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**AN INVISIBLE ENEMY: PATHOGENS, COLONIALISM, & ANCIENT DNA**

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**An Invisible Enemy: Pathogens, Colonialism, & Ancient DNA**

Nasreen Z. Broomandkhoshbacht

## Abstract

Pathogens and colonialism are interconnected threats. Pathogens are imperceptible to the naked eye and sometimes deadly. Colonialism similarly can cause death and long-lasting destruction while the structural violence often goes obscured or unacknowledged. Ancient DNA has the power to untangle stories where these "invisible enemies" intersect. This dissertation investigates the pathogens brought to South America during colonization, pathogens present in South America before colonization, alternative wet lab methodologies, and the involvement of colonialism in the field of paleogenomics itself. Using metagenomic and high-throughput ancient DNA methods, I report on pathogens found in Peru during the early period of Spanish colonization, a 5,500-year-old *Treponema pallidum*-like pathogen from the Sabana de Bogota, and human mitochondrial data recovered from ancient high-touch items such as moccasins. Connecting the past to the present, the impact of pathogens and colonialism on societies leaves a heavy imprint that can be traced through to the future.

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## Chapter 1: Introduction

The word “colonize” has multiple meanings. Among other definitions, it can describe both the violent act of subjugating people and the growth of an infectious disease agent. My dissertation examines both of these. The European colonization of the Americas impacted people, ecosystems, and landscapes. This colonization involved the transmission of disease, leading to large death tolls and health disparities that remain present in marginalized populations today (Mitchell et al., 2019; Walker et al., 2015). My dissertation is titled “An Invisible Enemy” because both the frameworks of oppression and the diseases I discuss fall into this category. Pathogens, invisible to the naked eye and often deadly, fit this description well. Infectious diseases and our bodies are mortal enemies in the most literal sense–pathogens infiltrate our bodies' defense systems and trigger our immune systems, which then find, battle, and hopefully destroy them. Pathogens are invisible enemies, but the category of “an invisible enemy” can accommodate less literal descriptions as well. For example, systems of colonialism both past and present too often go unacknowledged, in a sense “invisible”. However, the negative impacts of these colonial systems undoubtedly qualify them as an enemy.

While my doctoral degree program is listed as Biological Anthropology, my specific area of expertise is ancient DNA research, or paleogenomics. Paleogenomics itself is an interdisciplinary field; depending on the research question, it merges genomics with fields such as archaeology or paleontology. My dissertation deals specifically with the paleogenomics of pathogens. In the following chapters, I draw

upon the fields of genomics, archaeology, paleopathology, microbiology, disease ecology, and more. Interdisciplinary perspectives are necessary to fully investigate crucial questions regarding the connections between ancient and modern human health (Buikstra et al., 2022). Examining pathogen history from only one perspective results in a narrow understanding. For example, genomic analyses of ancient pathogens are useful, but they mean little without an in-depth understanding of the pathogen, the host, the ecosystem, and how biological and social systems may have been disrupted through an interconnected chain of events. Interdisciplinary contributions can also come from sources beyond the fields of Western science. For instance, oral histories and traditional knowledge are important contextualizing sources of information (Bader & Malhi, 2019; Echo Hawk, 2000; Wagner et al., 2020). The inclusion of additional knowledge pathways is necessary to broaden one's vision of pathogen history and the throughlines connecting this past with the present.

In the following chapters, I will discuss the genomics of ancient pathogens, the long-lasting impacts of colonialism on public health and Indigenous agency, and ways that ancient DNA research can be guided by and serve descendant communities rather than exploiting them.

In science, unethical research practices are also an enemy that lurks unseen. Chapter 2 discusses my ethical framework and explores the legacy and origins of ancient DNA research in its relation to white supremacy, racism, and colonization. Especially as someone who does not identify as Indigenous, "studying" the ancestors, bodies and stories of people who *are* Indigenous runs the risk of becoming

exploitative. While I do not identify as “Indigenous”, I am a woman of color, the descendant of both colonized and colonizers, and a member of an ethnic group that has survived multiple historic and ongoing genocide attempts. Furthermore, I was raised in an era of assimilation, which created a disconnect from my ancestral languages and cultural practices by harnessing shame. My positionality is not at all equivalent to that of the communities this research is about, but the shared experiences provide me with the ability to recognize injustice and the motivation to redistribute power. This chapter describes an approach to research that aligns with these values.

Chapter 3 is the overview and preliminary results of the Peruvian Pathogen Project. Broadly, this project investigates the presence and impacts of disease in Peru during Spanish conquest. Using interdisciplinary sources of knowledge, I synthesize a broad vision of the interactions between infectious diseases, ecosystems, and colonization in this context. The results here are preliminary and should be interpreted with that in mind. In the future, each of the initial findings described here will be investigated more thoroughly, and each result will likely be published as its own journal article.

Moving from a specific context with a broad pathogen focus to a broad temporal and geographic context with a specific pathogen focus, Chapter 4 takes a closer look at one particular group of pathogens: treponematoses. In the modern day, this category of diseases includes yaws, syphilis, bejel, and pinta. However, historical accounts suggest that clinical manifestations of treponematoses in the past differed

from what is seen today (Tognotti, 2009). Due to journal limitations on word count and space, I was unable to fit all of the historical and contextual background into the manuscript that became Chapter 5. To provide that richer context, I have included it here as its own chapter.

Chapter 5 investigates a *Treponema pallidum*-like ancestor. While from South America, it is not from the time of European colonization. At 5,500 years old, the genome of this pathogen predates all currently available *Treponema* genomes by millennia and provides a data point deep in time to help expand existing knowledge of the diversity of *Treponema pallidum*-like pathogens in the past. This genome may prove useful in future investigations pertaining to later *Treponema* species' divergence and evolution. This transdisciplinary project uses paleopathology, archaeology, and genomics to provide a well-rounded description of the pathogen and suggest potential characteristics of the resulting disease. Genomics analyses indicate this pathogen was distinct but closely related to all extant *T. pallidum* subspecies. The possibility that this ancient pathogen may have had a broad host range highlights the importance of screening archaeological faunal remains in the future to elucidate potential non-human reservoirs.

Chapter 6 takes a detour into a methodological discussion on the use of non-traditional DNA sources. These alternative sources and methods tend to be overlooked by ancient DNA researchers, but I argue that during the conception of a project, they should be presented to descendant communities as alternatives, albeit with some drawbacks. Implementation of these methods could combat the "invisible

enemies" of colonial frameworks and the resulting unethical research practices. However, they also have the potential to be used to perpetuate these harms. In this chapter, I explore the benefits, drawbacks, and potential ethical and legal concerns associated with these methods.

Chapter 7 is a specific example of the use of a non-traditional DNA source discussed in the prior chapter: moccasins, cordage, and mats from Promontory Caves. While there are no controversial results, we were unable to meet with tribal leadership from all implicated groups to discuss final results and/or publication. Consequently, this chapter is embargoed until we are able to meet with and gain approval from all stakeholders.

Chapter 8, my conclusion, is much like this introduction. While in this introduction I discuss how the concepts of each chapter are connected, in the conclusion I will discuss how the results and information presented all come together to form a new understanding borne out of my PhD research. Additionally, I've included an addendum of artistic expression connected to one of the deepest-running themes of my PhD: colonial museum collections as a source of injustice.

Colonization can kill. This is true whether through a pathogen's colonization of a body, a settler's destruction of the landscape, physical violence inflicted upon the marginalized, or colonial ideals embedded in the structures of our society. In the following chapters, I focus on the genomics of ancient pathogens, how the transmission of infectious diseases from colonizers to colonized is connected to present injustice, and the ways ancient DNA researchers can reduce their perpetuation

of colonial violence. Through multiple projects and perspectives, I aim to make visible these invisible enemies of the past and present.

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## Chapter 2: "Somebody Has To Do It"
## Ancient DNA as a New Frontier for Ethical Considerations

### Perspective and Introduction

I am by no means an expert on law, or tribal policies, or the Indigenous experience in North America. I do not identify as Indigenous, and am certainly not a member of a North American Indigenous group. I do not even consider myself an expert on NAGPRA[1], the one policy I actually interact with. This chapter is not meant to provide an authoritative source on any of these things. I live and work in the United States, a white supremacist settler colony; the dehumanization and violence inflicted upon marginalized people in this society is manyfold and takes various forms (Kalman, 2010). It is present in all aspects of our society, and is the foundational origin of many fields of research. While we might like to think we have moved past it, that we have reached a point where we can check off the to-do box marked "decolonize", it is still a prominent issue in ancient DNA research. Decolonization is an ongoing process, which means standards need to be continually re-assessed. Ethical standards in ancient DNA when working with marginalized communities are outdated, sometimes offensive, and often treated as an obstacle to "progress" and met with opposition. The following is an overview of those ethical standards and a discussion of how frameworks of white supremacy and colonialism are pervasive in paleogenomics.

[1] In short, NAGPRA, or the Native American Graves Protection and Repatriation Act, is a piece of US legislation mandating the return of ancestors, burial items, sacred items, and items of cultural patrimony.

In this chapter, I examine the ethics of using human remains (referred to here as ancestors or ancestral remains) and archaeological material culture for ancient DNA research (which could be extrapolated to advise other types of scientific analysis, particularly the archaeological sciences). I summarize a nonexhaustive list of legal obligations and general best practices gathered from the writings of Indigenous and allied scholars, and evaluate with a critical eye the implications and impacts of the modern practice of paleogenomics as well as the use of genetic information in general. I also detail some unresolved conflicts in archaeology between the most ethical course of action, common protocol, and U.S. law. I then discuss my own ethical framework used in the production of this dissertation.

**Background**

Ancient DNA (also known as paleogenomics) is a relatively new field. Beginning with experiments in the bacterial cloning and PCR eras (Cano et al., 1993; DeSalle et al., 1992; Hagelberg et al., 2015; Higuchi et al., 1984; Pääbo, 1985a; Pääbo, 1985b; Woodward et al., 1994), the results of many of these first experiments were highly publicized (Morell, 1992) before being shown to be in fact only contamination—often from the DNA of the researchers themselves (Allard et al., 1995; Austin et al., 1997; Hedges and Schweitzer, 1995; Henikoff, 1995; Lindahl, 1993; Zischler et al., 1995). For example, Woodward et al. claimed in 1994 to have extracted DNA from a Cretaceous-period dinosaur fossil. Even while the positive result was still being celebrated as the key to a new frontier of science, other

researchers promptly showed that the DNA was not dinosaur in origin, but unambiguously mammalian—likely the DNA of the human scientist performing the lab work (Allard et al., 1995; Hedges & Schweitzer, 1995; Henikoff, 1995; Zischler et al., 1995). Modern DNA is preferentially amplified using these old PCR methods (Pääbo, 1991), and it is still today difficult to eliminate modern human DNA while humans do all the lab work and sources of DNA have often been handled with bare hands (or have been licked, as often has happened in archaeology). As seen in the Woodward et al. (1994) example, it was difficult in this era to tell if a DNA sequence came from a human or a dinosaur, much less distinguish modern human DNA from ancient (Allard et al., 1995; Hedges & Schweitzer, 1995; Henikoff, 1995; Zischler et al., 1995). Researchers were interested in ancient human DNA projects from the beginning, but the potential for undetected human contamination was so high that it was often not financially feasible to pursue these projects, and so there was not an abundance of ancient or archaic human projects attempted during this period (Hagelberg et al., 2015).

