as a reservoir during their life. Rather we are making a case for the fact that plague has been identified at some point in certain rodent populations within the species included in this database, a fact which cannot be ignored when talking about long-term plague persistence and reintroduction.

The plague cycle is complex and reintroduction likely functions within a complex multi-host and multi-mechanism system, a fact further supported by statistical modeling

of rodent species (*Microtus californicus* - California vole, *Peromyscus maniculatus* - deer mouse) in California that shows plague persistence increases when more than one host population is involved (Foley and Foley 2010). When researchers try to oversimplify plague reintroduction into one distinct model or limit the dynamic role of rodents involved, they cannot possibly explain the process of long-term persistence in its entirety. These studies of North American rodent reservoirs of plague ultimately serve to exemplify a once plague free region where the disease was introduced in 1899 via human mediated transport and subsequently disseminated into rodent reservoirs, which have facilitated its persistence for over 120 years with cyclical outbreaks (Antolin *et al.* 2002). Therefore, a model that incorporates multiple mechanisms such as plague movement via trade and travel, rodent reservoir facilitated plague reintroduction on a local scale, and potentially some human-human transmission during epidemics should be considered moving forward.

2.6 The 'Local Reservoir' Model

In accordance with current comprehension of plague ecology, it is very likely that plague was being 'held' in local reservoirs during the second pandemic as well. This is not to say that it was not being transported between locations via human mediated trade or travel or spread amongst humans via non rodent-flea vectors at all. The systems that were at play during the second pandemic were likely much more complicated than a simple two-dimensional matrix involving solely trade routes and black rats or simply human to human transmission. The second pandemic reigned over human populations in many Afro-Eurasian regions for 500 years with several intermittent disease-free periods.

On the surface, this could indicate that the disease was continually being reintroduced from outside sources, however as mentioned previously, genomic evidence proves that early strains of *Y. pestis* were directly correlated to later epidemics indicating some form of local maintenance (Bos *et al.* 2016; Spyrou *et al.* 2016; Spyrou *et al.* 2019). Using North American plague dynamics as a model location where plague was introduced by trade and has persisted for 120 years in pockets of wild rodents, we can relate certain understandings of the plague cycle to other regions of the world that were long afflicted by plague during the second pandemic, such as the Mediterranean and Middle East, to garner a better understanding of how plague was able to persist for so long.

2.6.1 Plague in the Mediterranean: Kurdistan Plague Focus

The dynamics observed amongst North American rodent reservoirs is also well described in modern contexts in regions in the Mediterranean, particularly Iran. Iran is home to a very prominent natural plague focus which has been the subject of an increasing amount of research since the early 20th century, largely as a result of the Pasteur Institute of Tehran. The Kurdistan plague focus provides an opportunity to discuss plague research that is representative of the Mediterranean littoral as it stretches across western Iran, southern Turkey, northern Iraq, and northern Syria (Shahraki *et al.* 2016) (Fig. 2.2). This allows us to expand the focus of plague scholarship away from examples in western Europe and examine another region where plague continually affected human populations in the past and is still present in natural reservoirs. According to historical records, plague first appeared in the region now known as Iran in the 6th century during the first pandemic with recurring second and third pandemic outbreaks

spanning into the 20th century with the last reported human outbreaks occurring between 1947-1966 (Azizi and Azizi 2010; Shahraki *et al*. 2016; Mohammadpour & Mostafavi 2018).

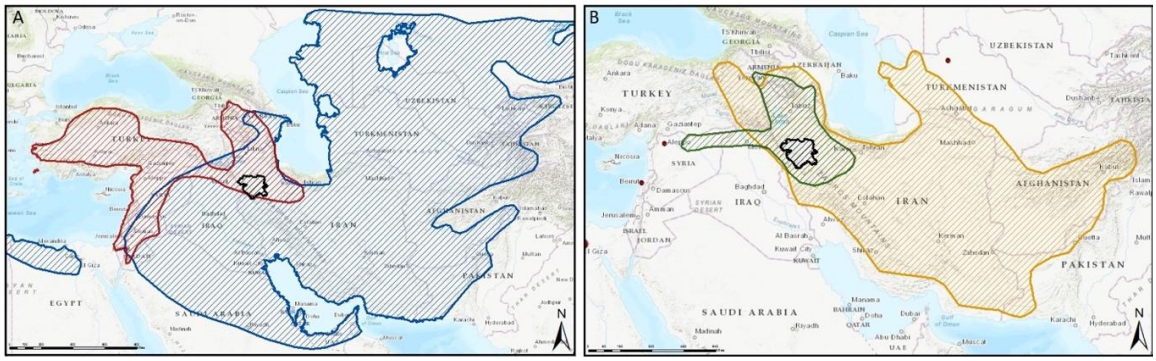

Figure 2.2: Overlap of rodent reservoir species with plague focus in Kurdistan province, Iran (outlined in black) and surrounding regions. (**A**) Demonstrates the species distributions of *M. tristrami* (red) and *M. libycus* (blue). (**B**) Demonstrates the species distributions of *M. vinogradovi* (green) and *M. persicus* (yellow). Maps were made in ArcMap and all data is available on interactive database, *Rodent Reservoirs of Plague: Modern Distributions and Host Characteristics* on ArcGIS Online.

Although no human plague cases were reported after the 1960s, plague surveillance efforts in western Iran documented 66/1402 (0.4%) plague positive rodents between 1966-1967 (Mohammadpour & Mostafavi 2018). More recently, a study carried out between 2011-2012 along the border between Kurdistan and Hamadan Provinces where 98 rodents (*Meriones persicus*, *Meriones vinogradovi*, *Meriones libycus*, *Microtus socialis irani*, *Ellobius lutescens*) and 117 sheepdogs were surveyed for the presence of plague, a total of 1.02% rodents and 3.42% of dogs tested positive for *Y. pestis* antibodies (Esamaeili *et al.* 2013). The role of such *Meriones* spp. as plague reservoirs in southwest Asia is further supported by the results of this study which identified *Meriones vinogradovi* (Vinogradov's jird), *Meriones tristrami* (Tristram's jird), *Meriones persicus* (Persian jird), and *Meriones libycus* (Libyan jird) as having the potential to host plague in regions overlapping with the Kurdistan plague focus (Fig. 2.2).

Although more recent surveillance studies have not been able to successfully identify plague positive rodents in Iran (Pourhossein *et al*. 2015; Ghasemi *et al.* 2017; Mostafavi *et al.* 2018), these examples demonstrate that plague was circulating within rodent populations during the last reported human outbreaks in the region and subsequently managed to remain strictly within enzootic cycles for decades. These results indicate that *Meriones* spp. present in Iran could have been a source of plague spillover that resulted in the sporadic plague outbreaks observed in these regions in the past. Therefore, while Kurdistan province has not reported human plague since the mid 20th century, it is evident that rodents in these areas are still maintaining the bacterium. Furthermore, these examples support the theory that plague is able to circulate in small enzootic foci during periods between human outbreaks and that these reservoir populations are able to maintain the disease for several years, even decades, without resulting in human plague epidemics.

2.6.2 Plague in the Mediterranean: Ottoman Empire

Modern examples of plague reservoirs in action, such as those in the United States and Kurdistan discussed previously are not uncommon. Despite the growing support for a rodent-based model of plague maintenance, however, there are still several researchers who stand in opposition to the potential role these rodents played in past plague ecology and instead believe that increasingly prevalent factors – such as climate change and increased land use – are the reason such reservoirs exist today but do not support their presence in the past. These trends, however, are mirrored in past plague outbreaks as well, for example, Varlık (2011) presents a strong case for a correlation between the increasing

urbanization and growth of the Ottoman Empire from 1453-1600 and the re-emergence and spread of plague.

The conquest of new regions and rise of new city centres described by Varlık (2011) would have inevitably resulted in the spread of plague as a direct result of increased human mediated trade and travel. Additionally, the increase in population size and density as well as changes in land use and arable agriculture would have certainly increased the odds of human-human transmission once the disease rooted itself within human populations. However, this burst of development and movement would also have rippled out into immense landscape changes that could have either seeded new or shifted pre-existing rodent plague reservoirs and allowed for interactions between maintenance foci and amplification species - such as *Gerbillus spp*. (gerbils), *Meriones spp*. (jirds), *Cricetulus migratorius* (grey dwarf hamster) documented in this study (see supplemental database).

Such interactions between reservoir populations would have allowed for the continued reintroduction of plague where it was already endemic and allowed for the emergence of plague in areas previously unafflicted. Thus, an argument can be made that landscape changes in the past similar to what we see today – along with other modes of plague transmission – could have fostered an environment suitable for an increase of rodent plague reservoirs. In fact, we have observed high diversity and adaptability amongst the rodent reservoirs in this study, so while ecological conditions could have varied historically there is no reason to believe that certain rodents were unable to adapt to their surroundings and maintain plague under variable conditions in the past.

2.7 Methods

We performed a comprehensive literature and database review to identify rodent species that currently host plague. These species, along with information on their host characteristics, were then compiled into an excel database in order to compare and contrast species information and identify possible trends in the data. Host characteristics that were assessed to determine what traits likely make for good plague reservoir species were climate, habitat, geographic range, elevation limits, generation length, reproductive rate, litter size, and sociability. The intention was to identify trends in ecological factors, population turnover rates, or social behaviours and interactions with other species that might indicate why certain species act as plague reservoirs and not others.

All data, along with geographic distribution shapefiles for each species acquired from the IUCN (2019) database, where then incorporated into a publicly available ArcGIS Online map to create an interactive and informative visual database entitled, *Rodent Reservoirs of Plague: Modern Distributions and Host Characteristics*. This interactive database also includes layers to inform users on ecological zones, elevation ranges, and locations of historically sourced mentions of second pandemic plague outbreaks in order to get a clear picture of where these rodent reservoirs exist today and how they might overlap with the history of plague.

2.7.1 Search Strategy and Selection Criteria

Searches for relevant publications on the ability of rodents to host plague were conducted primarily on Google Scholar and Scholars Portal Journals using key terms such as “plague reservoirs”, “rodent reservoirs of plague”, “rodents and plague”, etc.

References for this review were selected based on their association with a reputable source such as a public health organization (*i.e.* WHO, CDC) or a peer reviewed journal article. Information on species' host characteristics and geographic ranges were accumulated from the online IUCN red list database (IUCN 2019) as well as peer reviewed journal articles identified through searches on Google Scholar and Scholars Portal Journals using species specific key terms (*e.g.* "*Meriones vinogradovi* host characteristics"). Data points for historically sourced mentions of second pandemic plague outbreaks used in the ArcGIS Online interactive database were downloaded from the Schmid *et al.* (2015) publication. This dataset was first compiled by Jean-Noel Biraben (1975) and subsequently digitized by Atanasiu *et al.* (2008) and Buntgen *et al.* (2012). Schmid *et al*. (2015) curated this digitized data for use in their publication and expanded the dataset to include points in Russia, Constantinople, and Turkey.

2.7.2 Limitations of the Current Study

The most prominent limiting factor for this research was data availability and reliability. Firstly, the geographic distribution shapefiles used in ArcGIS programs were downloaded from the IUCN (2019) database, which could be limited to certain regions due to a lack of complete surveys in certain countries. Additionally, a lack of species-specific research on host characteristics resulted in gaps in the database that will not be filled until further studies are carried out on such rodents, which could limit the visible trends in the data.

Similarly, the Biraben (1975) plague dataset shows obvious bias towards western Europe and is known to contain many data points based on uncited mentions of plague

(Roosen and Curtis 2018). Moreover, it is unclear how Biraben (1975) distinguished plague from other diseases when including cases in the resulting dataset. Although the Biraben (1975) plague data used in this study was curated by Schmid *et al.* (2015) to favour – wherever possible – digitized data from the peer reviewed publication of Buntgen *et al.* (2012), as opposed to the earlier non-peer reviewed version by Atanasiu *et al.* (2008), and expanded to include points outside of western Europe - it is still not free of complications. However, as this collection is the only currently known and publicly accessible digitization of historically sourced mentions of second pandemic plague outbreak, it was still essential to incorporate it into this study while being completely transparent about its potential bias and using it instead as a general approximation of where plague was in particular regions during the second pandemic to demonstrate overlap with reservoir species.