The next-generation sequencing revolution was a huge breakthrough for ancient DNA studies. Because of this new technology, we can now rapidly sequence almost all the strands of DNA present in an extract, not just one or two short specified fragments (as it was in the PCR era) (Noonan et al., 2005). These advancements in technology have allowed us to sequence genomes in higher depth, higher throughput, and for lower cost (Bentley et al., 2008). We have been able to sequence older and older DNA, so much so that we now have reliable genetic data from a 1.2

million-year old mammoth tooth (Van der valk et al., 2021). We can now use damage profiles to more confidently call genetic sequences authentically ancient (Briggs et al., 2007; Skoglund et al., 2014), and we can use mitochondrial (Prüfer et al., 2014; Renaud et al., 2015), autosomal (Nakatsuka et al., 2020), X- and Y-chromosomal (Korneliussen et al., 2014; Meyer et al., 2012; Prüfer et al., 2014) data to accurately estimate contamination rates. All of these developments have facilitated the field of ancient DNA's explosion into the realm of human genetics (Sarkissian et al., 2015).

One might ask why this chapter is necessary to include in my dissertation. Why does paleogenomics need a specific set of ethics, and why is it important for me to discuss here? While it is possible to borrow ethical guidelines from related fields, those rules are often flawed, even for use within the field they were developed for (Cockburn & Cundill, 2018; Hodge, 2013). Additionally, paleogenomics is uncharted territory in a way—there are many situations comparable to those encountered in other disciplines, but blending those together creates a quagmire that any one field is unable to handle with their own ethical guidelines. This is not a situation that should be ignored. Ethical guidelines are critical to establish; while discussions and debate on this topic date back to the field's early days (i.e. Mirza & Dungworth, 1995; Weaver, 1997), a consensus in the field has been elusive, and much of the mainstream discourse between researchers in the field until recent years largely ignored the perspectives of descendant communities (i.e. Lalueza Fox, 1997). Additionally, norms change with the times. Like any other field, paleogenomics needs ethical guidelines to be not only laid out, but consistently reviewed and challenged in order to create a

more equitable and just discipline. Because I work in the aDNA field, and particularly because I focus on colonialism, it would be remiss of me not to include a discussion of the harm the field has and does perpetuate—even though paleogenomics is a relatively new field, its ethically concerning history extends well before its conception.

**Ancient DNA's Spectre of White Supremacy and Colonialism**

Genetics has a dark history, particularly within the subfield of genetics focused on humans. Before the world even suspected that deoxyribonucleic acid (DNA) was the encoding material for genetic information (Avery et al., 1944), genetics and evolutionary biology were already active fields (Clarke, 1989; Crow & Crow, 2002). The eugenics movement of the early 1900s was hailed as a brilliant and noble gift to humanity, a way to help society to become smarter, healthier, and overall improved (Galton, 1904). Aside from the ethical questions inherently raised by deciding who can and cannot reproduce (The American College of Obstetricians and Gynecologists Committee on Ethics, 2017), the ideas of who was "best fit" to reproduce were often rooted solidly in white supremacy (Lewis, 2017). For decades, "experts" with biased, limited and deeply flawed (generally outright false, see Bianculli, 2016) data promoted ideas about the inferiority of people who are not white men (such as claiming that Black people are not as smart as white people, see Williams, 2009) and were even called upon to testify about these ideas in court (Bianculli, 2016). Even while the Nazi party tortured and murdered millions of Jews, Slavs, Romani, and

other people labeled “undesirable” under the guise of eugenics (United States Holocaust Memorial Museum, 2019), scientists around the world still continued to promote these ideas until they largely fell out of favor in the 1970s (even though some forms of eugenics are still practiced today) (Bianculli, 2016; Ko, 2016). Even today, genetics still has a long way to go in shaking off the influence of this legacy: a 2013 study showed that race-related medical genetics papers often discuss “social implications” which appear to promote racist stereotypes and ideals (Phelan et al., 2013). Throughout genetics’ entire history, “sample collection” was (and often still is) done in a way that is structured through colonialism and imperialism. In the past, medical science (which human genetics can often be considered an extension of) often would often solicit medical “samples” from individuals who were Black, Indigenous, and/or people of color through graverobbing (Halperin, 2007), outright lying (Brandt, 1978) and without gaining informed consent (Brandt, 1978; Skloot, 2010). These methods took agency away from the studied group, and firmly centered the narrative around a white, colonialist, and eurocentric voice. Even the scientific wording used (such as “sample number XXX”) can actively work to dehumanize the people studied, leading to what McNiven & Russell (2005) refer to as “subjectation”, which is directly linked to a colonialist mindset.

However, genetics is not the only field guilty of these crimes. For example, anthropology is a field deeply rooted in white supremacy and colonialism. A field that has long been used to rewrite the past to fit a eurocentric view (Montón-Subías & Hernando, 2018), anthropology and archaeology are also notorious when it comes to

stealing things. From its beginnings in antiquarianism, archaeology has often been focused on collecting (Stiebing, 1994). Early ethnographers and cultural anthropologists "collected" ancestors, sacred items and material culture, sometimes through purchase, sometimes by plundering graves (Cole, 1995). While in the present day archaeological or ethnographic collecting does often happen in a relatively ethical manner after consulting or collaborating with descendant communities (McAnany & Rowe, 2015), for much of the field's history, researchers in these fields would often outright steal ancestral remains and material culture to hoard in museums and private collections (Cole, 1995). The majority of what was stolen still remains in these museums and private collections, even as the rightful stewards persistently push for their return (Little, 2018). In the context of the United States, legislation (NAGPRA) was enacted in the early 1990s in an attempt to facilitate repatriation of these ancestors, grave goods, and sacred items back to Native American groups (Native American Graves Protection and Repatriation Act, 1990). Other entities like the State of California and the University of California have added additional rules and guidelines to strengthen this intent (California Assembly Bill 275, 2020; California Assembly Bill 2836, 2018; California Native American Graves Protection and Repatriation Act, 2001; University of California Policy on Native American Cultural Affiliation and Repatriation, 2021). However, even with all these policies in place, museums and repositories have found ways to continue to cling to these stolen items with a death grip. For a tribe to make a legal request under federal NAGPRA for the return of their ancestors or material culture, they must be federally recognized

(Daehnke & Lonetree, 2011; Talbert, 2012). If a tribe is federally recognized (many are not, see Talbert, 2012), they can attempt to find the ancestors or material culture that was stolen from them to make that formal request. Given the large number of federally unrecognized tribes in California, California state law attempted to create an opportunity for unrecognized tribes to submit claims in specific situations (California Assembly Bill 275, 2020). In practice, other laws take precedence; this false promise to unrecognized tribes has only led to additional frustration and fraying of trust in the institutions tasked with repatriation. Even when recognized tribes have a clear path to repatriation, it is not always easy to know where to look—the way museums and repositories catalog their collections can be confusing. Whether intentional or not, this can make it difficult for those tribes to even know what a museum actually has (Anderson, 2018; Colwell, 2015). In addition, ancestral remains are often listed as "culturally unaffiliated" for various reasons (Daehnke & Lonetree, 2011). Many archaeologists either look the other way when encountering these practices or are actively in favor of them (Riding In, 1996), which I explain in more detail in a later section. As in genetics and other sciences, archaeology is also no stranger to "subjectation" of people through the use of distance-producing language (McNiven & Russell, 2005), and archaeologists have been called as "expert witnesses" in court and legislature (Rosen, 1977), which could result in groups of people losing land and water rights as well as the rights to collect their own cultural heritage and ancestors' remains. All of these practices serve to remove agency from Indigenous people and

descendant communities (Anderson, 2018; Colwell, 2015; Daehnke & Lonetree, 2011; Riding In, 1996; Rosen, 1977).

Archaeology and cultural anthropology are not alone in these problematic histories; biological anthropology carries the weight of a despicable past. Franz Boas, often referred to as the father of American anthropology, shamelessly admitted to robbing Native American graves, claiming:

> "it is most unpleasant work to steal bones from a grave, but what is the use, someone has to do it". (Boas, 1888, p.7)

Genetics and biological anthropology have been intertwined for a long time, particularly in regards to eugenics (Marks, 2012). In truth, biological anthropologists played a large part in purposely contributing to racist ideas and the efforts to corroborate ideas of white supremacy, often through the use of cranial measurements (Daehnke & Lonetree, 2011). Early biological anthropologists like Ales Hrdlicka and Samuel G Morton were obsessed with measuring skulls—they believed skull size and morphology could be used to determine a person's character and intelligence, and they intended to use these measurements to "prove" white supremacy (Blakey, 2022; Daehnke & Lonetree, 2011). Native American skulls for measuring were in such great demand at the height of these "experiments" in the United States that researchers offered people large sums of money for the skulls of Native Americans, encouraging the defilement of graves (and one could argue effectively putting a bounty on the literal head of every Native American) (Bieder, 1990; Daehnke & Lonetree, 2011). The government had long encouraged the murder of Native

Americans (Koehler, 2018), but beginning in 1867, the curator of the Army Medical Museum made the egregious and desecrate request that field doctors in the military send Native American remains from battlefields and nearby burials to him—a clear order from a government entity which resulted in the museum collecting around 4,500 Native American skulls (Daehnke & Lonetree, 2011). This frenzy for skull collecting was not confined to the United States either; it was a phenomenon in all European countries and colonies where there were biological anthropologists (Tharoor, 2016). While most of these specific practices are no longer accepted by the general research community, these legacies are not relegated to the past. Many researchers still use race-categorizing cranial measurements in forensic contexts, even though these methods have been shown to be inaccurate and do more harm than good (Elliott & Collard, 2009). This is the foundation of the field—unless we are vigilant about keeping it in check, it will seep into every crevice.

Paleogenomics is a merger between these fields that are steeped in white supremacy and settler colonialism. Due to its basis in genetics, archaeology, and biological anthropology, the foundation of paleogenomics is problematic. White supremacy and settler colonialism do not always express themselves in this field blatantly, but there are many subtle expressions of these white supremacist and settler-colonial ideologies frequently seen in the way ancient DNA research is conducted. For example, in 2017, a group of researchers published a paper on matrilineal relationships among burials at Chaco Canyon (Kennett et al., 2017). Because the remains studied were listed as “culturally unaffiliated” by the museum

where the bones were kept, the researchers did not make any attempt to even consult with Native American tribes in the area near Chaco Canyon, much less collaborate (Claw et al., 2017). In addition, researchers are often unethical when it comes to collecting and handling the bones themselves, regardless of their origin. There have been reports of researchers hoarding petrous bones (Makarewicz et al., 2017), and certain high-ranking archaeologists working in paleogenomics take whatever bones they want from museums (which were only in the museum because they were stolen in the first place), regardless of permissions (Lewis-Kraus, 2019)

Published ancient DNA research has also been misinterpreted and co-opted by multiple groups to justify various forms of discrimination and violence. While there are many examples of political figures misusing ancient DNA research to bolster problematic or violent ideologies (Jain & Lasseter, 2018; Wolinsky, 2019), here I will limit my examples to the topics this chapter centers on: white supremacy and colonialism. One example of dangerous public misinterpretation of ancient DNA research is ancient DNA's popularity in white supremacist circles. White nationalists' engagement on Twitter reflects this enamorment with the field of genetics (Carlson & Harris, 2020), but an examination of the far-right site Stormfront revealed a troubling amount of discussion linking ancient DNA research to ideas of racial purity, white superiority, and xenophobia (Hakenbeck, 2019). Another example is the use of ancient DNA by politicians to reinforce settler-colonial violence. In 2019, Israeli Prime Minister Benjamin Netanyahu took to Twitter to discuss an ancient DNA paper that had recently been published. The ancient DNA research studied ancestral remains

from the site of Ashkelon, a site in modern-day Israel, but did not say anything specific about modern Jewish or Palestinian people (Feldman et al., 2019). However, Netanyahu either misunderstood the research or willfully misinterpreted it, leading to a number of tweets where he used the research to dismiss the connection of Palestinian people to the land, arguably justifying the ethnic cleansing of Palestinians (Gannon, 2019; Wolinsky, 2019). The authors of the study reportedly "followed the debate, but chose not to comment" (Wolinsky, 2019, p.2), allowing a debate to continue when they could have settled the issue early on. This lack of action on the part of researchers is harmful. When ancient DNA research is co-opted by people with malicious agendas, researchers have a duty to act (Hakenbeck, 2019). Staying silent when others use your work to make false and harmful claims is complicity. Even if this is not the intent of the researcher, it still has that impact.

While disturbing, these sorts of unethical practices are not surprising, as (detailed above) the disciplines paleogenomics draws from are entrenched in these ideas of white supremacy and settler colonialism. However, the fact that the ethically questionable aspects of paleogenomics derive from its predecessors does not absolve today's researchers of fault. If anything, it could be argued that, because of the field's history, ancient DNA researchers have even more of a responsibility to be vigilant about righting wrongs and stopping the perpetuation of and actively combating these actions and ideas. As Nakatsuka (2020) puts it:

> "…if a research group is benefitting from past wrongs (e.g. studying human remains previously stolen from particular groups) without any attempt of

> repairing the situation, it can be argued that they are perpetuating the past wrongs and thus performing a wrong themselves." (p.30)

Unless these problems are faced head-on and continually exposed, they will continue to rear their ugly heads in ways that are perhaps less overt, but still undeniably harmful to communities.

**Genetic Data: Concerns and Considerations**

Entangled with the concerns discussed above, the implications of the use of genetic information in general are also necessary to consider. This is not meant to be an in-depth exploration of the ethics of producing, using and interpreting genetic data; there are many scholars much more knowledgeable than I who have written extensively on that subject (e.g. Fox, 2020; Hudson et al., 2020; Reardon & TallBear, 2012; TallBear, 2007; Tsosie, 2020). However, this topic is necessary to include in at least a cursory manner to more fully understand the context of criticisms leveled at the field. It is important to understand that the concerns many communities have with genetics are not unfounded, but are in fact justified due to the history of genetics, the history of how their ancestors were treated (and continued mistreatment), and the lack of security provided by genetic information.

The first concern that should be raised is the matter of consent. Informed consent is defined by the National Institutes of Health as:

> "The process of educating a person about the test and obtaining permission to carry out testing..."Informed" means that the person has enough information to make an educated decision about testing; "consent" refers to a person's

> voluntary agreement to have the test done." (National Library of Medicine, 2021)

This generally refers to the informed consent of an individual, but in many cases (such as in research involving remains of Ancestors and archaeological populations), it is necessary to get consent of a group as well (Nakatsuka, 2020). Sometimes this is because of the implications the research and future use of data could have for a particular group (Nakatsuka, 2020), and sometimes (as in the case often is in ancient DNA studies) the individual whom researchers would like to study is dead and there is not a stipulation in a will or clear next-of-kin designated to make these decisions (Wagner et al., 2020). Group consent relies on having an agreed-upon decision-making system within the group to speak for all its members (Dickert & Sugarman, 2005; Nakatsuka, 2020). However, if there is no such system in place, researchers will pursue consultation, which in the genetics realm refers to the process of starting a dialogue with community members about the project for the purposes of soliciting feedback or input, but without the possibility of group consent, as it is structurally impossible to obtain (Dickert & Sugarman, 2005). In archaeology, the word consultation holds a slightly different meaning. The Archaeological Resources Protection Act of 1979 as well as section 106 of the National Historic Preservation Act (1966) requires archaeologists with US federal funding to undergo consultations with federally-recognized tribes when excavation is proposed to take place on either US federal land or Native American reservations. As sovereign nations, each tribe generally has their own guidelines about what proper consultation entails, and this may include obtaining permission or consent from tribal leaders (Wagner et al., 2020;

Washington Department of Archaeology and Historic Preservation). Whether consultation in archaeology or informed consent in genetics, both should be the bare minimum; however, there are many examples of researchers in both fields failing to meet this requirement. As mentioned earlier, there are many recorded instances in relatively recent years of unethical sample collection and data use (Claw et al., 2018; Nakatsuka, 2020). Often this is due to improperly soliciting consent without fully informing the group or individuals about what the samples will be used for, using the samples for projects other than the ones disclosed, scientists claiming genetic data as their intellectual property, or researchers not communicating with the group after the scientists were allowed to take samples (Bardill et al., 2018; Cunningham, 1998; Dalton, 2002; Fleskes et al., 2022; Garrison, 2013; Hasian & Plec, 2002; Nakatsuka, 2020; Pacheco et al., 2013).

As noted earlier, individual and group consent are both important to consider. Depending on the context, one may be more appropriate to pursue than the other, and it is often necessary to obtain both. For example, in cultures where decision-making is more communal than individualistic, group consent would be more urgent to pursue (Nakatsuka, 2020), although individual consent should still be necessary in addition to that for each living individual being tested. This would also be the case for small groups that could be heavily affected by research implications, as it is important that decisions about the well-being of an entire group does not rest on one individual wanting to proceed with research (Fleskes et al., 2022). Depending on the legal and cultural structure, proceeding with the approval of a single person is an action that

could undermine the sovereignty of a nation. Often, group consent comes from a tribal council or a government (Wagner et al., 2020). However, depending on local politics and history, the government may not be the appropriate body to give group consent (for example, marginalized groups and oppressed people are often not represented appropriately by their country's government)—in this case it may be most appropriate to obtain consent from *both* the government and the community implicated (Fleskes et al., 2022; Kowal et al., 2023; Nakatsuka, 2020; Tsosie et al., 2020; Wagner et al., 2020).

Lastly, there is the concern about regulation, privacy and identification. It is important to consider how data is regulated—for example, can any person access it and use it post-publication? This could lead to the data being used in controversial projects it was never intended for. For example, the data could be used to shame and stigmatize people as well as cause undue anxiety (Fleskes et al., 2022; Nakatsuka, 2020; Tsosie et al., 2020), whether used by someone merely ignorant or with malicious intent. Even worse, it is possible that those with malicious intent could try to manipulate the data to justify eugenics. There is no good way to protect identities, because DNA is the most personal information possible (Ravenscraft, 2019). Data is often uploaded without identifiers in an attempt to overcome this problem (Shabani & Marelli, 2019), but especially in the context of small groups, this might not prevent people from figuring out what the data is, and using it for projects the data was not intended for (Shabani & Marelli, 2019). This is why we need a better system where data is not freely available, but instead is guarded by the studied group themselves

with assistance from the original researchers (Fleskes et al.,2022; Kowal et al., 2023; Tsosie et al., 2020). Frustratingly, this contradicts the rules of organizations like the National Science Foundation, where freely available and accessible data are touted as essential to equitable and just science (National Science Foundation, n.d.). As a researcher, someone else's bones do not belong to you, and neither does their genetic data. Requiring permission from the identified descendant group every time a researcher wants to use this data forces them to confront this fact, and puts the decision-making power back in the hands of the appropriate people.

**Archaeology's Unresolved Conflicts**

Archaeology is also rife with unresolved conflicts related to paleogenomics research. While the field does appear to be shifting as time goes on, there are sometimes still irreconcilable conflicts between the wishes of descendant communities, heritage laws, and common research practices in the archaeological sciences (including paleogenomics).

While there are different laws in all places in regards to how to handle ancestral remains, I am most familiar with the North American context, so here I will be focused specifically on NAGPRA. The Native American Graves Protection and Repatriation Act (commonly referred to as NAGPRA) became law in 1990. In an effort to make treating ancestral remains with "dignity and respect" the status quo, the United States Congress formalized into law a request that "human remains, funerary objects, sacred objects, and objects of cultural patrimony" be returned to the

appropriate federally-recognized tribes when identifiable (Native American Graves Protection and Repatriation Act, 1990). NAGPRA details the bare minimum of what we should be doing to move toward properly and respectfully working with descendant communities, but a large proportion of archaeologists strongly opposed even setting those minimal rules in place. The Society for American Archaeology (SAA), the largest association of archaeologists in the world, publicly opposed NAGPRA when it was still being debated and developed in Congress (Steponaitis & Davis, 2008) and lobbied against it even many years into implementation (Society for American Archaeology, 2007).

Today the SAA publicly supports NAGPRA (Society for American Archaeology, 2020), with many archaeologists going above and beyond these legal requirements. The shift in overall opinions in the field may be representative of the demographic shift in archaeology, as the field becomes more diverse and inclusive (White & Draycott, 2020). However, a surprising number of archaeologists do still seem to view NAGPRA as a thorn in their side—something to try to skirt around so their work can progress. Based on personal experience, it appears most (but certainly not all) of the archaeological scientists holding negative views of repatriation belong to the older, less diverse generations of archaeologists, many of whom are approaching retirement. However, even if this attitude were entirely absent in the next generation of archaeologists, simply ignoring these hostile opinions would be a mistake—these views are currently doing great harm in the present, and, unless there is a widespread effort to combat them, will continue to do so until these individuals retire.

To be clear, few of these academics are the stereotypical racist image we might like to imagine (although those people certainly also do exist). As with most bigotry, much of the bias is subconscious (Greenwald & Krieger, 2006; White & Draycott, 2020). Especially for those of us living in the heart of the colonial empire, we are immersed in racist, patriarchal and settler-colonial ideals; those who benefit from the current system have little to gain by critically examining these biases that have been instilled. Because of this, some researchers may lack the ability to empathize with people of differing positionalities and different opinions on topics like repatriation. These researchers may even feel that approaches that differ from their own are in fact unethical, because the ancestors and science belong to "the world" (Nakatsuka, 2020).

While there are those who simply lack awareness, the appeal of "progress" and leaving one's mark on the field may also drive many to *knowingly* engage in unethical practices. Like Boas (1888), they may view the process as "most unpleasant", but that the work is so important that "someone has to do it". Similarly, some may feel pressured into unethical practices by the threat of losing their job or the idea that engaging in these practices while advocating for their group to adopt some more ethical practices (and thus having an overall net positive impact) is the "lesser of two evils". If "somebody has to do it", it is easy to justify. This sense of inevitability can be tricky to escape, especially by those trying to change the field. What is "right" or "wrong" in these situations is often ambiguous, but the existence of the dilemma itself necessarily requires those of us in these positions to buy into the inevitability of the status quo. It is predicated on the permanence of the colonial machine, and collapses

when we take radical accountability for our own actions as individuals. A seemingly unrelated event can help explain this idea: in 2021, Yaakov Fauci, a settler from New York, forcibly kicked Muna El-Kurd out of her home in occupied East Jerusalem and took it as his own ("If I don't", 2021). When Muna exclaimed "you are stealing my house!" Yaakov responded "and if I don't steal it, someone else is gonna steal it". Muna, exasperated, replied "No! No one is allowed to steal it!". In the settler-colonial reality, it is true that perhaps someone else would have stolen her house. However, that does not absolve Yaakov of his participation in the act. He is the one who stole her house. That action has impact. While the intention behind the excuse is different in the two scenarios–Yaakov in reality likely does not actually care about what happens to the El-Kurd family, whereas a researcher in a position of low power may truly care about the people being impacted by their actions– it is still an attempt to shift the blame off oneself, to wipe one's hands clean.

Recognizing the impact of our actions, taking accountability, and refusing to participate in unethical systems of research may be an unattainable ideal, but that does not mean researchers should accept current norms. There are many situations where the concrete actions researchers can take to prevent harm are unambiguous, straightforward, and simple.