2.8 Results

Table 2.1: Breakdown of key traits identified in this study including proportions of total species in database (n=45) and mention of species within Mediterranean region associated with each trait.

Key traits	# rodent species	% rodent species	Mediterranean species
Habitat			
Open rural areas (*i.e.* grasslands, savannas, mountain steppes, shrubland)	37	82%	*C. migratorius, G. gerbillus, G. nanus, M. libycus, M. persicus, M. shawi, M. tristami, M. vinogradovi, M. arvalis*
Mixture of urban and rural	8	18%	*A. sylvaticus*
Climate			

Temperate	12	27%	*A. sylvaticus, M. arvalis*
Arid/hot	12	27%	*G. gerbillus, G. nanus, M. libycus, M. persicus, M. shawi, M. tristrami, M. vinogradovi*
Moist/cold	2	4%	
Variable climate	16	36%	*C. migratorius*
Associated with agricultural areas (fields, pastureland, etc.)	31	69%	*A. sylvaticus, C. migratorius, G. gerbillus, G. nanus, M. libycus, M. shawi, M. tristrami, M. arvalis*
Hunted	14	31%	
Threatened	14	31%	
Generation length			
<1	2	4%	
1-2	31	69%	*A. sylvaticus, C. migratorius, M. libycus, M. persicus, M. shawi, M/ tristrami, M. vinogradovi, M/ arvalis*
>2	12	27%	*G. gerbillus, G. nanus*

Although the plague cycle is commonly perceived as a human-black rat system (Royner 2015), we identified 45 other rodent species throughout Afro-Eurasia that have the ability to host plague (see supplemental database). The diversity of rodent reservoirs indicates that the reliance placed predominantly on the black rat in plague studies has neglected the potential roles of other rodents. Analysis of the habitat information collected in this study demonstrates that unlike black rats, many rodents that can host plague are not commensal species found directly in urban centers. Instead, habitat types were most commonly non-commensal open areas (37/45 - 82%) – *i.e.* grassland, shrubland, deserts, cropland, etc. – with certain rodents persisting in less populated mountainous regions, such as *Marmota himalayana* (Himalayan marmot) in China, or in

remote desert regions, such as *Meriones meridianus* (Midday jird) in the Middle East, indicating that the range of viable hosts is widespread and diverse (Table 2.1). Climate and habitat preferences are seen to range, both between and within species, with 12/45 (27%) in temperate regions, 12/45 (27%) in arid/hot regions, 2/45 (4%) in moist/cold regions and 16/45 (36%) persisting in more than one climate zone (Table 2.1). Such diversity lends further support to the idea that certain species, aside from *R. rattus*, could have been locally maintaining plague. If these species were indeed maintaining the pathogen, they would not have spilled over into human populations until certain conditions forced them into contact with humans or encounters with an intermediary amplification species enabled the transfer of plague infected fleas to other populations.

Further analysis showed that a total of 14/45 (31%) rodents are reported as being threatened by habitat degradation, while another 14/45 (31%) rodents are commonly hunted for food, fur, display, or pest control (Table 2.1). Such factors would certainly increase contact between these rodents and human populations and could facilitate the spread of disease between reservoir populations and less susceptible hosts. Additionally, 31/45 (69%) of the species are common to agricultural areas in some capacity, which may be suggestive of their potential to have 'held' plague nearby during the second pandemic until certain conditions or interactions between rodents that persist in more natural habitats and those in closer association with human populations escalated into spillover events (Table 2.1). The majority of species (33/45 - 73%) also had a generation length, or the time between the birth of an individual and the birth of their first offspring, of less

than 2 years indicating that faster population turnover replenishment may aid in long-term plague persistence (Table 2.1).

Finally, upon mapping the geographic distributions of the rodent reservoirs included in this study in ArcGIS we observed overlap with approximate locations of second pandemic plague outbreaks mentioned in historical records (Biraben 1975), as well as modern plague foci in the Mediterranean littoral (WHO 2016) (Fig. 2.3).

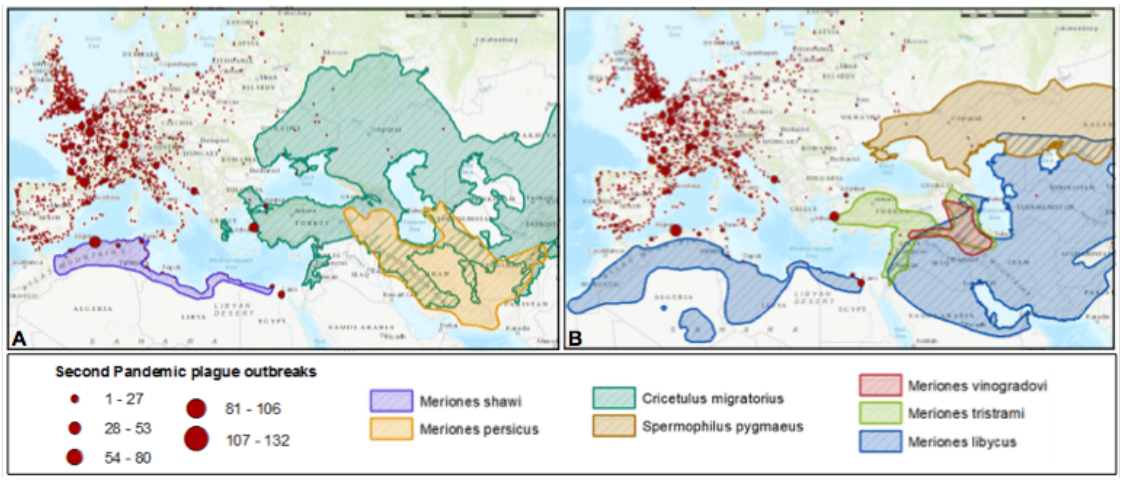

Figure 2.3: Overlap of reservoir species (**A**: *M. shawi, M. persicus, C. migratorius*; **B**: *S. pygmaeus, M. vinogradovi, M. tristrami, M. libycus*) with Mediterranean and Middle East and historical mentions of second pandemic plague (red) (Biraben 1975). Created in ArcMap, all data is available on interactive database, *Rodent Reservoirs of Plague: Modern Distributions and Host Characteristics* on ArcGIS Online.

The overlap of these species with regions where plague is currently, or was in the past, endemic could indicate that these rodents are acting as reservoirs and contributing to the re-emergence of the disease. Additionally, not only are these species known to exist within these regions, but modern plague and rodent surveillance studies have confirmed that certain species have been infected with plague or infested with fleas that are known to be plague vectors, adding support to the theory that they are playing a role in plague ecology and epidemiology within those regions.

2.9 Conclusion

The results presented in this study highlight that many rodents other than *R. rattus* have the ability to host plague, however, until now it has been difficult for researchers to access detailed information on such species. This study offered the first comprehensive, interactive, and publicly available database – *Rodent Reservoirs of Plague: Modern Distributions and Host Characteristics,* found on ArcGIS Online – on rodent reservoirs of plague which can be used to help future researchers target specific species of interest to hopefully identify these reservoirs in the past. Although several studies have negated the importance of rodent reservoirs in past plague ecology (Hufthammer and Walløe 2013; Schmid *et al.* 2015; Yue *et al.* 2016; Yue *et al.* 2017; Dean *et al.* 2018), they have failed to provide a conclusive argument that supports either the 'trade route' model or the less popular 'human ectoparasite' model. A model that incorporates rodent reservoirs as key drivers behind long-term plague maintenance and reintroduction is much more likely to provide answers to questions still remaining about the long-term maintenance of plague exhibited in the past.

This study offers a starting point for more intensive research into plague reservoirs – both in a modern and medieval context – by providing a comprehensive list of modern rodents known to host plague along with essential host characteristics that can help inform on their suitability to act as successful reservoir species. Using this information to identify reservoir species in the zooarchaeological record is essential to confirm their historical presence in the regions they are found today and verify their role in past plague. Overall, the compilation of host information provided here will not only have significant

impacts on how we view past plague maintenance but has implications for our understanding of modern plague as well as the distribution and species diversity of rodent reservoirs remains relatively unknown to the general public, whereas *Y. pestis* remains focalized in more than thirty countries worldwide (WHO 2016).

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Chapter 3.0: Laboratory Methods and Results

3.1 Introduction

Several studies, such as those conducted by Bos *et al.* (2016), Spyrou *et al.* (2016), Spyrou *et al.* (2019), Keller *et al.* (2019), Smith *et al.* (2010), and Malek *et al.* (2015), have identified plague within human remains from past plague pandemics or rodent remains within a modern context. However, there is no known study to date that has successfully identified *Yersinia pestis* DNA, the etiologic agent of plague, within premodern rodent remains. This has resulted in a bias within the study of plague's evolutionary history towards its human hosts as opposed to its "natural" rodent host. Plague is typically a disease of rodents and their associated fleas, however, as rodent remains are often too small to be noticed or well preserved in the archaeological record they are often overlooked or disposed of (Morlan 1994; Lyman 2012), which has resulted in a large gap in the history of the pathogen. Thus, the second objective of this study was to locate, acquire, and screen premodern rodent samples from the Mediterranean littoral for the presence of *Y. pestis*. The intent of this research was to identify past rodent reservoirs of plague to add to the genetic history of the disease and increase our understanding of past plague dynamics between its rodent and human hosts.

Samples were obtained from collaborators in Istanbul, Turkey and Haifa, Israel for this project and were processed in the Ancient DNA Centre at McMaster University in Hamilton, Ontario. In order to assess these samples for the presence of *Y. pestis* I employed a protocol adapted by the McMaster Ancient DNA Centre for use on damaged

(*i.e*. ancient) DNA and utilized various bioinformatic tools to analyze the sequenced data. I assessed these samples for endogenous host (*i.e.* rodent) DNA preservation as well as endogenous pathogen (*i.e. Y. pestis*) DNA preservation. This chapter outlines the laboratory procedures carried out on these samples from subsampling to sequencing of both shotgun and enriched libraries and discusses the results of the screening process.

Although *Y. pestis* was not identified in any of the samples processed, several samples showed high endogenous rodent DNA preservation, which serves to validate the efficacy of the aDNA retrieval methods employed by the McMaster Ancient DNA Centre. In the field of aDNA research where the target DNA often makes up less than 1% of the total DNA recovered within the sample, negative results are not uncommon (Margaryan *et al.* 2018; Spyrou *et al*. 2019). Nevertheless, discussing negative results is still important – however scarcely done – in the study of ancient DNA as they can help improve future methods and analyses and as such this thesis will present such results to highlight the various difficulties that accompany aDNA research and provide suggestions for future studies such as this.

3.2 Samples

A total of 72 rodent samples from Byzantine period (4^{th}-15^{th} century) archaeological sites were obtained from collaborators Vedat Onar in Istanbul, Turkey (42) and Guy Bar-Oz in Haifa, Israel (30). These samples were ideal to identify rodent plague reservoirs as they originate from key areas in the Mediterranean littoral (Fig. 3.1), a region that was repeatedly afflicted by past plague pandemics. Additionally, these regions of the Mediterranean littoral have long been underserved in plague studies as the focus

has continually landed primarily on western Europe, thus by selectively sampling from these regions we can begin to create a more inclusive and representative body of literature within plague studies.