**Best Practices and Legal Requirements**

A list of best practices in ancient DNA has previously been put forth for how archaeologists and geneticists should interact (Sirak & Sedig, 2019), but the inclusion

of a discussion of descendant communities' concerns was largely side-stepped. A much more thorough list of best practices was also published by Prendergast & Sawchuck (2018), although it was mainly focused on an African archaeology context. Authors from both of these papers, others from the same research group, and their collaborators later published a short list of ethical guidelines (Alpaslan-Roodenberg et al., 2021), but this received an overwhelmingly negative reception. The accomplishment of getting all the co-authors to agree to abide by the ethics rules they laid out was likely not a small feat, given the contentious past practices of some of the coauthors; however, the article was widely criticized as milquetoast, researchers "writing their own rules", and not acknowledging prior criticism and scholarship (Kowal et al., 2023; Tsosie et al., 2021). This case is not an outlier; ethics standards are often shaped and defined by researchers themselves, without the input of those the standards are supposedly set up to protect (Fox, 2020; Kouritzin & Nakagawa, 2018). This is a clear conflict of interest. However, many scholars who center extractive research's impact on descendant communities (with Indigenous scholars in particular at the forefront of this work) have contributed detailed recommendations for researchers working in genomics and paleogenomics (Bardill et al., 2018; Claw et al., 2018; Fleskes et al., 2022; Kowal et al., 2023; Nakatsuka, 2020; Tsosie et al., 2020; Wagner et al., 2020) as well as research more broadly (Tuhiwai Smith, 2021). The following heavily draws upon or echoes these sources of existing scholarship. In many cases, these are legal obligations put forth by the nation the descendant community belongs to; ignoring the laws of the nation in question is tantantamount to

an attack on their sovereignty. Understanding the laws and cultural norms of the community is critical–rigidly and forcibly applying standards of one group onto another can be a colonial action, even if the groups are both Indigenous. Every nation and every community will have their own regulations, standards and processes researchers should follow. The communities should hold the decision-making power, not the researchers. While each project or situation has to be tailored to fit on a case-by-case basis, I have compiled a list of general best practices I try to use as a framework:

1. **Collaborate with all parties that are implicated by geographic space, historic range, or research questions—regardless of federally-recognized status.** The dead cannot give consent, and the complications of who can give consent post-mortem become even more complicated when remains are thousands of years old. There may be multiple different groups that need to be involved, depending on the project. Depending on the context, these groups might include the local government, groups whose traditional land the burial was found on (even if they are relative "newcomers" to the land due to forced migration or if they do not claim the remains as an ancestor, they should still be included in this process because they are stewards of that land (Bardill et al., 2018; Claw et al., 2017; Fleskes et al., 2022), groups who can plausibly claim the deceased as an ancient ancestor, and any group that might be implicated by research questions or results. Figure out what the appropriate forms of group consent and individual consent are for the project, and make sure that all parties who are implicated are involved in all decisions made.

These groups (not the research team) should start with all the power. Researchers should ensure they are not just "consulting" the groups, but truly collaborating. Consider how to tackle potential harm before it becomes a problem—determine what research questions are ok (and not ok) to ask, what methods should be pursued, and determine what language choices to make early on (Tsosie et al., 2020). Lab methods should be chosen by weighing levels of destructiveness with efficacy—explain the pros and cons of each method to all stakeholders, and listen to their decision. If the descendant community requests a minimally destructive method (such as Harney et al., 2021) or a nontraditional DNA source (see Chapter 6), respect that and do not try to argue your way into a more destructive method, even if it produces better data. Research question creation should be led by descendant communities—if they are not interested in asking a question, you should not be asking that question.

Work with community leaders within their legal and cultural systems to set up culturally-appropriate forums such as regular town halls to get community input, to report back to the community with results, and to educate the community about what those results do and do not mean. This is an important step to take regardless of the group (i.e. even in European contexts)—even though the government might represent people, it is still good to have the option of community input if culturally appropriate and allowed by the protocols of the community leadership. This is not a one-time thing; these engagements are something that should happen consistently throughout the research process (Claw et al., 2018).

Ideally, there would be members of the community involved in the study at every level (leading the study, doing lab work, writing papers for publication, etc), but forcing this on anyone is even more problematic—the best course of action is to make it an option, to provide capacity-sharing support and give community members the opportunity to get involved. This is not unheard of (Claw et al., 2018; Wagner et al., 2020), but it *is* something that will improve the research—all people have biases (whether explicit or implicit) that can skew how data is collected or interpreted, and the inclusion of people from the studied community in the research process can help prevent that from happening by adding in perspectives (Bardill et al., 2018; Claw et al., 2018; Tsosie et al., 2020; Wagner et al., 2020).

2. **Come to an agreement early on about how to handle results that might be unexpected or appear to contradict the worldview of some or all group members.** It is important to make sure group members have a full understanding that ancient DNA research has a high probability of resulting in no data at all, and they should be prepared for that potential outcome. It is also possible that results could appear to contradict a group's worldview or origin story (even though, as mentioned earlier, often more than one thing is true at once). This has the potential to deeply upset some people, or even challenge a group's land or water rights (Nakatsuka, 2020; Wagner et al., 2020). It is important to come to an agreement on how to deal with these potential issues before they are encountered. For example, are there findings that the group would ask not be published to avoid shame or a legal battle to keep their traditional lands? While it is important that scientific data not be tampered

with or manipulated to fit whatever story a group wants, is there a way these findings could be presented in a way where both the results are reported and harm is minimized?

3. **Consider the cultural views regarding ancestral remains and material culture.** Many cultures hold views about the body being sacred, and this may even extend to portions most western cultures consider disposable (Fox, 2021; Sahota, 2014). Similarly, some cultures may also view certain material culture as a member of the community, not a commodity (Smith, 2016). Because of this, researchers should discuss these views with the collaborating communities early on to develop a respectful research plan that takes these beliefs into consideration. In these early discussions, any plans for return or reburial post-lab-work should also be detailed as much as possible.While sometimes overlooked, less prominent aspects of research like word choice are also important to consider here. In archaeology, ancestral remains and material culture are often referred to in terms of "artifacts" and "samples" (Claw et al., 2017). While in many contexts these terms are appropriate ways to describe material culture, not all cultures view objects in the same way. Some cultures may view certain objects (such as baskets) as members of the community or as having agency (Smith, 2016). This is a prime example of why collaboration with implicated communities to determine language choice and develop a research plan is so essential. For a collaborating group, perhaps it is fine culturally to destroy a shoe for isotope analysis, but not okay to do residue analysis on a pot. Referring to a basket as an object or a sample may be offensive. These points of view are important to

recognize early on in order to minimize harm. While one could delve deep into the criticisms of science as a colonial and othering mechanism, that is outside the scope of this chapter and has already been addressed by respected scholars (e.g. Arteaga & El-Hani, 2012; McNiven & Russell, 2005; Mohr, 2009).

4. **Consider the source of "culturally unaffiliated" remains and material culture.** If remains are listed as "culturally unaffiliated", researchers should still collaborate with the tribes or traditional groups that live in the area where the remains were found. Regardless of whether or not they even claim the deceased as their ancestor, they are the stewards of the land and should be included in the research and decision-making processes. In the context of the United States, some might argue that the tribes currently in the surrounding area are not the exact tribe this person may have belonged to, and therefore it is not appropriate to involve those tribes. Those people may or may not be right that those tribes are not the *ideal* group to collaborate with, but it is better to collaborate with that group than no community stakeholders at all.

5. **Give back to the community with both results and positive outcomes.** Is the research going to provide some benefit for the community other than knowledge? Research "partnerships" that do not benefit all parties may be extractive and not partnerships at all in anything but name. Researchers should consider providing training workshops and paid research internships to interested members of the community, and consistently organize and participate in community engagements.

Research should benefit everyone involved, not only those of certain education levels or socioeconomic status.

6. **Commit to education and cultural translation.** While informed consent and dissemination of results within the community studied might be difficult in some cases due to lower levels of western science education in a community, this does not give researchers a pass—researchers have the responsibility to educate involved community members (and "culturally translate" for them, if needed [Nakatsuka, 2020]) on the basics they need in order to understand the questions, the implications, and the results.

7. **Commit to the respectful housing and appropriate return of remains and cultural materials.** Currently, studied remains are often housed jumbled up in boxes far away from home, often after being stolen from their graves many decades earlier (Nakatsuka, 2020). Ideally, a lab should have a system set in place for the respectful and culturally-appropriate storage of all ancestral remains (and cultural materials, if relevant) with an additional special space available upon community request for sacred materials or ancestors who need to be placed in a separate area. Researchers should be respectful when working with ancestors and cultural material, always be aware of who the "sample" is that they are working with, and choose their terminology with intention. Other protocols (such as greeting ancestral remains upon entering the lab every morning) should be considered as well on a case-by-case basis when requested by community stakeholders.

8. **Make access to data something the community has power over.** All the work done to determine appropriate research questions and data handling should not be abandoned once the research is published. As noted earlier, other researchers should have to request permission to use this data, and the community should have the final say on whether or not they are interested in collaborating on this project and allowing their ancestors' data to be used. It is best to establish this agreement early on in any project, as it may require negotiation with the funding agency or university that claims ownership of the research.

## Approaches in Action

Limitations such as lack of time and funds, disputes regarding group identity, and data-sharing mandates from funding sources all impact how (im)perfectly these ethical guidelines can be implemented. Descendant communities were involved in all three projects included in this dissertation, but the described framework had to be adjusted to accommodate each circumstance. The three chapters corresponding to these projects each have an ethics statement detailing all measures taken, including explanations of any deviations from the framework described here.

## Conclusions

When approaching research in a colonial institution, navigating these issues is like navigating a minefield. If ancient DNA researchers want to avoid perpetuating white supremacy and settler colonialism, we need to examine our biases and carefully consider the impacts of each action we take or neglect to take. This may be difficult

work at times, but it is necessary. Where appropriate, we should let the descendant communities and implicated parties lead the way when asking research questions, rather than taking information simply for our own interests. Quite frankly: if the descendant communities implicated are not interested in a question, we should not be asking it. If descendant communities are purposefully disempowered and prevented from having any input on a project that is actively harmful to them (whether it affects land/water rights, their spiritual beliefs, the restfulness in death of their ancestors, etc), this amounts to a crime. However, if we build bridges in good faith and follow best practices, both our research and relationships with communities will benefit greatly.

In this chapter's introduction, I stated that decolonization is an ongoing process and that ethical standards for the field need to be continuously reassessed and updated. Similarly, my own perspectives have changed over the years and will likely continue to change. In the future, I may disagree with some of the recommendations I have presented here. These standards will continue to evolve, but prioritizing the well-being and empowerment of marginalized people must always be the driving goal. Decolonization is not simply a buzzword, it is the shifting of our mindsets *and* our corresponding actions. This idea is not a standard with an endpoint (Ritskes, 2012; Sium et al., 2012), but rather an invitation to continually reflect on our approaches and adjust accordingly. This shifting of mindsets and methodologies will take effort in the field of ancient DNA, but it is the only ethical way forward.

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## Chapter 3: An Invisible Enemy
## Investigating the Ancient Pathogens of Conquest-Era Peru through Genomics

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### Abstract

Among other outcomes of the Columbian exchange, many diseases were transmitted from Europe to the Americas, sweeping rapidly through Indigenous populations. These diseases are thought to have led to mass mortality and likely influenced the outcome of European colonization attempts. The far-reaching repercussions of both colonialism and this transmission of disease are interconnected and are still being dealt with today. We analyzed ancestral remains (n= 87) from three strategically selected early contact-era sites in Peru in a focused ancient pathogen genomics study. We detected agents of infectious diseases including tuberculosis, diphtheria, and Epstein-Barr virus. We will validate these initial findings in future publications by obtaining higher coverage of the genomes of interest using genome enrichment methods where needed, characterizing those genomes and analyzing their relation to modern strains. Including contextual factors in data interpretation, I aim to investigate how these pathogens behaved and evolved. While the evolutionary history of these diseases is interesting, this data can also contribute to multiple fields of research, importantly those pertaining to public health. The threat of evolving, emerging and reemerging diseases is widely recognized; the environmental and social crises looming on the horizon are potential contributing factors and there is no widely-accepted plan in place for management. Ancient pathogen data could be used

to improve predictive models to foresee how emerging and reemerging diseases might act, highlighting the importance of this type of research. As the world enters an era characterized by multiple chaotic crises, this information is needed more than ever.

**Introduction and Background**

Colonialism has left deep and long-lasting scars throughout the history of the region known today as South America, from the violence and brutality endured during early European conquest to modern-day transgressions (Gabbert, 2012). Perhaps one of the most tragic and long-lasting of these is the transmission of diseases.

In 1492 CE, European "explorers" embarked on a voyage that would change the course of history— a convoy of three ships from Spain sailed across the Atlantic Ocean, resulting in one of the earliest recorded encounters between European colonizers and the peoples indigenous to the land that would come to be known Eurocentrically as the "New World." This voyage inspired other voyages in a rush to conquer, throwing the Americas into a state of upheaval, violence, and disease.

The list of diseases thought to have been brought to the Americas is long, including smallpox, whooping cough, and the European variant of tuberculosis, among other diseases (Brynildsrud et al., 2018; Nunn & Qian, 2010). Disease transmitted by European contact in the 1500s is estimated to have been a driving force in the deaths of up to (and some estimates even exceeding) 90% of the population in the early days of colonization (Denevan, 1992a; Nunn & Qian, 2010;

O'Fallon & Fehren-Schmitz, 2011). While population size estimates vary considerably (Denevan, 1992a; Nunn & Qian, 2010), many scholars believe disease paved the way for Europeans to much more easily take control of the land by decreasing estimated population levels in the "New World" from 54 million people in 1492 to just six million merely a century and a half later (Denevan, 1992a; Denevan, 1992b).

In 1526 CE, drawn by the rumors of gold, Francisco Pizarro and his 12 accompanying *conquistadores* became the first Europeans to reach Peru (Hemming, 2012). By this time, the Inca empire had reached its peak as a society and, according to historians, was possibly already in decline (Fernández-Armesto, 2009; Hemming, 2012; Hopkins, 2002). Preceding the arrival of European humans, the first epidemic of European origin thought to reach Peru was an alleged smallpox outbreak starting around 1524 CE (Hopkins, 2002). Many other deadly epidemics reportedly followed in rapid succession (Dobyns, 1963). Although the identity of the disease is still disputed, in the mid to late 1520s the Inca emperor Huayna Capac died of what many modern historians believe was European-introduced smallpox (Hopkins, 2002; McCaa et al., 2004). His death subsequently led to a civil war as two of his sons both vied for the position of power (Livi-Bacci, 2006). Unaware of these developments, Pizarro obtained permission from Spain in 1529 to conquer Peru (Hemming, 2012), leading to even more violence and disaster. In the case of Peru, disease likely amplified an already unstable situation. Scholars estimate a population decline during this time of up to 63 deaths to every one birth in some regions of Peru (Smith et al.,

1970), so while it is indeed possible that the Inca were already in a decline as a society, the spread of disease truly gave European invaders an advantage.

Many of these diseases continue to be a significant problem for Indigenous populations in Amazonia today (Walker et al., 2015). Perhaps due to better access to modern medicine or higher levels of genetic diversity, other modern-day South American groups may fare better against some of these diseases (Walker et al., 2015), but even those groups may still be experiencing the effects of the decimating interactions with diseases their ancestors endured. Disease has undoubtedly contributed to the devastating social and economic effects colonialism has had on societies as a whole. In addition, it is likely that modern-day groups whose ancestors endured these colonially-introduced diseases also carry the tragedy with them as a story told through their genes (Lindo et al., 2016).

Due to geographic barriers, "Old World" and "New World" populations did not encounter the same pathogens before Europeans crossed the Atlantic. This difference in pathogen exposure over thousands of years means both groups may have been "immunologically naïve", with no protective immunity towards any foreign diseases (Crosby, 1976), but it also means they may not have endured the same selective pressures from (or coevolution with) pathogens over hundreds or even thousands of generations (Lindo et al., 2016; Walker et al., 2015). A serial founder effect is also thought to have drastically reduced genetic diversity in the Americas, leading to potentially less efficient immune responses (Walker et al., 2015), although this is contested by those who hypothesize the deaths were due instead to the

settler-colonial disruption of sociological factors, referred to as the "Black Legend Hypothesis" (Archer, 2016; Collen et al., 2022; Livi-Bacci, 2006). The disruption of social infrastructure combined with the physical and mental stress of enduring violence and famine create an environment ideal for pathogen spread (Gayer et al., 2007; Ramenofsky et al., 2003). All of these aspects together may have left "New World" populations in a situation of high transmission risk and without the defense mechanisms present in European populations, making them potentially more susceptible to the diseases commonplace in the "Old World" that were unleashed upon them during European contact (Walker et al., 2015). Individuals in the "New World" who survived these diseases hypothetically left subsequent generations some of the genes that protected them, meaning it may be possible to see which genes were selected upon by this disease pressure by studying the genomes of modern-day populations. While this type of work has been given adequate attention by medical researchers (for example, Black & Pandey, 1997; Rempel et al., 2011), ancient research data is severely lacking. If desired by affected communities, data from different time periods could help add detail to the story, increasing our understanding of the past, present, and future while potentially improving health outcomes. However, this type of research is complicated both technically and ethically, and is beyond the scope of my current work, which focuses on the pathogens themselves.

Ancient pathogen DNA studies have proliferated greatly in the past few years as methods have improved (Spyrou et al., 2019). Often focused on Eurasian disease, this research has helped us understand the origins and spread of the bubonic plague

(Spyrou et al., 2018), and treponemal diseases (Majander et al., 2020), among others. Ancient pathogen studies in the Americas have also detailed impressive discoveries. For example, research of colonial-era burials in Mexico has identified a potential causative agent in a mysterious epidemic known as cocolitzli (Vågene et al., 2018), as well as reconstructed the genomes of Parvovirus B19 and Hepatitis B Virus strains of that time (Guzmán-Solís et al., 2021). Ancient pathogen research across South America has also proven to be successful; in Peru alone, researchers were able to identify a unique and now extinct strain of HBV circulating in Andes prior to European invasion (Kocher et al., 2021), reveal previously unknown details on the history of malaria in this region (Michel et al., 2024), and reconstruct the genome of a pre-Columbian type of tuberculosis-causing pathogen, *Mycobacterium pinnipedii*, circulating in ancient Andean populations (Bos et al., 2014). Further research of tuberculosis in the ancient Americas has revealed this pathogen to be more widespread in the region than previously thought (Vågene et al., 2022), which begs the question of why the European L4 lineage of tuberculosis appears to be the predominant lineage remaining in the region today (Gagneux et al., 2006; Santos-Lazaro et al., 2021).

We can learn a great deal from disease genomes, including phylogenetic placement, geographic origins, divergence time, diversification events, and even how variations between strains may have related to disease spread through increased virulence, mortality rates, or pathogenicity. When combined with contextual data, this information has the power to paint a detailed picture of past events. In order to gain a

deeper understanding of the diseases in early contact-era Peru, I used paleogenomics methods to investigate samples taken from 87 individuals obtained from three strategically selected sites in Peru. The genetic data combined with the great depth of the archaeological and historical records for contact-era Peru (including large amounts of data concerning burial practices, the local environments and demography of that time and place) can uncover a clearer understanding of how and why the diseases spread and affected the population.

Early contact-era Peru is an ideal area of focus due to the detailed contextual information, and because it can be viewed as a model for a society undergoing a period of drastic, stressful change. As such, conclusions drawn from ancient Peruvian pathogen data may possibly be applied in some aspects to the rest of South America, as well as to other parts of the world where similar situations of upheaval were experienced. The potential of using this data to help inform models for predicting emerging and re-emerging diseases makes this research valuable. As human populations grow larger, more densely populated, and encroach on wild animal habitats, the risk of an emerging, re-emerging, or antibiotic-resistant disease outbreak potentially increasingly becomes a more urgent concern (Garrett, 1994). Climate disasters may exacerbate the rate of transmission of these epidemics as people move en masse through our highly interconnected world. All of these factors indicate research of ancient pathogen evolution and behavior during times of upheaval will be beneficial as we navigate through this upcoming era of rapid change and uncertainty.

## Research Questions and Approach

Based on what is known from historical accounts, disease ecology, and archaeological research, I began with several relevant research questions. These can be separated into three general topics:

### 1) Identification: Which pathogens were causing the widespread deaths of Indigenous people during this time period in Peru?

While there are historical accounts of European diseases decimating Indigenous South American populations, the accounts tend to be vague. This has resulted in rampant speculation and uncertainty surrounding the identity of the causative agents (Vågene et al., 2018). Ancient DNA results can confirm historians' suspicions, but in some cases researchers may stumble across something unexpected. It is possible that some outbreaks were caused by European pathogens other than the ones historians have named, or even endemic diseases that seized the opportunity to ravage the local population. This latter possibility could be due to factors such as stress on the Indigenous people's immune systems (potentially stemming from factors like the psychologically stressful situation, lack of resources, famine, and the physical stress of fighting off both endemic and imported infectious diseases), mass migrations leading to environmental changes beneficial to a pathogen, or even disease hybridization with related European pathogens. To avoid erroneously excluding unexpected diseases from the pool of potential suspects, I used metagenomic approaches to broadly analyze shotgun data for these initial investigations rather than

using PCR-based approaches or jumping straight to capture enrichment. While more costly, the shotgun approach has become the gold standard for researchers in pathogen paleogenomics in recent years (Spyrou et al., 2019). Once a pathogen has been identified, specific capture enrichment can be performed to obtain more complete coverage of its genome.

**2) Phylogenies and characterization: How did the pathogens evolve in this novel environment?**

While pathogens brought from Europe were allegedly encountering the highly susceptible Indigenous populations, local pathogens may have also been encountering new "environments" of sorts due to the upheaval caused by European invasion (these situations are explored further in topic three). Regardless of the reason for a new environment, a pathogen in novel conditions is subject to a new set of selective pressures that may lead to the pathogen adapting to the new environment. Genomic characterization and phylogenetic placement of these pathogens may help inform us of traits that were affected by these selective pressures.

When combined with published modern and ancient disease genomes, the newly reconstructed genomes characterized through this research have the potential to offer a better understanding of phylogenetic evolutionary relationships between subspecies or strains. These analyses can potentially elucidate the geographic origin of a strain, its placement on the phylogenetic tree in relation to other strains, and even detect hybridization events (Martin et al., 2021; Stamatakis, 2014). The inclusion of

Bayesian approaches analysing genomic data with radiocarbon dates can help us determine divergence dates and diversification events (Suchard et al., 2018). The information generated from these analyses can inform on the geographic, temporal, and evolutionary history of a pathogen, allowing us to trace its presence across time and place. It also allows us to see if there was a diversification event in a disease already present in the Americas coincident with European arrival (the cause for which could be inferred using contextual information–see research question number three below). Even if European in origin, we might expect to see a diversification event in the Americas, as a rise in the number of susceptible people means more infected people, which means more opportunities for the disease to mutate and spread.