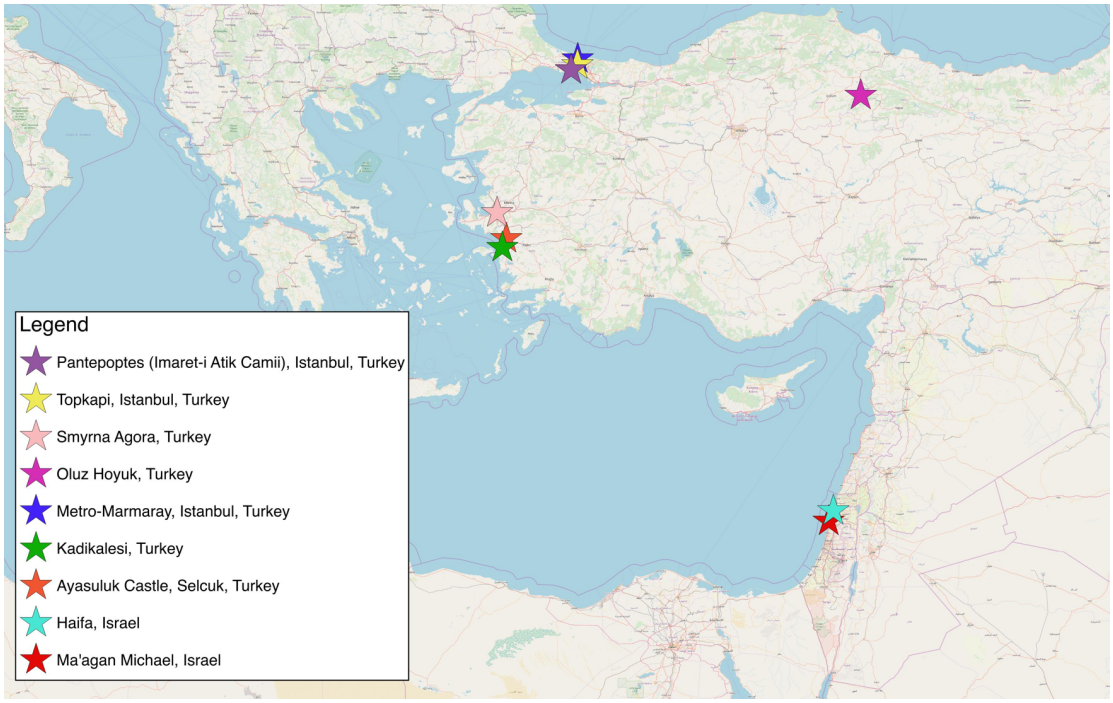


Figure 3.1: Map demonstrating the distribution of sites from which samples included in the study were obtained.

The samples originating from Turkey consisted of 31 rodents identified as *Rattus spp.*, 9 rodents identified as *Spalax spp.*, and 2 lagomorphs (Table S3.1). The samples were provided from Istanbul University and were obtained via archaeological excavations in various locations in Turkey such as Istanbul, Izmir (formerly Smyrna), Amasya (site: Oluz Hoyuk), Kadikalesi, Selcuk (site: Ayasuluk Castle), and Enez (Table S3.1., Fig. 3.1). Out of the 42 samples obtained, 23 consisted entirely of bone material, while 19 were composed of bone and teeth (Table S3.1). The samples provided from Haifa University in Israel consisted of 16 *Meriones* spp., 6 *Mus* spp., 6 *Gerbillus* spp., and 2 *Rattus* spp. from Nahal Bega in the Negev Desert and from a shipwreck off the coast of

Ma'agan Michael, Israel (Table S3.2., Fig. 3.1). All 30 samples consisted of both tooth and bone material that was used for subsampling (Table S3.2).

3.3 Laboratory Procedures

3.3.1 Subsampling

As *Y. pestis* is a blood-borne pathogen and the root of the tooth houses the pulp cavity, which is highly vascularized, this is the most beneficial sample type for aDNA pathogen analysis as opposed to bone (Margaryan *et al.* 2018). This is because the dense enamel and cementum layers on the exterior of the tooth, as well as the position within the jaw bones, protects the interior of the tooth from external contamination and allows for prolonged preservation of endogenous DNA (Adler *et al.* 2011). Typically, when subsampling from a tooth a Dremel tool is used to excise a portion of one root leaving the majority of the tooth intact. However, as rodent teeth are usually very small, the entirety of the tooth – if not multiple teeth – were used in order to maximize and standardize subsample mass (between 50-100 mg). In cases where no teeth were present, a portion of bone was excised using a Dremel tool and used as the subsample. When both tooth and bone were present, but the teeth were too small to provide sufficient material for further analysis I utilized the entirely of the sample material provided (*i.e.* combined an incisor, molar, and portion of maxilla or mandible). The tooth, bone, or a combination thereof was then pulverized into a fine powder and placed into a 2 mL collection tube.

3.3.2 aDNA Purification and Extraction Protocol

The mineral and collagen matrices within the subsample were further broken-down using demineralization and digestion buffers in order to release the DNA into the solution. The buffer solutions were administered to the subsamples twice each in 0.5 mL on alternate days for a total of 4 days allowing for an incubation period of 24 hours between each buffer change. The supernatant was removed each day and ultimately combined to total 2 mL. Following this, a silica-based extraction protocol, optimized for aDNA designed by Dabney *et al.* (2013), was used to purify the DNA from the supernatant collected during the demineralization and digestion stage. This was accomplished by using a guanidinium hydrochloride (GuHCl) based extraction buffer, which helps the DNA bind to a silica filter while the remaining solution is washed away, thus purifying the DNA out of the solution. The supernatant produced from the demineralization and digestion stage was combined with the extraction buffer, flushed through the silica filter, and rinsed with a wash buffer to remove excess remnants of the buffers previously used. This extraction procedure ultimately resulted in 50 uL of DNA extract suspended in an elution buffer to support long term storage.

3.3.3 Plague Screening via PCR Assay

Following the extraction, the samples were then screened for the presence of *Y. pestis* DNA using a *pla* qPCR assay. In this assay, primers specific to the plasminogen activator (*pla*) gene are added to the DNA extract. The DNA is then denatured from its double stranded to single stranded form allowing the primers to anneal to the corresponding single stranded DNA sequence and the primers are subsequently extended.

Thus, if the DNA fragments within the sample possess the *pla* gene sequence the primers will anneal properly and we would expect to see a noticeable amplification within a similar range of the *pla* standards run at the same time (*i.e*. amplification around 30-40 cycles and a melt temp within 0.5 degrees of the standards) (Fig. 3.6a-b). The *pla* gene is used as an indicator of the presence of *Y. pestis* DNA within a sample because it resides on the pPCP1 plasmid, which is found in high copy number within *Y. pestis* (Schuenemann *et al.* 2011). Using this assay to identify samples that screen positively for *pla* allows us to selectively target which samples we want to take through to further analysis in terms of enrichment and sequencing. Each sample was run in 4 replicates (1:10 dilutions of extracts in the PCR master mix) for the *pla* assay: 2 straights (*i.e.* the 1 mL of template DNA used in the reaction was taken directly from the extract) and 2 dilutions (*i.e.* the 1 mL of template DNA used in the reaction was a 1:10 dilution of the extract with a buffer).

3.3.4 Library Preparation, Indexing, and Sequencing

Double stranded library preparation was carried out following the protocol designed by Meyer and Kircher (2010) and updated by Kircher *et al.* (2012) to convert the samples into Illumina sequencing libraries. Following this protocol, the DNA extracts were subjected to 3 sequential reactions designed to properly ligate the Illumina adapters to the DNA within each sample. After combining the DNA extracts with the master mix for each sequential library preparation reaction, they were incubated in a thermocycler and purified prior to each subsequent reaction. The libraries were then indexed using unique combinations of forward and reverse indexing primers for each sample and pooled

at equimolar concentrations. Sequencing was then performed on the Illumina HiSeq 1500 platform at the Farncombe Metagenomics Facility (McMaster University).

3.3.5 Targeted Enrichment

Samples that screened positively (*i.e.* had 2-4 replicates amplifying within the proper range) in the qPCR *pla* assay were enriched using a targeted capture protocol following a modified version of the Mybaits Manual Version 3.0. The enrichment strategy designed by MYcroarray outlined in the MYbaits manual follows an in-solution hybridization protocol using biotinylated RNA baits (MYcroarray 2015). The RNA baits are introduced to the double stranded DNA libraries and upon denaturation of the libraries they are hybridized to the complimentary target DNA (MYcroarray 2015). Streptavidin-coated magnetic beads are then used to collect the biotinylated baits, and the now associated target DNA, while the remaining DNA is removed (MYcroarray 2015). The target DNA is then amplified and sequenced (MYcroarray 2015).

3.4 Bioinformatic Analysis of Sequenced Data

3.4.1 Processing and mapping the data

In the Ancient DNA Centre, sequencing data is processed via a pipeline designed for use on aDNA that serves to filter and sort short sequencing reads into the proper format in order to be mapped and run through BLAST in preparation for downstream analysis. Upon retrieving the Illumina sequencing data, it was first trimmed of adapter sequences, merged to account for overlapping regions, filtered for a minimum length of 24 base pairs, rid of duplicates, and mapped to a reference genome of interest.

For fast results, it is beneficial to choose a small reference genome when mapping and as such I chose the mitochondrial genome of the host organism being studied. Thus, for the rodent samples processed I chose to map first to the *Rattus rattus* mitochondrial genome (RefSeq: NC_012374.1) and subsequently mapped the samples to other genomes of interest, such as the *Mus musculus* (RefSeq assembly: GCF_000001635.26) and *Yersinia pestis* CO92 (RefSeq assembly: GCF_000009065.1) whole genomes for example, to compare mapping quality and statistics and to identify reads mapping to the pathogen of most interest within the samples. Following this, the data was filtered to include only mapped sequences that had a minimum of 35 base pairs and a mapping quality – a filter which retains only reads with a high probability of being correctly mapped – of 30 to show the most conservative and on target results. The mapped data was then visualized in Geneious (version 6.1.8) to assess the coverage and depth of the mapped reads along the desired reference genome.

3.4.2 Taxonomic analysis

At this point, the .fasta files that contained all merged, or properly paired, unique reads with a minimum of 24 base pairs that had been properly trimmed of adapter sequences were then run through the BLAST nt (nucleotide) database with the megablast algorithm, which retrieves highly similar sequences. The files were returned in .blast format, which were then converted to a krona.html (Fig. S3.1 & S3.2) format to visualize the BLAST results and demonstrate the relative proportion of reads that classify to particular organisms with respect to total reads classified. It is from this data that we can get a good idea of the major constituent DNA within the samples and develop a plan for

how to move forward in the bioinformatic analysis. Additionally, the .fasta files for each sample were also run through Kraken, an exceedingly fast and more conservative algorithm compared to blast (Wood & Salzberg 2014; Wood *et al.* 2019), in order to compare and validate bacterial classifications made in blast.

The overall preservation of the samples was assessed by comparing the proportions of endogenous host species (*i.e.* rodent) DNA versus non-endogenous bacterial, non-rodent Eukaryote, virus, and Archaea DNA in the metagenomic profiles generated in BLAST and visualized through Krona (Fig. 3.2-3.4). When the host species' DNA content is relatively high in comparison to non-endogenous DNA, it indicates that the skeletal material - and thus the DNA within the sample - was well preserved. Not only does this support the taxonomic classification of the host species, but this also provides better support for any pathogen findings within the sample being present at the time of death as opposed to being post-mortem contamination.

3.5 Results: Turkey Sample Set

3.5.1 Plague Screening via qPCR Assay

The results of the qPCR showed that 12 samples had 1 replicate amplify and 1 sample had 2 replicates amplify indicating weak and moderate *pla*-positives respectively (Table S3.6). None of these samples were enriched for *Y. pestis*, however, all were shotgun sequenced in order to evaluate the DNA composition of the samples and further assess the presence or absence of *Y. pestis* in the overall metagenomic profile.

3.5.2 Taxonomic Analysis (Shotgun Data)

Set 1 consisted of 16 samples from the order *Rodentia* that show poor preservation for all but 3 samples: MT12a with 90% *Rodentia*, MT19a with 91% *Rodentia*, and MT21 with 95% *Rodentia* (Fig. 3.2). Set 2 consisted of 26 samples from the order *Rodentia*, which show mixed levels of endogenous preservation with 10/26 having >50% of the total reads classify as *Rodentia*, while several show low amounts of total classified reads that are predominantly taken up by bacterial species (Fig. 3.2). All 42 shotgun sequenced samples from the Turkey collection showed less than 0.1% of *Y. pestis* out of the total sequenced reads mapping to or classifying as *Y. pestis* in the taxonomic analysis, thus these samples were not enriched for *Y. pestis*.

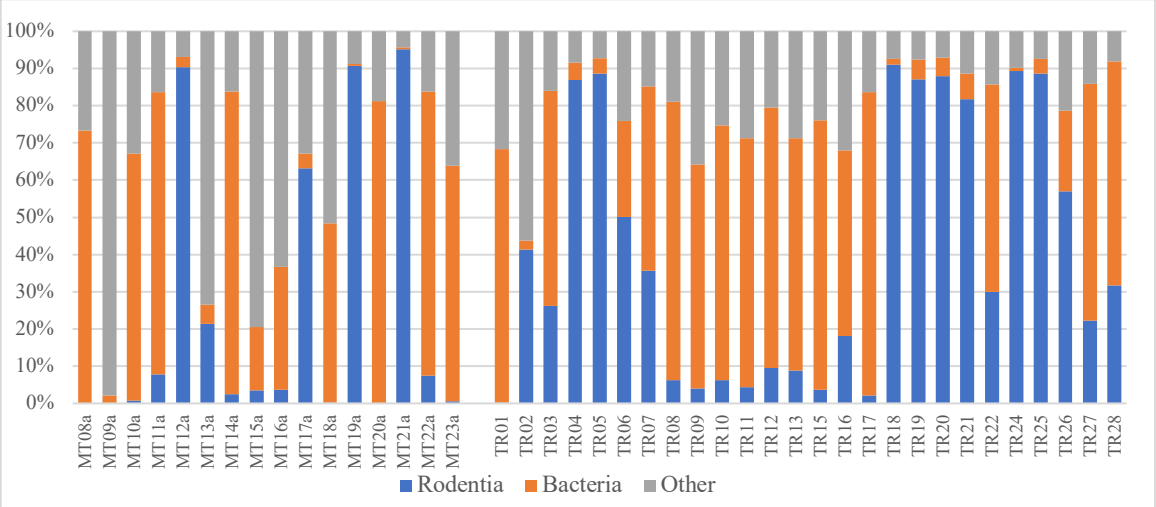


Figure 3.2: Total classified reads from metagenomic datasets (set 1: MT08a-MT22a and set 2: TR02-TR28) classified through BLAST and visualized through Krona to represent overall endogenous preservation within the samples by comparing Rodentia, bacteria (*i.e.* predominantly non-endogenous soil contaminants), and other (*i.e.* non-host species eukaryotes, viruses, and archaea).

3.6 Results: Israel Sample Set

3.6.1 Plague Screening via qPCR Assay

The results of the qPCR showed that 17 out of the 30 samples had no replicates amplifying, which indicates that plague is likely not present (Table S3.7). However, 10 samples had 1 replicate amplify and 3 samples had 2 replicates amplify, indicating weak and moderate *pla*-positives respectively (Table S3.7). Although none of the samples were strongly positive for *pla* (ex. 3-4 replicates amplifying within the proper range) all were shotgun sequenced in order to evaluate the overall metagenomic content and further corroborate or negate the presence of *Y. pestis* via taxonomic analysis.

3.6.2 Taxonomic analysis

Overall, the 30 samples from the Israel collection showed mixed levels of preservation with 13/30 having >50% of the total reads classify as *Rodentia*, and the majority of the remainder with <10% classify as *Rodentia* with high non-endogenous bacterial components (*i.e*. soil dwelling species) (Fig. 3.3). Samples ISR03 and ISR08 appear to have the highest rodent DNA content and the best preservation, followed by samples ISR01, ISR04-ISR07 (Fig. 3.3). *Y. pestis* was only identified at proportions of less than 0.007% of the root and as such no samples from this set were enriched.

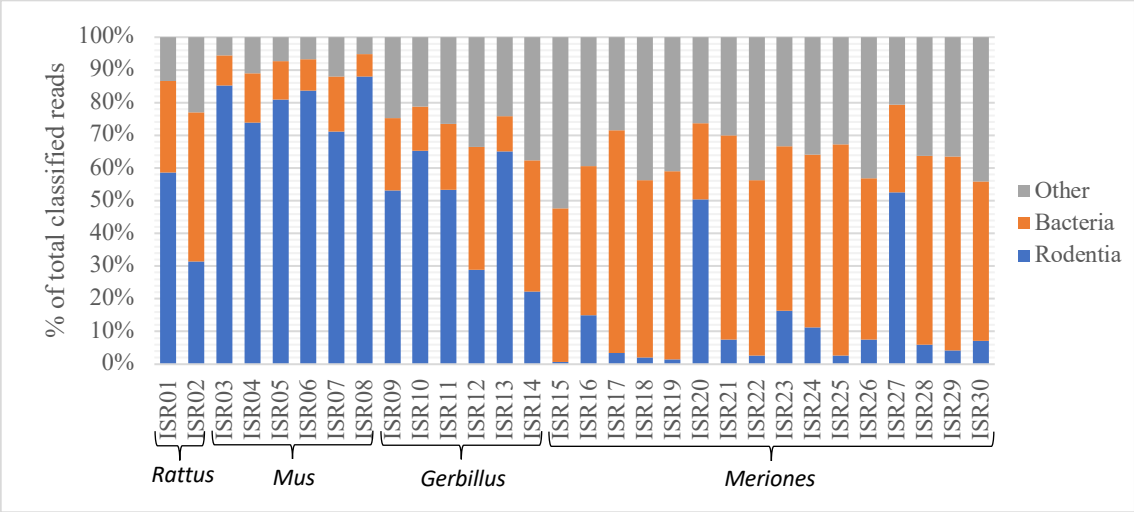


Figure 3.3: Total classified reads from metagenomic dataset of Israel samples classified through BLAST and visualized through Krona to represent overall endogenous preservation within the samples by comparing Rodentia, Bacteria (*i.e.* predominantly non-endogenous soil contaminants), and Other (*i.e.* non-rodent Eukaryotes, Viruses, Archaea).

3.7 Additional Laboratory Work

3.7.1 Canis lupus familiaris (Istanbul University, Turkey)

In addition to rodents, plague is also known to affect canids when infected rodent fleas undergo a host switch when in search of a new food source (Boone *et al.* 2009; Brown *et al.* 2011). As such, when a set of 7 samples from the genus *Canis* became available for aDNA sampling it was imperative to assess them for the presence of *Y. pestis* to identify potential alternate plague hosts in the past. These samples were processed using the same laboratory and bioinformatic methods as described above to assess the overall metagenomic content. Overall, these samples show poor preservation as the highest proportion of host specific DNA is 43% of the total classified reads in sample MT02a, with the majority of the samples from this set showing high proportions of non-endogenous bacterial DNA, such as soil bacteria or reagent contaminants, thus indicating that *Y. pestis* was not present (Fig. 3.4).

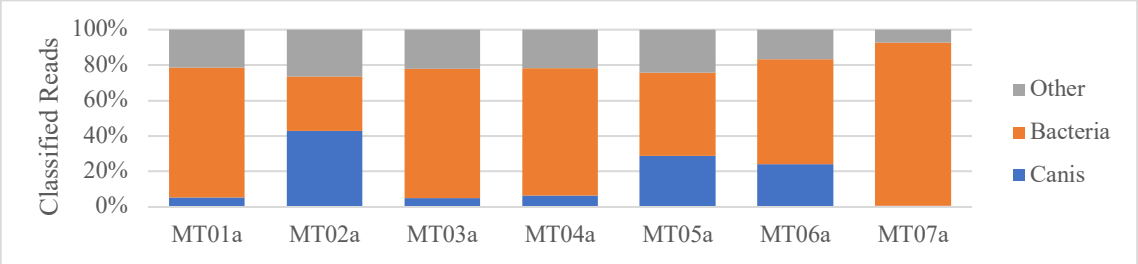


Figure 3.4: Total classified reads from metagenomic dataset of Turkey set 3 classified through BLAST and visualized through Krona to represent overall endogenous preservation within the samples by comparing Canis, bacteria (*i.e.* predominantly non-endogenous soil contaminants), and other (*i.e.* non-host species eukaryotes, viruses, archaea).

3.7.2 Homo sapiens (Istanbul University, Turkey)

A total of 19 *Homo sapiens* samples collected in Turkey were similarly assessed for the presence of *Y. pestis* aDNA and ultimately showed varying levels of endogenous preservation. Sample MT27a showed the best host DNA preservation with 77% of the total classified reads matching *H. Sapiens*, whereas the remainder of this set showed proportions of *H. sapiens* that were less than 40% of the total classified reads (Fig. 3.5).

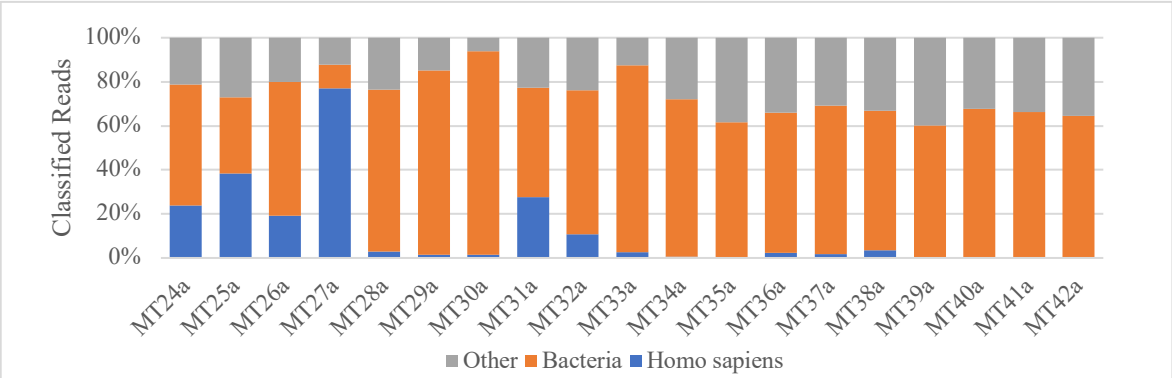


Figure 3.5: Total classified reads from metagenomic dataset of Turkey set 4 classified through BLAST and visualized through Krona to represent overall endogenous preservation within the samples by comparing Homo sapiens, bacteria (*i.e.* predominantly non-endogenous soil contaminants), and other (*i.e.* non-host species eukaryotes, viruses, archaea).

The results of the qPCR *pla* assay for this sample set showed that 3 out of the 19 samples had replicates amplifying within the proper ranges, which indicates that *Y. pestis* was potentially present (Table S3.3, Fig. 3.6a-b). Of these 3 *pla* positive samples, MT24a

was considered a weak positive as it only had 1/4 replicates amplify, MT27a was considered a strong positive as all 4 replicates amplified, and MT35a was a moderate positive as 2/4 replicates amplified (Table S3.3, Fig. 3.6a-b).