If a recovered pathogen genome has high enough coverage, functional analyses may also be warranted. This has the potential to identify gene losses or gains important for virulence or pathogenicity. If horizontal gene transfer or hybridization between strains is suspected, further analyses should also be run to view this in greater detail. These analyses might clarify exactly how the disease adapted to its new environment: Was there a reduction in virulence over time? Did it take genetic material from a related strain to become even more virulent? In short, these functional analyses can help us determine whether the high mortality rates were due to disease virulence, environmental variables, or factors concerning the host.

**3) Demographic, cultural, and ecological impacts: Did different environments and/or cultural practices lead to differences in disease spread or mortality outcomes?**

Well-documented ecological, cultural and demographic records in the studied region and time period offer a unique framework for interpreting the genomic data. The already-existing contextual data combined with genomic data could be used in an epidemiological manner to exclude or confirm different scenarios based on how likely it is that a particular type of disease might thrive in that particular situation. For example, simple demographic information recorded by bioarchaeologists (e.g. age, sex, and physical health and stress markers) could potentially shed some light on how a disease was affecting different segments of a population. Additionally, population density estimates could help determine how ideal conditions were for disease spread. Recorded cultural information such as differences in burial customs may also pertain to the level of disease spread, making it important to take into consideration from an epidemiological perspective. Ecological differences due to geographic environments (such as the striking altitude gradient seen in Peru) can also play an important role due to varying climates and potential reservoir species. Interactions with fauna in contexts such as hunting or animal domestication are important. Many of the pathogens from the "Old World" are correlated with zoonosis in farming contexts; while animal domestication occurred in the Americas as well, the domestic animal reservoirs of these "Old World" diseases did not exist in the Americas before European arrival (Collen et al., 2022). In line with this, the presence or absence of

animal reservoirs or disease vectors in a region is evidence to consider during data interpretation for certain pathogens. All of this contextual data should be examined and taken into consideration when interpreting genetic data from ancient pathogens.

**Site and Sample Background**

For this project, I sampled remains from three sites of interest circa colonial invasion in areas of modern-day Peru that are thought to lie along the routes taken by the Spanish (Figures 1 & 2):

1) Kanamarka is a highland archaeological complex in the Espinar Province, approximately 150 kilometers south of Cusco, Peru. A site with pre-Inca, Inca, and colonial-era structures, Kanamarka shows evidence of human occupation over impressive lengths of time (Aparicio Laucata, 2009). However, this study focused only on human burials recovered from the early colonial-era churchyard. This church is estimated to have only been in use for approximately 50 years (Elsa Tomasto, personal communication, 2020), making it the likely resting place of contact-era disease victims. Radiocarbon dates obtained from these human skeletal remains indicate the cemetery was in use from approximately 1530 to 1580 CE, supporting this idea. In all, about 30 individuals buried in this churchyard lived during the early period of Spanish contact, but we selected 13 individuals to include in this study. Separate genomic investigation of individuals at Kanamarka at the UCSC Human

Paleogenomic Lab resulted in up to 60% endogenous DNA, indicating excellent levels of DNA preservation at this site.

2) Iglesia Colonial de Huanchaco is a site on the north coast of Peru (close to Trujillo, Peru) containing an early colonial church. Huanchaco is well-known as the site of the largest child sacrifice found in Peru (Prieto, 2019), but our research focused instead on the site's colonial-era church cemetery. Many of the individuals buried in the cemetery were children, but they appear to have met a much less violent fate than those found in the more famous area of the site. The approximately 100 individuals found in the church cemetery were likely alive during Spanish conquest, and therefore can provide valuable information about disease (Prieto, 2019). Of these, we selected 34 individuals for the study. We began the investigation optimistic about obtaining high-quality DNA, as the preservation at this site is remarkable; many of the skeletons were found with hair and well-preserved clothing (Prieto, 2019).

We also had high hopes of finding pathogens based on prior research–the individuals buried here underwent a paleopathological analysis where indications of diseases were identified (Tschinkel, 2022). Thirteen of the individuals selected for ancient DNA analysis were chosen due to the presence of osteological signs of infection. While all of these signs were nonspecific indicators insufficient for identifying specific infectious agents, three of these individuals were young children with indicators of viral infection (potentially smallpox) on their elbow joints. Two other individuals, one an infant and one a woman over the age of 60, had vertebral

characteristics that researchers suspected may be due to tuberculosis. Other maladies were listed as sinusitis, avascular necrosis of the hip, genu valgum ("knock knees"), osteomyelitis of the ankle, a maxillary infection, and three cases of "endocranial new bone formation". One additional individual was a woman with an atrophied leg, although this presumably was not why she was selected for pathogen DNA analysis.

3) Marcajirca, a highland site in Peru's Ancash region, is approximately 6 kilometers from Huari, Peru. In addition to the many individuals with paleopathological evidence of tuberculosis found in typical burials at this site, at least 46 deceased individuals were found buried in the ground (Ibarra, 2013). In this region during this period, the dead were generally buried in either rock shelters or *chullpas*, making these in-ground "mass burials" highly unusual (Ibarra, 2013). Because of the abnormal style of burial and lack of evidence of physical trauma (E. Washburn, personal communication, 2019), we hypothesized these individuals died in rapid succession due to an epidemic of infectious disease. Radiocarbon dates indicate these individuals died circa European contact, which lends credence to this idea. Including 14 individuals from earlier typical burials and 22 individuals from these atypical burials, we included 36 individuals from this site in this study.

Collecting samples from the deceased was done with the full permission and endorsement of Peru's Ministry of Culture. Peru has a system in place guided by *Ley General del Patrimonio Cultural de la Nación* (Law No. 28296) in which each site is managed by the district archaeologist local to the area; this archaeologist is a

representative of the local community of which they are a member, and are responsible for the dissemination of research results to the community through educational materials, museum exhibits, and community meetings. This system ensures that local Peruvians are in charge of all projects and permissions at various stages, which provides some representation of community interests. These district archaeologists are part of the team working on this project. Before final results are published, we aim to participate in town meetings with the broader local communities to give individual community members an opportunity to get involved and contribute their thoughts on how continuing research should be conducted. This project is a new facet of a longstanding collaboration, so these meetings will be an iteration of meetings that have been carried out before.

**Methods**

To reduce the risk of sample contamination, we followed all typical aDNA decontamination and working protocols (Fulton & Shapiro, 2019). This includes working in a dedicated positive-pressure, HEPA-filtered, multi-chamber cleanroom with a dedicated gowning chamber for cleanly donning a hair cover, surgical mask, Tyvek coverall suit with a hood, eye protection, a bottom glove layer that is secured within the suit, and a top glove layer. Top gloves and surroundings were frequently cleaned with bleach, DNA-away, or Alconox as per standard aDNA protocol. Tooth or bone samples, as well as reusable instruments, were subjected to UV light before powder collection in order to crosslink surface contaminant DNA.

Due to time and financial constraints, a subsection of the available individuals were selected for sampling based on preservation and contextual information, resulting in samples from 87 individuals overall. Well-preserved teeth were targeted for sampling, but highly vascularized bones or remains with paleopathological signs of disease (such as collapsed vertebrae) were occasionally sampled instead on a case-by-case basis (see Table 1). The sampling approach in pathogen paleogenomics studies may differ due to sample anomalies or requests from the community or scientific stakeholders. In general, our strategy followed a specific protocol: Once brought into the clean-room, a sample was subjected to UV light and then "surface cleaned" using a sterilized circular saw drill attachment to remove environmental surface contamination in the region including and surrounding the area to be powdered. The working area was then cleaned, and the drill bit was interchanged with a new decontaminated drill-bit best suited for the sample. For tooth samples, DNA from the tooth pulp was targeted, as the likelihood of bloodborne pathogen DNA preservation is often greatest in the tooth pulp (Spyrou et al., 2019). To do so, the pulp was generally drilled out as powder using a small handheld drill, although for some the tooth root was cut off and milled to a powder before extraction. For non-tooth samples (generally a vertebra with visible pathology), the area thought to contain pathogen DNA was drilled into to create powder. Instruments and workspaces were thoroughly decontaminated between samples.

Due to the potential of false positive pathogen hits stemming from the presence of closely related soil bacteria, we collected soil samples from Marcajirca.

To establish a baseline metagenomic profile, one "grave soil" sample was taken from each of the two burial sites. Because it is possible that pathogen DNA found in grave soil was shed by the body during the decomposition process (and therefore authentic; Thomas et al., 2019), one "proximal soil" sample was taken from 50-75 meters away from each burial site as well. Ideally, both "grave" and "proximal" soil samples should be collected from the same depth as the burials, but this is sometimes difficult to obtain. This was the case in Marcajirca, so the soil samples were collected from at least 45 cm below the soil surface in order to minimize modern contamination potential. While extractions optimized for soil do exist, soil samples for our purposes were subjected to the same extraction protocols as the bone and tooth samples in order to minimize variables and provide a more accurate view of how soil bacteria contamination may present in the bone and tooth sample data. We were unable to collect soil samples from the sites of Kanamarka or Iglesia Colonial de Huanchaco to establish those baselines, so preliminary results from those sites must be interpreted with additional caution.

Once approximately 50 mg of soil, bone or tooth powder was collected in a 2 mL Safe-Lock Eppendorf tube, we proceeded with extractions. In general, we performed extractions in batches of 15 samples and one negative control, following the protocol described in Dabney et al. (2013), which is optimized for ancient DNA samples.

We then partially UDG-treated most extracts, a process described by Rohland et al. (2015) which repairs the cytosine to uracil damage characteristic of ancient

DNA on all but the first and last bases of each fragment. Our lab has modified this process to work optimally with our chosen single-stranded library protocol, the SCR method (Kapp et al., 2021). In addition to being time- and cost-efficient, this library-building protocol offers the ability to transform single-stranded DNA into libraries and results in higher complexity than other methods, which improved our chances of producing informative data (Kapp et al., 2021). Samples were then dual-indexed and shotgun-sequenced for screening (approximately 2 million reads per sample) on an Illumina NextSeq platform.

To begin drylab analysis, the sequencing data was run through the initial stage of "BATpipe," the UCSC Human Paleogenomics Lab's bioinformatics pipeline (https://github.com/mjobin/batpipe). This consists of trimming barcodes and filtering of low-complexity sequences and reads shorter than 30 basepairs in length. This process generates the adapter trimmed files of our raw sequencing data that serve as the input for our screening tool of choice, HOPS (Hubler et al., 2019); we used a minimum identity threshold of 90% and a custom RefSeq Genome database of: all complete virus genomes without 'Other' or 'unknown' in names [2017-10-30]; all bacterial assemblies at 'chromosome' [with WGS data] or 'complete' completeness level, without 'Other' in names [2017-11-03]; a selection of pathogenic eukaryotic genomes; and the Homo sapiens subsp. sapiens reference sequence GRCh38). The HOPS pipeline uses a modified version of MALT (Vågene et al., 2018), a metagenomic alignment tool that will taxonomically bin reads, providing a taxonomic ranking based on similarity of sequence reads to reference. The second major tool of

the HOPS pipeline, MaltExtract, further assesses taxonomic assignments for a selected list of taxa, in this case pathogens of interest, for characteristics of ancient DNA (Hübler et al., 2019). This tool provides hierarchical results falling into three categories: reads passing the expected edit distance distribution, reads demonstrating ancient DNA damage, and reads possessing damage that demonstrate the expected edit distance. Results reported in Table 2 are separated and labeled to reflect these categories.

When the initial shotgun data appeared to potentially contain pathogen DNA, the associated libraries were sent to another institution for deeper sequencing on a HiSeq or NovaSeq Illumina platform. Once this new data was merged with the initial shotgun data for each individual, the previously mentioned pipeline including HOPS was run again. We report results from this stage for all individuals (Table 2).