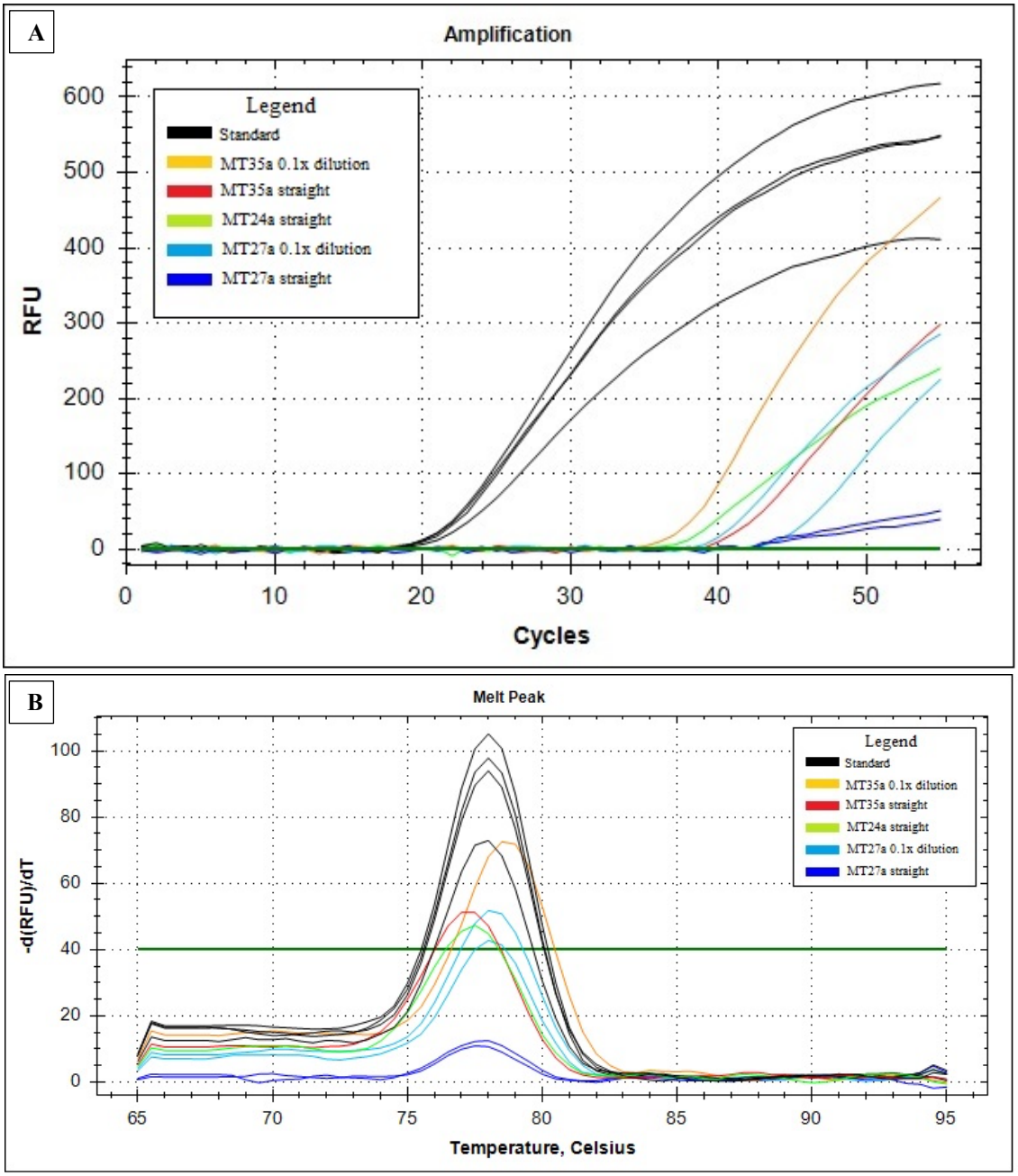


Figure 3.6: Amplification plot (a) and melt peak (b) from qPCR pla assay performed on human samples from Turkey set 4. The amplification plot (a) shows replicates for samples MT35a 0.1x dilution (yellow), MT35a straight (red), MT24a straight (green), and 1 MT27a 0.1x dilution (light blue) amplifying between

30-40 cycles and the other MT27a 0.1x dilution (light blue) as well as both MT27a straights (dark blue) amplifying past cycle 40. The melt peak (b) shows all samples falling within a close range of the pla standards with both MT27a straights (dark blue) falling below the threshold.

Although only 3/19 samples screened positively during this qPCR assay, all samples were shotgun sequenced in order to evaluate the overall metagenomic content and further corroborate or negate the presence of plague. In addition to shotgun sequencing, the 3 samples that did screen positively in the pla assay were enriched for Y. pestis prior to sequencing.

Samples MT24a, MT27a, and MT35a were enriched first for the entire plague genome with the Megapestis baitset and then for just the pPCP1 plasmid where the *pla* gene is located. The second enrichment, targeting just the pPCP1 plasmid, was done in an attempt to verify the plague signal that appeared during the *pla* assay by focusing our efforts on the plasmid where that gene is located. All iterations of the samples showed similar metagenomic profiles to the shotgun sequencing profile in terms of the proportion of human, bacterial and "other" components, which can be seen in Figure 3.7, and there was only a slight increase in the proportion of reads mapping and classifying through BLAST to *Y. pestis* post enrichment (*i.e.* <0.1% of the total blast classified or sequenced reads) (Table S3.4). Thus, it is unlikely that *Y. pestis* is present in these samples as the signal seen in the qPCR *pla* assay was unable to be corroborated in either the shotgun or the enriched sequenced datasets. Further research here to assess the samples for inhibition, repeating the Megapestis enrichment, or sequencing the samples to a greater extent may allow us to uncover *Y. pestis* if it is present within these samples, as it is still unclear why the qPCR assay yielded positive results whereas the sequencing data did not.

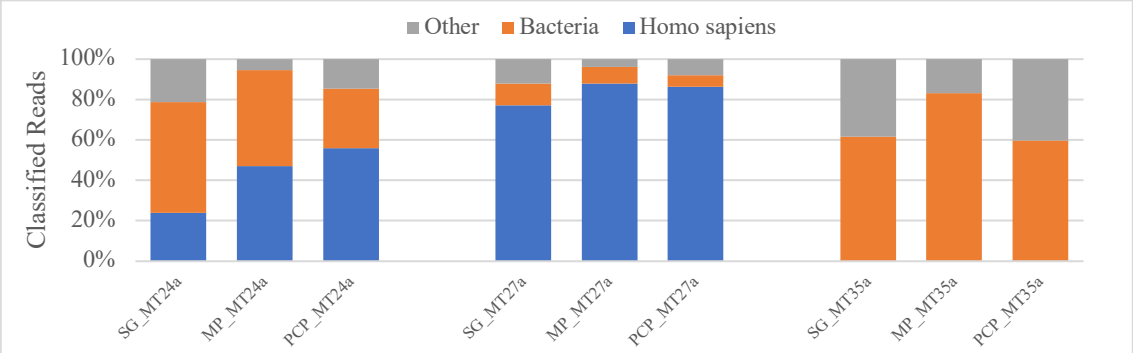
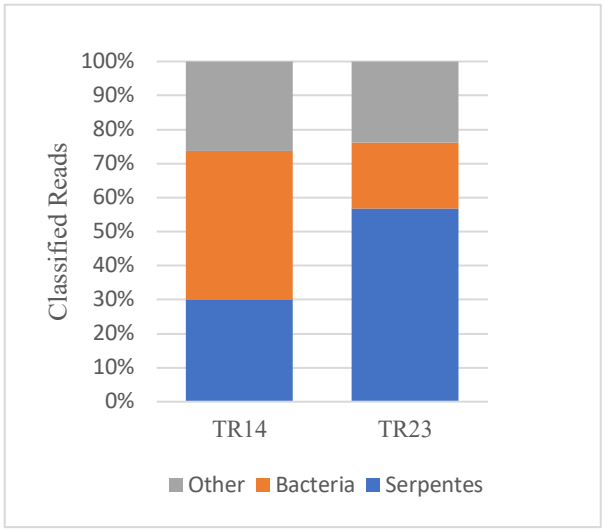


Figure 3.7: Comparison between shotgun (SG), Megapestis enriched (MP), and pPCP1 enriched (PCP) metagenomic datasets of Turkey set 4. Total classified reads from metagenomic dataset classified through BLASTn and visualized through Krona to represent overall endogenous preservation within the samples by comparing Homo sapiens, Bacteria (*i.e.* predominantly non-endogenous soil contaminants), and Other (*i.e.* non-host species Eukaryotes, Viruses, Archaea).

3.7.3 Serpentes (Istanbul University, Turkey)

The second set of rodent remains received from Istanbul University, Turkey (n=26, discussed above) originally consisted of 28 samples identified as being within the order *Rodentia*, however, 2 samples – TR14 and TR23 – were ultimately classified through the taxonomic analysis as being within the suborder *Serpentes* (Fig. 3.8a). Samples TR14 and TR23 were identified as snakes as they showed high proportions of *Serpentes* classified reads in the metagenomic profile created in BLAST, 30% and 57% respectively, and very small rodent components (*i.e.* <5%) (Fig. 3.8a). The material used for these samples was a single vertebra and due to its small size and close association with other small rodent bones, it is unsurprising that it appeared at first glance to be a rodent vertebra as opposed to a snake vertebra (Fig. 3.8b).



A

B

Figure 3.8: A) Total classified reads from metagenomic dataset of Turkey set 2 classified through BLASTn and visualized through Krona to represent overall endogenous preservation within the samples by comparing Serpentes, Bacteria (*i.e.* predominantly non-endogenous soil contaminants), and Other (*i.e.* non-host species Eukaryotes, Viruses, Archaea); B) Skeletal material used for sample TR14 demonstrating the small size of the snake vertebra used.

3.7.4 Plasmodium Vinckeia spp. (Istanbul University, Turkey)

Two partially mummified rodent samples from the first set sent from Turkey, MT19a and MT21a (Table S3.1, Fig. 3.9a-b), presented with interesting results in their metagenomic profiles such that several reads were classifying as *Plasmodium berghei*, which is known to cause malaria in rodents. The identification of *P. berghei* in ancient samples has yet to be done, and in fact very few modern studies have even focused on this parasite, making this an interesting and potentially very important find. Initially, the metagenomic profile showed 2% and 1% of the total classified reads being assigned to *P. berghei* for MT19a and MT21a, respectively, indicating very strong support for its presence within the samples (Fig. S3.1 & S3.2).

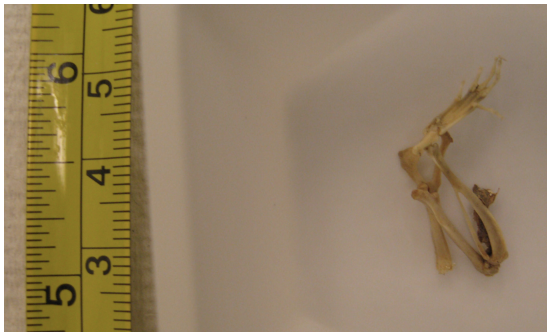
A  B 

Figure 3.9: Samples MT19a (**a**) and MT21a (**b**) comprised of bone and mummified tissue of the lower extremity.

Following this, I performed a targeted enrichment on these samples using a *Plasmodium* spp. baitset designed in the McMaster Ancient DNA Centre. This baitset consisted of human specific species *P. falciparum, P. vivax,* and *P. malariae*, which were all aligned with the *P. berghei* genome prior to enrichment and found to have ~10-15% divergence from *P. berghei*, which was deemed acceptable to capture *P. berghei* DNA within the samples. Unfortunately, the enrichment yielded very poor results where the proportion of reads mapping and classifying (via BLAST) to *P. berghei* declined. Following this, the shotgun sequencing data was reanalyzed with an updated version of the BLAST database and showed a drastic drop to 0.0003% and 0% of reads classifying as *P. berghei* in samples MT19a and MT21a, respectively. Interestingly, we did see a shift in preference for *P. yoelii* - another rodent specific malarial parasite - over *P. berghei* in the updated database with 0.4% and 0.3% of total classified reads in MT19a and MT21a, respectively.

These inconsistencies led to extensive troubleshooting, which included first applying an edit distance filter to ensure there were no residual adapters that could have resulted in non-specific mapping or classifications in BLAST of adapter sequences with

low complexity regions of the *P. berghei* genome. Following this I filtered out any reads mapping to the *M. musculus* whole genome and both remapped the remaining sequences to *P. berghei* as well as reclassified the remaining sequences with BLAST. These initial troubleshooting attempts showed no significant decrease in reads mapping and classifying to *Plasmodium* (*Vinckeia*) spp., *i.e.* rodent malarial parasites, indicating that the high proportion of reads initially classifying as these species were not simply misclassified reads from either adapter or *M. musculus* sequences.

Following this, I assessed the mapped data (filtered for *M. musculus*) in Geneious (version 6.1.8) and observed that the majority of reads were mapping to low complexity regions, which resulted in the discovery that *Plasmodium* spp. are typically notorious for their low complexity genomes, thus making it difficult to successfully identify without long and well preserved reads. I then ran the sequence data through another metagenomic classifier, Kraken, and re-mapped the mouse-filtered reads to the *P. berghei* mitochondrial genome, which resulted in drastic reductions of reads classifying and mapping to *P. berghei*.

Further troubleshooting resulted in the discovery that the change in reads classifying as *Plasmodium* (*Vinckeia*) spp. observed in the updated BLAST database was due to the removal of many *P. berghei* sequences as a consequence of low complexity and unusually high similarities to *M. musculus*. Why these low complexity reads had such a high affinity for mapping or classifying as *Plasmodium* (*Vinckeia*) spp. even when *M. musculus* reads were filtered out is still unclear, however, as further investigation into this

phenomenon was not within the scope of this MA thesis these answers will unfortunately remain unanswered until potential future research can shed light on these issues.

3.7.5 Pox Project Rodents (Chicago Field Museum)

A total of 147 DNA extracts were sent to the Ancient DNA Centre from collaborators Dr. Molly McDonough and Dr. Adam Ferguson at Chicago State University and the Chicago Field Museum, respectively. This sample set consisted of various rodents, predominantly *Gerbillus* spp., and a small number of snakes from Africa and the Middle East collected between 1909-2016. I converted these extracts into indexed libraries and subsequently sequenced them at 2,000,000 reads per sample to allow for other researchers involved in the project, namely Dr. Ana Duggan, to assess the metagenomic profiles in an effort to identify potential rodent reservoirs of Orthopoxviruses.

3.8 Discussion

3.8.1 Turkey Sample Set

Overall, the Turkey sample set showed variable levels of preservation with 15 samples (14 rodents, 1 human) having >50% endogenous DNA. These well preserved samples can be seen in Fig. 3.2-3.3 with high levels of host specific DNA that is able to be successfully classified through BLAST in comparison to low proportions of sequences classifying as soil bacteria, viruses, etc. When samples have good preservation of host species specific DNA, we would expect that the DNA from any pathogens that were present at the time of death would also have a higher chance of being preserved and

identified through aDNA analysis. However, when searching through the sequence data in an attempt to identify *Y. pestis*, we were unable to confidently identify a strong DNA signal for the presence of the pathogen. Interestingly, all 15 samples from Turkey that showed >50% endogenous DNA preservation were extracted strictly from bone material.

The samples extracted from teeth did not perform as well in terms of endogenous DNA preservation, with proportions well below 50%, even though the exterior of the tooth and placement in the jaw typically acts as a protective barrier allowing DNA to preserve within teeth for long periods of time (Adler *et al.* 2011). Of the 15 samples that performed well in terms of endogenous DNA preservation, 8 were sourced from portions of the femur including the metaphyseal and epiphyseal regions. A recent study by Antinick & Foran (2019) in fact demonstrates – in a modern context – that distal regions of long bones outperform the diaphyseal region in terms of DNA quantity retrieved. In this study, Antinick & Foran (2019) postulated that DNA might be present in higher quantities in regions that experienced higher levels of bone remodeling during life, such as points of articulation like the femoral head, which could indicate why such regions yield more DNA after death (Antinick & Foran 2019). Thus, although teeth are typically the preferred sample material for identifying blood-borne pathogens in ancient remains, it appears as though well-preserved weight bearing long bones – particularly the metaphyseal and epiphyseal regions – can be useful for extracting endogenous host DNA.

As *Y. pestis* was of high interest in this research I performed multiple methods in an effort to identify it within the samples such as a *pla* qPCR assay, assessing for classified reads through BLAST and Kraken, and mapping to the *Y. pestis* CO92 whole

genome. None of these attempts were successful as the majority of samples had less than 2 PCR replicates amplify indicating that the *pla* gene and thus the pPCP1 plasmid of *Y. pestis* was not identified. Upon assessing the metagenomic profiles of all samples, less than 0.1% of total reads classified as *Y. pestis* in BLAST. Finally, all samples were mapped to the *Y. pestis* CO92 genome with a minimum of 35 base pairs and a mapping quality of 30 in order to verify its presence or absence in the samples, which resulted in 0 mapped reads for the majority of cases. Even in samples with good endogenous DNA preservation we were unable to successfully find *Y. pestis* DNA, meaning that even under the best conditions it was still not identified. Thus, as all of these attempts were unsuccessful, it can be concluded that *Y. pestis* was either not present in these samples or unable to be identified at this time.

3.8.2 Israel Sample Set

Several samples within the Israel set showed very good DNA preservation as can be seen in the high levels of rodent DNA that was able to be successfully mapped with strict filters for fragment length and mapping quality (Table S3.5). Good endogenous preservation is also supported for 13/30 samples with >50% sequences classifying as *Rodentia* after being run through BLAST. (Fig. 3.3). Interestingly, the samples that have the highest rodent content overall in the Israel collection (in the metagenomic classifications) are from the genus *Mus* followed by those from the genus *Rattus* and *Gerbillus*, with *Meriones* having the lowest rodent content (Fig. 3.3).

This could indicate that the different species occupied the site at different time periods, with the better performing samples being much more recent, or perhaps there

were environmental differences in the locations the samples were found (*i.e.* acidic soil pH or flooded sections of the site where the poorer performing samples were found). Another potential explanation for the difference in rodent content between the samples is that there could be a database bias where *Gerbillus* and *Meriones* species could be underrepresented in the NCBI BLAST database. However, unless the genomes of these species are highly diverged from other rodent species in the database (such as *Rattus spp.* or *Mus spp.*) we would still expect a larger portion of unspecified *Rodentia* to be identified at the very least. I am currently unaware of any such differences in recovery location, environmental conditions in those regions, or issues with the BLAST database and am therefore unable to make any concrete conclusions about the endogenous DNA preservation trends we see between the different samples.

Of the 13 samples that performed well in terms of endogenous content, 11 were comprised of an amalgamation of bone and teeth (*i.e.* mandible/maxilla, molar, and incisor) as opposed to just teeth. Of the remaining 17 samples that did not perform well in terms of endogenous content (*i.e.* <50% classifying as *Rodentia*), 12 were comprised solely of teeth and 5 were again a combination of bone and teeth. This could indicate that the teeth were poorly preserved or, as was the case with certain molars utilized, perhaps the teeth were too small to yield ample concentrations of endogenous DNA.

As with the Turkey sample set, I performed multiple methods in an effort to identify *Y. pestis* within the samples from Israel. None of these attempts proved successful as no samples had 3-4 PCR replicates amplify, indicating that the *pla* gene and thus the pPCP1 plasmid of *Y. pestis* was not identified (Table S3.6). Moreover, less than

0.007% of the total metagenomic dataset classified as *Y. Pestis* when run through BLAST and less than 0.0004% of each sample successfully mapped to the *Y. Pestis* CO92 whole genome (Table S3.5). Therefore, in line with the Turkey sample set, it can be concluded that *Y. pestis* was either not present or simply not able to be identified in the samples originating from Israel.

3.8.3 Challenges with aDNA

There are several factors that can affect the success of aDNA research projects including sample availability, preservation, contamination, and quality of the aDNA recovered (Spyrou *et al.* 2019). For blood borne pathogens, such as *Y. pestis*, it is best to target highly vascularised skeletal material such as the pulp cavity of the tooth, however, tooth material was only available for 49/72 samples. The remaining samples consisted primarily of long bones, the interior of which does not preserve as well as teeth that are protected from the external conditions by their placement in the jawbones and layer of enamel (Higgins and Austin 2013). Furthermore, several samples – both bone and teeth - showed signs of poor preservation as they were extremely dry and brittle. Thus, even if *Y. pestis* had been present in those samples it was likely to have been degraded beyond identification and perhaps could have been located had better preserved sample material been available.

On top of sample availability and preservation issues, contamination is yet another difficulty with aDNA research, particularly when the target DNA in question is that of endogenous pathogens present at the time of death of the individual being studied. The pathogen target DNA often makes up less than 1% of the total DNA present within the

sample (Spyrou *et al.* 2019), leaving the rest of the sample to be comprised of contaminant DNA introduced from the surrounding environment, laboratory reagents, or human handling.

Several of the samples in this project demonstrated high proportions of non-endogenous (*i.e.* contaminant) DNA from common soil bacteria, which could indicate that *Y. pestis* was either not present and the sample was poorly preserved or that it was not successfully extracted from the sample in favour of contaminant DNA present at higher concentrations. DNA quality is another challenge with ancient samples as chemical processes that occur within the body after death as well as thermal and UV damage over time can alter and degrade the DNA within the sample (Mouttham *et al.* 2015). Although the laboratory methods used in the aDNA centre have been adapted to favour short molecules that have experienced such damage, the samples processed here were filtered for reads that were a minimum of 35 base pairs and had a mapping quality of 30, meaning that we could have lost *Y. pestis* reads - had they been present - if they were degraded below these thresholds.

3.9 Conclusions

Of the 72 rodent samples processed in this research project, none yielded successful *Y. pestis* DNA thus indicating that *Y. pestis* was not present in these samples or was unable to be successfully identified. Although *Y. pestis* was not identified in these samples, the methods that we use at the McMaster Ancient DNA centre are seen to be of value in extracting the endogenous DNA present in many samples and thus if future

samples contain sufficient amounts of endogenous pathogen DNA, I am confident that these methods would be successful in positively identifying it.

In addition to the rodent samples, a variety of other samples were processed as new, non-rodent, samples became available or interesting pathogens/parasites, other than *Y. pestis*, were identified within the rodent samples being studied. Although these results were not the intended goal of the current research project, they were still interesting to investigate and ultimately amass to an incredible amount of experience and intrigue. Although no positive results were harvested from these additional samples, there still remain some unanswered questions that may be addressed if further research is performed on this material, such as new bioinformatic troubleshooting techniques, deeper sequencing, or further and more refined targeted enrichments.

Overall, throughout my thesis research I processed a total of 242 samples in the lab and analyzed the sequencing data for 100 of those samples. Although no pathogens/parasites of interest were successfully mined from these samples, the negative results curated over the last two years of research presented here resulted in an invaluable set of skills and immense experience in aDNA research and further demonstrates the need for future and more extensive research into rodent reservoirs of plague. Successfully identifying *Y. pestis* in ancient rodent remains is incredibly important as it would add to the genomic history of plague and answer long-standing questions about how the disease was able to be maintained and reintroduced over extended periods of time in the past.

3.10: Supplementary Tables and Figures

Table S3.1: Overview of samples processed from collection sent from Istanbul, Turkey.