Plausible hits for pathogens of interest based on HOPS results were compiled and data from all individuals were mapped to references corresponding to those pathogens of interest to obtain mapping statistics. Some pathogens not detected by HOPS were included as additional references in mapping analyses due to historical context and MALT results. To begin the mapping step, sequencing files were concatenated by individual and processed using AdapterRemoval with a minimum adapter overlap of 1, minimum length cutoff of 30, minimum quality cutoff of 20, and consecutive strings of Ns and low-quality bases trimmed from the 5’ and 3’ ends (Schubert et al., 2016). The resulting files were then mapped to the respective pathogen reference genome (for references used, see Table 3) using bwa aln (Li &

Durbin, 2009;  -n 0.1, -l 32 -q 37 and a samtools quality filter of 37). Duplicates were removed using MarkDuplicates (https://broadinstitute.github.io/picard/), and the resulting bam files were visualized in Geneious v9.1.8 (Kearse et al., 2012) to determine evenness of coverage. Geneious provides an easy-to-use visual display, which helps determine if the mapped reads are truly endogenous, highly conserved regions, or simply mapping onto contamination in the reference used. Mapping statistics were reported for all HOPS detections as well as any individuals whose mapped data covered more than 0.2% of the reference genome (this threshold was chosen arbitrarily) or if an individual appeared to have many more reads mapping to the reference in question than other individuals. In cases where mapped viral data resulted in a small number (<10) of reads mapping, we used the nucleotide BLAST (blastn) web interface (https://blast.ncbi.nlm.nih.gov/Blast.cgi; we used standard databases, megablast, and default parameters with the taxon of interest excluded) as a sanity check to see if the reads mapped to anything other than the pathogen of interest.

## Preliminary Study Results and Interpretation

### *Excluded Taxa*

A number of taxa identified by HOPS were identified as unreliable hits during the mapping process (see Table 4) and excluded from downstream analyses. The first category of these are the false positives. In these cases, the reference genomes for the

representative taxon are contaminated with adapters or sequences from other taxa (often human DNA contamination). The second category is taxa that could potentially be true positives, but are complicated to authenticate as they are also known to be present in burial and/or lab environments. All of these taxa that were originally included in the HOPS analysis and were later excluded are listed with the reason they were excluded in Table 4.

It is also worth noting that many of the individuals not flagged by HOPS that met the arbitrary 0.2% reference coverage reporting threshold also tended to be the individuals with higher shotgun sequencing investment. The higher numbers of overall reads correlate to higher coverage, but when investigated further these will likely prove to be higher coverage restricted entirely to conserved regions. As such, these mapping-only results (shaded gray in Table 3) should not be considered positive hits, but are reported here for transparency.

*Oral Microbiome and Red Complex Bacteria*

While not investigated thoroughly or reported in detail here, one of the many benefits to sampling teeth is the potential to detect commensal and pathogenic oral taxa. Many of the individuals in this study were flagged by HOPS as having DNA from oral taxa present. This was often the case when teeth were sampled, but they were generally not detected (or only passed the edit distance filtering stage, see full results in Table 2) in vertebrae samples and negative controls. However, this was not the case for one of the vertebrae sampled (the vertebra from individual MAR_50);

many bacteria (especially *Streptococcus* species) were detected in this individual, with genus-level *Streptococcus* reads passing all filters. Whether these taxa were necrobiome species or present due to modern contamination, it provides a reminder that extra caution should be taken when interpreting results indicative of "oral" taxa.

*Tannerella forsythia*, *Porphyromonas gingivalis*, and *Treponema denticola* are the three oral bacteria the make up the "red complex", a grouping strongly associated with advanced periodontal disease (Socransky et al., 1998; Suzuki et al., 2013). All three "red complex" bacteria were present in multiple individuals at all three sites. In addition, other oral taxa associated with dental caries (i.e. *Streptococcus mutans*, see Conrads et al., 2014) and periodontal disease (i.e. *Fusobacterium nucleatum*, see Socransky et al., 1998) were present in many of these and other individuals. However, many of these other bacteria not in the red complex are opportunists also found in normal oral flora (Lenartova et al., 2021); this means the proportion of the microbiome these bacteria make up is important information for interpretation. Because not all DNA may be converted into libraries, be sequenced, or survive at the same rates (Parker et al., 2020; Poulsen et al., 2022), information on relative prevalence is inappropriate to derive from sequencing data.

While we do not have information on the reliability of these hits, the prevalence of paleopathologically-visible oral disease at these sites, or the proportions of these bacteria within the oral microbiomes, the discovery of red complex bacteria is still an interesting finding. As such, individuals with all three red complex bacteria detected by HOPS are indicated by an "X" in the appropriate column of Table 1.

*Marcajirca Overall Results*

Using the methods detailed above, I processed 8 individuals from Structure 7, 14 individuals from Structure 10, 6 individuals from elsewhere on site with paleopathological indication of disease and 14 individuals randomly selected from traditional burials. Thus far, our investigations at this site have unearthed a few potential disease suspects. The findings produced by HOPS that were not excluded taxa include: *Bartonella bacilliformis*, a species in the family *Borreliaceae*, *Corynebacterium diphtheriae*, *Coxiella burnetii*, *Haemophilus influenzae*, bacteria within the *Mycobacterium tuberculosis* complex, *Mycobacterium leprae*, *Neisseria meningitidis*, *Salmonella enterica* subsp. *enterica, Streptococcus pneumoniae*, and *Streptococcus pyogenes*.

*Kanamarka Overall Results*

High-quality libraries from all 13 individuals sampled were successfully prepared and sequenced. At this site, detected pathogens that are most plausible include: *Corynebacterium diphtheriae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, bacteria within the *Mycobacterium tuberculosis* complex, *Neisseria* species, *Plasmodium vivax*, *Salmonella enterica* subsp. *enterica, Shigella* species, *Streptococcus pyogenes*, and *Streptococcus pneumoniae*. There are additionally three potential *Bordetella pertussis* cases and one potential *Orthopoxvirus* case, discussed

in the relevant sections below. HOPS detections and mapping statistics can be found in Tables 2 & 3 respectively.

*Iglesia Colonial de Huanchaco Overall Results*

Of the 34 individuals sampled from the early colonial site of Iglesia Colonial de Huanchaco, DNA libraries from two did not pass our standard wetlab quality checks. The 32 other libraries were sequenced and screened for pathogens in the manner previously described. At this site, *Human gammaherpesvirus 4* (also known as Epstein-Barr Virus), *Corynebacterium diphtheriae*, *Human alphaherpesvirus 1* (also known as Herpes Simplex Virus 1) and an *Orthopoxvirus* (most likely *Variola virus*) were highly likely present. Other findings include: *Bartonella quintana*, *Bordetella pertussis*, *Brucella* species, *Haemophilus influenzae*, species within the *Mycobacterium tuberculosis* complex, *Mycobacterium leprae*, *Mycoplasma* species, *Neisseria* species, *Salmonella enterica* subsp. *enterica, Shigella* species, *Streptococcus pyogenes*, and *Streptococcus pneumoniae*.

While prior paleopathological study of individuals at this site suggested a number of infectious agents were present (Tschinkel, 2022), one highly significant finding at this site was the tentative diagnosis of smallpox in some of the young children (Tschinkel et al., 2024). HOPS did not detect *Orthopoxvirus* DNA in any of the individuals, but did detect *Human gammaherpesvirus 4* in these individuals. This initial finding led us to consider that the recorded skeletal changes indicative of viral infection may have instead been caused by *Human gammaherpesvirus 4*. However, a

more targeted investigation revealed *Orthopoxvirus* DNA was in fact present in individuals at this site, supporting the paleopathological conclusions. The presence of *Human gammaherpesvirus 4* as a possible co-infection or as a false positive in some samples is discussed in the relevant section below.

*Bartonella*

HOPS detected *Bartonella quintana* and *Bartonella bacilliformis* in individuals IG_130 and MAR_28 respectively. *Bartonella quintana* is a bacterium spread by body lice that causes a form of bartonellosis referred to as "trench fever" (Ohl & Spach, 2000). It was first identified by Western scientists during World War I, as European soldiers in trenches experienced cyclic relapsing fevers with headaches, shin pain, and other symptoms (Ohl & Spach, 2000). It is today found worldwide and is most often associated with unhoused populations and the living conditions of poverty (Ohl & Spach, 2000). *Bartonella bacilliformis* is a related species endemic to Peru that is associated with the form of bartonellosis known as "Oroya fever", as well as the subsequent "verruga peruana". If untreated, up to 88% of individuals with Oroya fever die, often due to complications with secondary infections (Minnick et al., 2014). The disease is most common in Peru, in regions of the Andes where the sandfly vector thrives (Minnick et al., 2014). While the disease is named after an 1871 outbreak of deadly febrile hemolytic anemia in railroad construction workers moving through the Peruvian Andes, the disease was well-known to Indigenous

people living in the mountainous valley regions of the Andes since at least before the Inca period (Alexander, 1995; Minnick et al., 2014).

While not directly associated with colonization, further research into the *Bartonella bacilliformis* infection detected at Marcajirca could provide insight into the diseases present before colonization and the evolutionary history of this bacterium. While Marcajirca's elevation is about 400 meters higher than the recorded habitat of the sandfly vector, modern outbreaks in areas outside of the sandfly's typical altitude range have been recorded (Minnick et al., 2014). It is also likely that the people living at Marcajirca at least occasionally ventured down into the valleys where the sandflies thrive; the context suggests this finding is highly plausible and should be investigated further.

The *Bartonella* finding at Iglesia Colonial de Huanchaco was classified as the species *quintana* by HOPS, but mapping results show longer average read lengths and a higher number of reads when mapped to the reference for *Bartonella bacilliformis*. Contextually, either species would make sense–infected body lice certainly could have been present in the context, and travel to or from the highland valleys where infected sandflies live would not have been impossible for people living at this site. Based on the HOPS results and mapping statistics, IG_130 also had a likely co-infection with *Shigella*. As this is a common secondary infection seen in *Bartonella bacilliformis* patients (Karem et al., 2000; Minnick et al., 2014), future investigations should include both *Bartonella* species in analyses.

*Bordetella pertussis*

*Bordetella pertussis*, a vaccine-preventable pathogen that causes a disease commonly known as whooping cough, has been dangerously gaining resurgence in recent years due to growing anti-vax sentiments (Grignolio, 2018). Like many pathogens, it is thought to have been first introduced to people in South America by colonial explorers, resulting in deadly consequences (Nunn & Qian, 2010). At the genus level, HOPS identified *Bordetella*; however, there are environmental species of this genus present in soil and water (Hamidou Soumana et al., 2017), meaning these identifications could be false positives. Additionally, one of these genus-level identifications was in a negative control. Given the historical context, the presence of *Bordetella pertussis* is possible, but reads assigned to the species level did not pass the HOPS filters. Mapping statistics show that individuals from all three sites have reads covering more than 0.2% of the *Bordetella pertussis* reference genome, with one individual's data covering upwards of 3%. However, given the environmental context, this mapping data is not sufficient to suggest the presence of pathogenic species.