Set #	Original ID	Sample ID	Sample Type	Genus
Set 1	A8	MT08a	Bones	Rattus
	A9	MT09a	Skull	Rattus
	C1	MT10a	Skull	Rattus
	D2	MT11a	Mand+bone	Rattus
	D3	MT12a	Femur	Rattus
	D4	MT13a	Mandible	Rattus
	D5	MT14a	Mandible	Rattus
	E1	MT15a	skull+femur	Rattus
	E2	MT16a	Mandible	Rattus
	E3	MT17a	Femur	Rattus
	E4	MT18a	Humerus+vertebra	Rattus
	I9	MT19a	left extremity	Rattus
	I10	MT20a	right extremity	Rattus
	I11	MT21a	right extremity	Rattus
	D1	MT22a	Skull; mole rat	Spalax
	A30	MT23a	Coxae-left	Rabbit
Set 2	C32	TR01	Humerus left	Rabbit
	D59	TR02	Femur right	Rattus
	D60	TR03	Femur right	Rattus
	D61	TR04	Femur right	Rattus
	D62	TR05	Femur left	Rattus
	D63	TR06	Antebrachium right	Spalax
	D64	TR07	Coxae right	Rattus
	D65	TR08	Mandible right	Rattus
	D66	TR09	Skull	Spalax
	D67	TR10	Mandible left	Spalax
	D68	TR11	Skull	Spalax
	D69	TR12	Mandible left	Spalax
	D70	TR13	Skull	Spalax

	D71	TR14	Vertebrae	Serpentes
	D72	TR15	Mandible left	Spalax
	D73	TR16	Mandible left	Spalax
	D74	TR17	Skull	Rattus
	D75	TR18	Humerus left	Rattus
	D76	TR19	Coxae left	Rattus
	D77	TR20	Femur left	Rattus
	D78	TR21	Tibia right	Rattus
	D79	TR22	Femur right	Rattus
	D81	TR23	Vertebrae	Serpentes
	D82	TR24	Tibia left	Rattus
	D84	TR25	Tibia left	Rattus
	D85	TR26	Femur left+Vertebrae+skin	Rattus
	F15	TR27	Skull	Rattus
	J4	TR28	Skull	Rattus
Set 3	A1	MT01a	Tooth	Canis
	A2	MT02a	Tooth	Canis
	A3	MT03a	Tooth	Canis
	A4	MT04a	Tooth	Canis
	A5	MT05a	Tooth	Canis
	A10	MT06a	Bone	Canis
	H3	MT07a	Tooth	Canis
Set 4	A31	MT24a	Bone	Homo
	A32	MT25a	Tooth	Homo
	A33	MT26a	Bone	Homo
	A34	MT27a	Bone	Homo
	A35	MT28a	Bone	Homo
	B1	MT29a	Tooth	Homo
	B2	MT30a	Tooth	Homo
	B3	MT31a	Bone	Homo
	B4	MT32a	Tooth	Homo
	B5	MT33a	Tooth	Homo
	B6	MT34a	Tooth	Homo
	B7	MT35a	Tooth	Homo
	D6	MT36a	Bone	Homo

	F1	MT37a	Bone	Homo
	F2	MT38a	Tooth	Homo
	F3	MT39a	Bone	Homo
	F4	MT40a	Bone	Homo
	H1	MT41a	Bone	Homo
	H2	MT42a	Bone	Homo

Table S3.2: Overview of samples received from Haifa University, Israel and type of sample material processed in the McMaster aDNA lab. Mu = Mus; Me = Meriones; G = Gerbillus.

Original ID	Sample ID	Sample Type	Genus	Material Used
MMB-714	ISR01	Bone: skull fragments	Rattus	portion of maxilla and teeth
MMB-717	ISR02	Bone: skull fragments	Rattus	incisor and molar
A-7658-1Mu	ISR03	Bone: mandibles	Mus	mandible, molar and incisor
A-7658-2Mu	ISR04	Bone: mandibles	Mus	mandible, molar and incisor
A-7658-3Mu	ISR05	Bone: mandibles	Mus	mandible, molar and incisor
A-7658-4Mu	ISR06	Bone: mandibles	Mus	mandible, molar and incisor
A-7658-5Mu	ISR07	Bone: mandibles	Mus	mandible, molar and incisor
A-7658-6Mu	ISR08	Bone: mandibles	Mus	mandible, molar and incisor
A-7658-1G	ISR09	Bone: mandibles	Gerbillus	mandible, molar and incisor
A-7658-2G	ISR10	Bone: mandibles	Gerbillus	mandible, molar and incisor
A-7658-3G	ISR11	Bone: mandibles	Gerbillus	mandible, molar and incisor
A-7658-4G	ISR12	Bone: mandibles	Gerbillus	mandible, molar and incisor
A-7658-5G	ISR13	Bone: mandibles	Gerbillus	mandible and molars
A-7658-6G	ISR14	Bone: mandibles	Gerbillus	mandible, molar and incisor
A-7658-1Me	ISR15	Bone: mandibles	Meriones	incisor and molar
A-7658-2Me	ISR16	Bone: mandibles	Meriones	mandible, molar and incisor
A-7658-3Me	ISR17	Bone: mandibles	Meriones	incisor and molar
A-7658-4Me	ISR18	Bone: mandibles	Meriones	incisor and molar
A-7658-5Me	ISR19	Bone: mandibles	Meriones	incisor and molar
A-7657-6Me	ISR20	Bone: mandibles	Meriones	incisor and molar
A-7658-7Me	ISR21	Bone: mandibles	Meriones	incisor and molar
A-7658-8Me	ISR22	Bone: mandibles	Meriones	incisor and molar
A-7658-9Me	ISR23	Bone: mandibles	Meriones	mandible, molar and incisor
A-7658-10Me	ISR24	Bone: mandibles	Meriones	incisor and molar
A-7658-11Me	ISR25	Bone: mandibles	Meriones	incisor and molar

A-7658-12Me	ISR26	Bone: mandibles	Meriones	mandible, molar
A-7658-13Me	ISR27	Bone: mandibles	Meriones	incisor and molar
A-7658-14Me	ISR28	Bone: mandibles	Meriones	incisor and molar
A-7658-15Me	ISR29	Bone: mandibles	Meriones	incisor and molar
A-7658-16Me	ISR30	Bone: mandibles	Meriones	incisor and molar

Table S3.3: Results from the pla qPCR assay of Turkey sample set #4 showing the number of replicates amplifying for each sample (out of 4 total; 2 straights and 2 1:10 dilutions) indicating the presence or absence of the pla gene on the pPCP1 plasmid of Yersinia pestis.

Sample ID	Number of replicates amplifying
MT24a	1
MT27a	4
MT35a	2

Table S3.4: Shotgun sequencing data vs enrichment data of Turkey sample set #4 with the megapestis and pPCP1 baitsets showing the percent of unique reads mapping to the human mitochondrial genome (rCRS) and Y. pestis C092 whole genome out of the total sequenced reads for each sample at a minimum of 35 basepairs and a map quality of 30.

Shotgun data		**Human mitochondrial genome (rCRS)**		**Ypestis C092 whole genome**	
Sample ID	**Sequenced clusters**	**min35MQ30 BWA**	**% of total**	**min35MQ30 BWA**	**% of total**
MT24a	1099258	1221	0.11%	3	0.0003%
MT27a	661622	1541	0.23%	0	0%
MT35a	1314594	0	0%	5	0.0004%
Megapestis Enrichment data		**Human mitochondrial genome (rCRS)**		**Ypestis C092 whole genome**	
Sample ID	**Sequenced clusters**	**min35MQ30 BWA**	**% of total**	**min35MQ30 BWA**	**% of total**
MT24a	1159078	22103	1.91%	51	0.005%
MT27a	968398	11491	1.19%	9	0.001%
MT35a	699317	3	0.0004%	21	0.003%
pPCP1 Enrichment data		**Human mitochondrial genome (rCRS)**		**Ypestis C092 whole genome**	
Sample ID	**Sequenced clusters**	**min35MQ30 BWA**	**% of total**	**min35MQ30 BWA**	**% of total**
MT24a	1729436	15	0.001%	2	0.0001%

MT27a	970800	25	0.003%	10	0.0010%
MT35a	1283426	0	0%	3	0.0002%

Table S3.5: Shotgun sequencing data for Israel sample set showing the percent of unique reads mapping to the *Mus musculus* and *Yersinia pestis* CO92 whole genomes out of the total sequenced reads for each sample at a minimum of 35 basepairs and a map quality of 30.

		Mapped to Mus musculus whole genome		**Mapped to Ypestis C092 whole genome**	
Sample ID	**Sequenced clusters**	**min35MQ30 BWA**	**% of total**	**min35MQ30 BWA**	**% of total**
ISR01	1686989	16363	0.9700%	5	0.0003%
ISR02	1341626	4059	0.3025%	5	0.0004%
ISR03	1224917	218796	17.8621%	1	0.0001%
ISR04	1081455	79703	7.3700%	1	0.0001%
ISR05	1168584	116999	10.0120%	0	0.0000%
ISR06	1137965	151467	13.3103%	4	0.0004%
ISR07	1510314	92268	6.1092%	0	0.0000%
ISR08	1181056	198268	16.7873%	3	0.0003%
ISR09	1514267	6472	0.4274%	1	0.0001%
ISR10	1453409	8747	0.6018%	0	0.0000%
ISR11	1147671	4636	0.4039%	0	0.0000%
ISR12	1166919	1783	0.1528%	2	0.0002%
ISR13	1828560	12768	0.6983%	0	0.0000%
ISR14	1275604	1783	0.1398%	1	0.0001%
ISR15	1212590	50	0.0041%	2	0.0002%
ISR16	2129999	1770	0.0831%	3	0.0001%
ISR17	1469814	356	0.0242%	1	0.0001%
ISR18	1444391	127	0.0088%	2	0.0001%
ISR19	1140905	72	0.0063%	2	0.0002%
ISR20	1104216	4944	0.4477%	1	0.0001%
ISR21	3771855	1856	0.0492%	7	0.0002%
ISR22	865374	128	0.0148%	1	0.0001%
ISR23	1973385	2334	0.1183%	5	0.0003%
ISR24	1153803	673	0.0583%	1	0.0001%
ISR25	1051088	159	0.0151%	2	0.0002%
ISR26	1263931	497	0.0393%	2	0.0002%
ISR27	1141121	6306	0.5526%	1	0.0001%

ISR28	1361388	507	0.0372%	2	0.0001%
ISR29	1167341	246	0.0211%	1	0.0001%
ISR30	2354661	939	0.0399%	2	0.0001%

Table S3.6: Results from the *pla* qPCR assay for the Turkey sample set showing the number of replicates amplifying for each sample (out of 4 total; 2 straights and 2 1:10 dilutions) indicating the presence or absence of the *pla* gene on the pPCP1 plasmid of *Yersinia pestis*.

Sample ID	# straight replicates amplifying	# dilution replicates amplifying	total # of replicates amplifying
MT01	0	0	0
MT02	1	0	1
MT03	0	0	0
MT04	0	0	0
MT05	0	0	0
MT06	2	0	2
MT07	0	0	0
MT08	0	0	0
MT09	0	0	0
MT10	0	0	0
MT11	0	0	0
MT12	0	1	1
MT13	0	1	1
MT14	0	1	1
MT15	0	0	0
MT16	0	0	0
MT17	0	0	0
MT18	0	0	0
MT19	0	0	0
MT20	0	0	0
MT21	0	0	0
MT22	1	0	1
MT23	0	0	0
MT24	1	1	2
MT25	0	0	0
MT26	0	0	0
MT27	2	2	4
MT28	0	0	0

MT29	1	0	1
MT30	0	1	1
MT31	0	0	0
MT32	0	0	0
MT33	0	1	1
MT34	0	0	0
MT35	1	1	2
MT36	0	0	0
MT37	0	0	0
MT38	0	1	1
MT39	0	0	0
MT40	0	0	0
MT41	1	0	1
MT42	0	1	1
TR01	0	0	0
TR02	0	0	0
TR03	1	0	1
TR04	0	0	0
TR05	0	0	0
TR06	0	0	0
TR07	0	0	0
TR08	0	0	0
TR09	0	0	0
TR10	0	1	1
TR11	0	1	1
TR12	0	0	0
TR13	1	0	1
TR14	0	0	0
TR15	1	0	1
TR16	1	0	1
TR17	2	0	2
TR18	0	0	0
TR19	0	1	1
TR20	0	0	0
TR21	0	0	0
TR22	0	1	1
TR23	0	0	0

TR24	0	0	0
TR25	0	0	0
TR26	0	0	0
TR27	0	0	0
TR28	0	0	0

Table S3.7: Results from the pla qPCR assay for the Israel sample set showing the number of replicates amplifying for each sample (out of 4 total; 2 straights and 2 1:10 dilutions) indicating the presence or absence of the pla gene on the pPCP1 plasmid of Yersinia pestis.

Sample ID	# of straight replicates amplifying	# of dilution replicates amplifying	total # of replicates amplifying
ISR01	1	0	1
ISR02	0	0	0
ISR03	0	1	1
ISR04	0	1	1
ISR05	0	0	0
ISR06	0	0	0
ISR07	0	0	0
ISR08	1	1	2
ISR09	0	1	1
ISR10	0	0	0
ISR11	0	1	1
ISR12	0	1	1
ISR13	0	0	0
ISR14	0	2	2
ISR15	0	0	0
ISR16	1	0	1
ISR17	0	0	0
ISR18	0	0	0
ISR19	0	0	0
ISR20	0	1	1
ISR21	1	0	1
ISR22	0	0	0
ISR23	0	0	0
ISR24	0	0	0

ISR25	0	0	0
ISR26	0	0	0
ISR27	1	1	2
ISR28	0	0	0
ISR29	0	1	1
ISR30	0	0	0

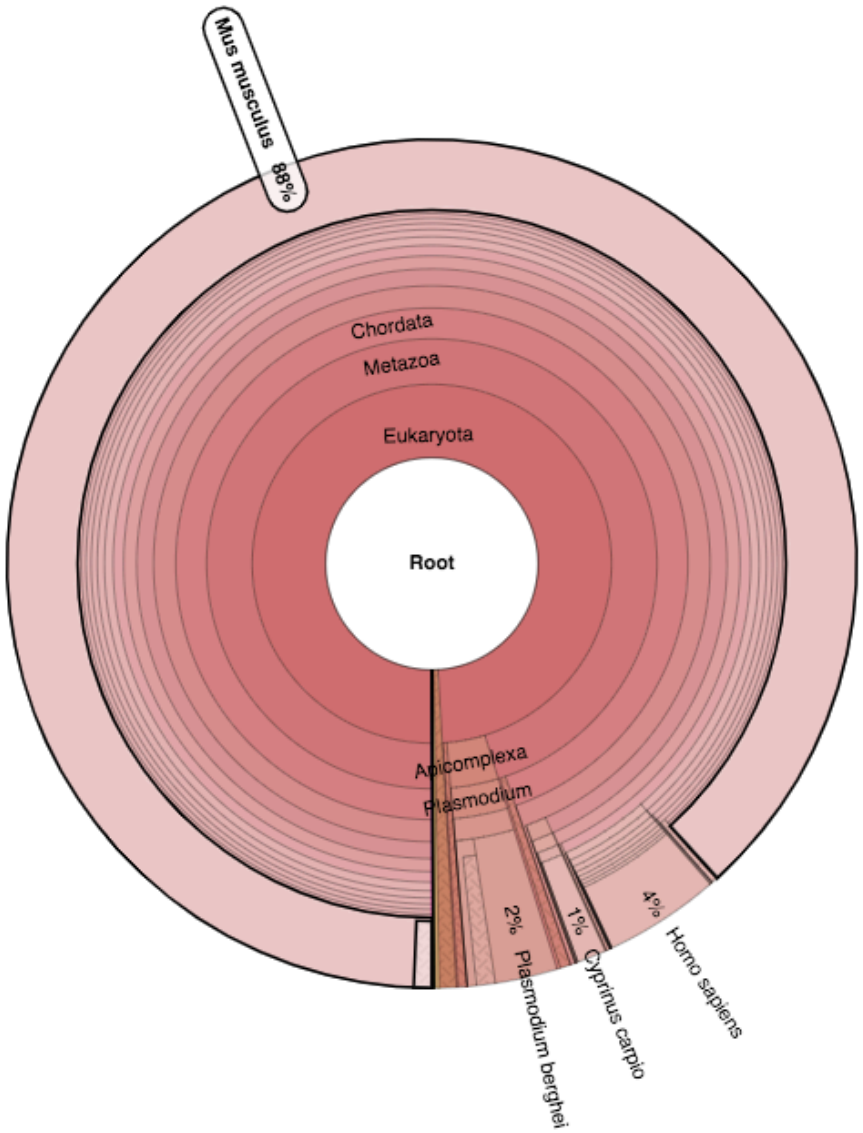


Figure S3.1: Krona plot of sample LMT19a from Turkey collection demonstrating the relative proportions of reads classified to M. musculus (88%) and P. berghei (2%).

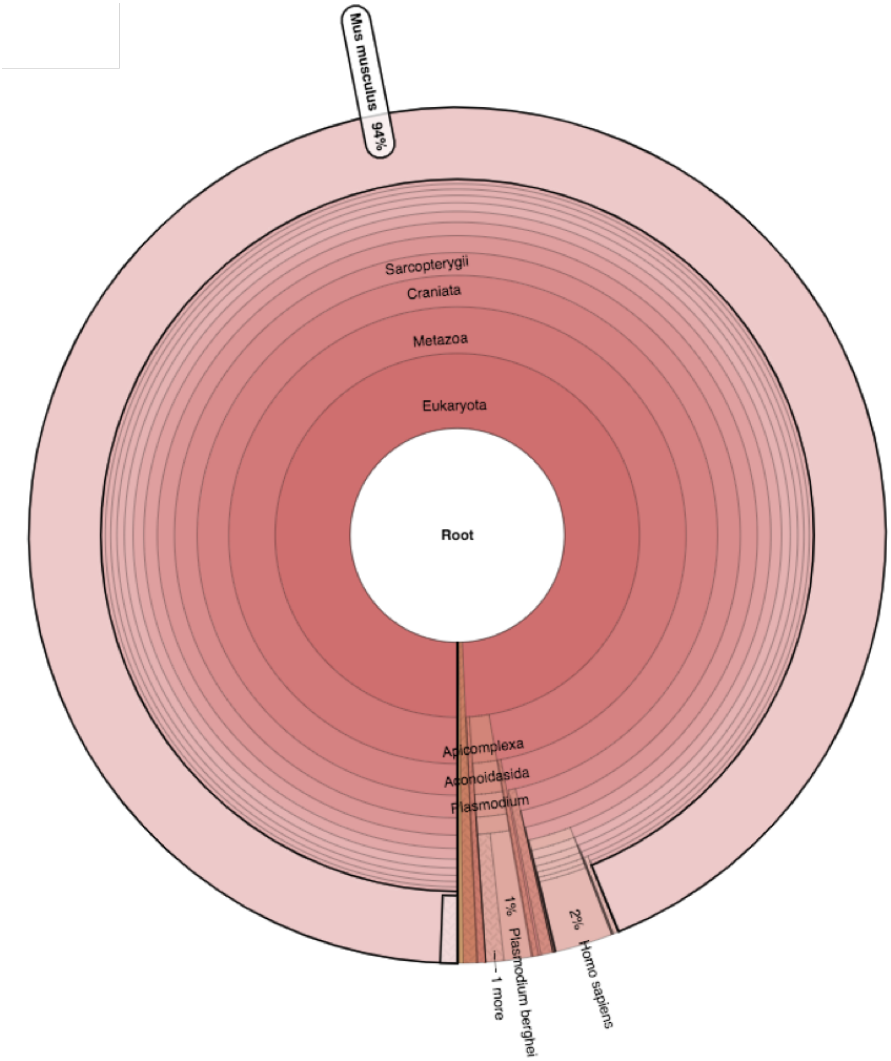


Figure S3.2: Krona plot of sample LMT21a from Turkey collection demonstrating the relative proportions of reads classified to M. musculus (94%) and P. berghei (1%).

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Chapter 4.0: Conclusion

4.1 Summary and Contributions

4.1.1 Rodent Reservoir Database

The research presented in this thesis aimed to shift the perspective of plague scholarship towards a model of plague maintenance which incorporates a rodent-reservoir mechanism of disease reintroduction. First, we curated a list of 45 non-*Rattus* rodent species capable of hosting plague and accumulated host characteristics to observe trends in the traits of reservoir species. This research culminated in the development of the first publicly available and interactive database of modern rodent reservoirs which includes embedded species and environmental information (*Rodent Reservoirs of Plague: Modern Distributions and Host Characteristics*, available at ArcGIS Online).

This database will not only allow us to target potential rodent reservoirs in the past but will also aid in research on modern rodent reservoirs that persist in natural foci in more than 30 countries to this day (WHO 2016). To our knowledge, no recently updated comprehensive database of rodent reservoirs including host characteristics has been published, which represents a weak point in plague scholarship as it leaves the focus to be placed predominantly on the black rat (*R. rattus*). However, by presenting 45 other rodent reservoirs species we hope to eliminate that bias in plague scholarship and emphasize the complexities of the sylvatic plague cycle, such that many species can be involved in maintaining and reintroducing the bacterium.

4.1.2 Shifting away from Eurocentrism

Additionally, the limited historical attention to plague in the Mediterranean and Middle East has led to the exclusion of this region in modern analysis in terms of the level and quality of research devoted to this area as opposed to that conducted on mainstream Europe. By incorporating examples of plague from Mediterranean and Middle Eastern regions, we sought to include them in the discussion and demonstrate their lengthy history of plague. With this we hope to spur future research into these regions so we can work towards a better understanding of the complete history of plague, not just the Eurocentric experience with the disease.

4.1.3 aDNA Analysis of Ancient Rodents

Finally, we presented the first known aDNA investigation into identifying *Y. pestis* within ancient rodent remains. As cycles of plague in rodent populations are known to directly influence exposure and prevalence of the disease in humans, we hoped to improve our understanding on the spread of plague between ancient human populations and determine what allowed for the many second pandemic reintroductions. Although we did not identify plague within any of the ancient rodent samples in this thesis, we did demonstrate the ability of our laboratory methods to retrieve high quantities of endogenous DNA and ultimately reinforce the need for future and more extensive aDNA research into past rodent reservoirs.

Cumulatively, this thesis represents a large body of work that emphasizes the need to investigate the history of plague within its primary hosts, *i.e.* rodents, and place the focus on a multi-mechanism model of plague reintroduction. This study sought to

demonstrate the startling gap in plague scholarship where research on the potential role of rodent reservoirs in past pandemics has been lacking and facilitate a starting point for future and more intensive investigations to identify such reservoirs in the past.

4.2 Future Research

Moving forward, it would be beneficial to perform thermal niche modeling of the rodent reservoirs identified in this study based on past climate patterns. Modeling of this sort would allow us to determine the feasibility of these rodent reservoirs to have persisted in regions where plague was continually re-emerging during the second pandemic. Further exploration into regions without modern plague where local reservoir species have been eradicated is also necessary as the relationship between these factors has not yet been thoroughly researched and may support the connection between rodent reservoirs and local plague maintenance.

Furthermore, future research aimed towards identifying and screening ancient DNA from rodent samples is essential in order to verify their ability to host plague in the past and add to our understanding of the pathogen's history. There are many challenges that accompany aDNA research such as low abundance, poor preservation and quality, external contamination, and limited sample availability. Future research is still required to identify rodent plague reservoirs in the past via the identification of ancient *Y. pestis* DNA within them. In doing so, such studies can mitigate some of the common challenges associated with aDNA by 1) accumulating a larger sample set of rodents from key regions and time periods afflicted with plague in the past through the use of the database presented in this thesis (*Rodent Reservoirs of Plague: Modern Distributions and Host*

Characteristics available at ArcGIS Online), 2) identifying well preserved tooth material for sampling, and 3) sequencing the samples to a deeper extent or performing targeted enrichments.

Plague has devastated human populations for centuries via mechanisms not entirely understood and continues to this day in several regions around the world. As this study has demonstrated, it is imperative that we move towards a multi-host and multi-mechanism model when working to answer questions about long-term plague maintenance and reintroduction. By conducting this suggested future research into past rodent reservoirs, we can begin to piece together the complete story of how plague was able to have such a widespread impact, both spatially and temporally, in the medieval world.