*Corynebacterium diphtheriae*

While the story of Balto may be what comes to mind for many Americans today when they think of diphtheria, the deadly disease was certainly not restricted to 1920s Alaska. Caused by a bacterial infection most often colonizing the throat and nose, diphtheria is thought to have been brought to the Americas by European

colonizers, and infections are still a concern in many regions of the world today (Truelove et al., 2020). *Corynebacterium diphtheriae*, the causative agent of diphtheria, is thought to be nearly exclusively a human pathogen (Parveen et al., 2019). The most severe effects of the disease are caused by a potent toxin produced by the bacteria, which can actually only be produced if the bacteria themselves are infected with a specific bacteriophage (Parveen et al., 2019). *Corynebacterium diphtheriae* was detected by HOPS in three individuals from Marcajirca, one individual from Kanamarka, and one individual from Iglesia Colonial de Huanchaco. Four additional individuals from Marcajirca were not flagged by HOPS, but mapping revealed over 0.2% of the genome covered. Given some other *Corynebacterium* species occur naturally in the environment and in the skin microbiome (Grice et al., 2009; Oliveira et al., 2017), these additional four are most likely not true infections. However, the five individuals flagged by HOPS may warrant further investigation, including competitive mapping and capture enrichment. The case in the individual from Iglesia Colonial de Huanchaco (IG_497) in particular is of interest, as the reads passed all HOPS filters and mapping of the shotgun data resulted in over 1% of the genome covered.

*Human alphaherpesvirus 1*

Formerly known as *Herpes simplex virus 1*, *Human alphaherpesvirus 1* is the herpesvirus responsible for cold sores (Wald & Corey, 2007). While sometimes sexually transmitted, it is most often acquired during childhood through non-sexual

contact (Wald & Corey, 2007). HOPS detected this virus in two individuals from Iglesia Colonial de Huanchaco (IG_453 and IG_497). Almost 9% of the reference genome is covered by the data from IG_497 and reads are distributed across the genome, strongly indicating the presence of this virus. Taking mapping results and subsequent BLAST results into consideration, both of these individuals plus two additional individuals from the same site appear to be infected with *Human alphaherpesvirus 1*.

*Human gammaherpesvirus 4*

*Human gammaherpesvirus 4*, also often referred to as "Epstein-Barr Virus", is a common infection in humans worldwide and is thought to have co-evolved with humans since the emergence of our species (Ba Abdullah et al., 2017). Infection with the virus often results in infectious mononucleosis ("mono"), and is also associated with a number of cancers (Palser et al., 2015). No individuals from Kanamarka or Marcajirca were flagged by HOPS as containing reads assigned to *Human gammaherpesvirus 4*. In contrast, 15 individuals from Iglesia Colonial de Huanchaco were. While it is possible that this represents a large outbreak of the virus or a difference in viral prevalence between the different populations, it is also possible that some of these putative infections are actually a side-effect of our sequencing strategy. In one individual (IG_466), ~80% of the genome is covered, with ~11x fold coverage on average. The library from this individual was sequenced deeply, and pooled with other libraries from individuals at this site. It is possible these reads found in some of

the individuals at minimal levels are in fact due to index contamination or "index-hopping" where reads from one library are assigned to a different one (van der Valk et al., 2020). Two individuals from a different project (the individuals are from a site in a different country and lived thousands of years before the individuals in this study) were sequenced on the same flow cell and were also flagged by HOPS due to a few reads assigned to *Human gammaherpesvirus 4*, supporting the index-hopping hypothesis. However, based on read numbers, there are at least two individuals that likely were infected: IG_466 (the individual mentioned above) and IG_497.

Notably, data from one child (IG_497) strongly indicated the simultaneous presence of *Corynebacterium diphtheria*, *Human alphaherpesvirus 1*, and *Human gammaherpesvirus 4*. Unlike other instances where multiple low-level false positives are present in one individual simply due to the sequencing depth, there is strong evidence that this child was indeed infected with all three. The HOPS and mapping data together provide robust support for this conclusion. Additionally, this co-infection would make sense from an ecological perspective. While fairly distantly related, both *Human alphaherpesvirus 1* and *Human gammaherpesvirus 4* are herpesviruses (Cohen, 2020). Herpesviruses enter into a latent phase after the initial infection, and can be reactivated during times of stress on the immune system (Cohen, 2020; Suzich & Cliffe, 2018). While *Human gammaherpesvirus 4* establishes latency in B cells, often in the tonsils and adenoids (Seishima et al., 2017), *Human alphaherpesvirus 1* most frequently establishes latency in nerve cells connected to the original infection site (Wang et al., 2023). Given the individual's age and the fact we

sampled a tooth, it is most likely that this original infection site was in the oral region (ie a cold sore). We hypothesize that this child had a diphtheria infection, which created an environment conducive for the reactivation of both latent herpesviruses.

*Mycobacterium Tuberculosis Complex*

*Mycobacterium tuberculosis* complex bacteria (MTBC) are human pathogens of interest with a historic connection to European conquest in South America (Walker et al., 2015). These bacteria cause tuberculosis, still an incredibly widespread and deadly disease today (Kidder, 2009). This is a major public health issue in Peru; Peru has the highest incidence rate of tuberculosis of all South American countries and the highest number of antibiotic-resistant cases (World Health Organization, 2023). Paleopathological evidence of pre-columbian tuberculosis is also widespread in Peru (Nelson et al., 2020). Genomic data derived from ancient individuals in the Andean region has confirmed MTBC was present in Peru before European colonizers embarked on their first voyage across the Atlantic, although this differs from the lineages seen in the region today (Bos et al., 2014; Vågene et al., 2022). The identified species, *Mycobacterium pinnipedii*, found in modern seals and sea lions, appears to have been endemic in South America by the time of European contact (Bos et al., 2014; Vågene et al., 2022), but was replaced as the dominant cause of tuberculosis following European colonization by the lineage 4 of *Mycobacterium tuberculosis* complex (Gagneux et al., 2006; Santos-Lazaro et al., 2021).

In line with prior paleopathological evidence of possible tuberculosis in multiple individuals at Marcajirca (E. Washburn, personal communication, 2019), MTBC was detected by HOPS in multiple individuals from this site. Interestingly, only one of these findings was in an individual with recorded pathology. One other individual with pathology yielded a finding of *Mycobacterium leprae*, a related species that is one of two known causative agents of Hansen's Disease (commonly known as "leprosy"; Deps & Collin, 2021). Multiple individuals from Kanamarka and Iglesia Colonial de Huanchaco were also flagged by HOPS for MTBC (Broomandkhoshbacht et al., 2023), and *Mycobacterium leprae* was detected in one additional individual from Iglesia Colonial de Huanchaco. While these results provide an exciting opportunity for further research, the data must be interpreted with caution. Many closely related mycobacterial species are abundant in the environment (Primm et al., 2004), making additional verification of any *Mycobacterium* DNA crucial.

*Orthopoxvirus*

While it was not flagged by HOPS, we felt the prior paleopathological diagnosis of smallpox in individuals from Iglesia Colonial de Huanchaco warranted further investigation. Data from all individuals from all three sites were mapped against the reference genome for *Variola virus* (NC_001611.1) using the parameters outlined in the methods section above. The mapping statistics for all individuals with any reads aligning were recorded in Table 3 and the resulting bams were visualized in Geneious. Due to the limited numbers of reads, we used the BLAST approach

described in the methods section above. Based on the distribution of reads across the genome and the specificity of the reads inferred from BLAST results, some individuals from Iglesia Colonial de Huanchaco–including IG_124, who was diagnosed in the prior study (Tschinkel et al., 2024) –do appear to have been infected with an orthopoxvirus. One individual from Kanamarka was also putatively positive, but notably no reliable reads were detected in any individuals from Marcajirca. This of course does not exclude the possibility that *Variola virus* or a related *Orthopoxvirus* was present, but does reduce the likelihood that it was the causative agent of death for the individuals in the mass graves at Marcajirca. Based on historical context, it is likely the virus detected at Iglesia Colonial de Huanchaco (and possibly Kanamarka) was smallpox brought by European colonizers. Further verification would require additional work; in line with the research restrictions surrounding these viruses, future analyses will only be conducted after communication and approval from the US Centers for Disease Control and Prevention and any additional authorities whose approval they deem necessary.

*Shigella*

As noted in the earlier section on *Bartonella* infections, *Shigella* infections are a common secondary infection seen in patients infected with *Bartonella bacilliformis* (Maco et al., 2004). Infections of *Shigella* species were detected by HOPS in two individuals: IG_130 (the individual from Iglesia Colonial de Huanchaco who likely also was infected with a *Bartonella* species), and KMA_19-1 (a subadult from

Kanamarka). *Shigella* species are transmitted through the fecal-oral route and are a major cause of dysentery worldwide (Strockbine & Maurelli, 2015; Williams & Berkley, 2018). Humans are the main reservoir of *Shigella* species, although other non-human primates can be infected (Strockbine & Maurelli, 2015). Mapping statistics showed multiple other individuals had reads covering more than 0.2% of the *Shigella* reference genomes. However, given Shigella species share much of their genome with more widespread close relatives (Strockbine & Maurelli, 2015), it is likely these mapped reads are from related bacteria present in the gut microbiome, necrobiome and soil.

*Streptococcus pyogenes & Streptococcus pneumoniae*

While *Streptococcus pneumoniae* and *Streptococcus pyogenes* can both be found as normal human microbial flora, they can also act as major pathogens (Patterson, 1996). *Streptococcus pneumoniae* causes pneumococcal disease, which can include pneumonia, meningitis, and other serious types of infection (Patterson, 1996). Streptococcus pyogenes is the causative agent of "strep throat", scarlet fever, and other types of infections (Patterson, 1996). HOPS detected both *Streptococcus* species in individuals from both sites. The mapping statistics show coverage of *Streptococcus pneumoniae* too low to draw conclusions from, but mapping data of two individuals from Iglesia Colonial de Huanchaco and one individual from Marcajirca appear to confirm the presence of *Streptococcus pyogenes*, with 28-38% of the genome covered. Scarlet fever is one of the diseases hypothesized to have been

brought to the Americas by Europeans (Nunn & Qian, 2010), making this a notable result.

*Other Pathogens Detected*

HOPS also detected reads assigned to multiple other categories, including: the family *Borreliaceae*, the genus *Brucella*, *Coxiella burnetii*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Mycoplasma pneumoniae*, *Neisseria* species, *Plasmodium vivax,* and *Salmonella enterica* subsp. *enterica.* While each of these is interesting, for brevity I will not be focusing on these with the depth provided to the other findings. In this section, I will briefly describe each pathogen and the relation to the findings.

*Borreliaceae* is a family of pathogenic vector-borne spirochetes (Barbour & Gupta, 2021). While detected by HOPS in two individuals from Marcajirca (MAR_8 and MAR_98), mapping to references for *Borrelia burgdorferi* (NZ_CP019767.1) and *Borrelia recurrentis* (NC_011244.1) resulted in coverage of less than 0.3% of the genomes. Mapping statistics from two additional individuals from Marcajirca (MAR_9 and MAR_45) are also listed, as they each passed the 0.2% coverage threshold for *Borrelia recurrentis*; however, like most of the low-coverage findings, these additional detections could be due entirely to reads mapping to conserved regions.

*Brucellaceae* is a family containing many environmental species (Kämpfer et al., 2014), so HOPS identifications at the family level were ignored. However, the genus *Brucella* was identified by HOPS in one individual (IG_121). *Brucella* species,

commonly associated with brucellosis, can survive in the environment, but generally they thrive in animal hosts and are associated with livestock (Kämpfer et al., 2014).

*Coxiella burnetii* is a pathogen that causes Q fever, a zoonotic infection associated with farm animals (Abeykoon et al., 2021). One individual from Marcajirca (MAR_45) was flagged by HOPS for this bacterium. This bacterium can also persist for long periods in the environment (Abeykoon et al., 2021), which draws suspicion upon the source and antiquity of the reads detected.

*Haemophilus influenzae* was detected by HOPS in two individuals from Kanamarka, two individuals from Marcajirca, and four individuals from Iglesia Colonial de Huanchaco. *Moraxella catarrhalis* was detected by HOPS in one individual from Kanamarka and one individual from Iglesia Colonial de Huanchaco. Certain strains of these two groups of bacteria can cause severe and sometimes life-threatening infections, but others are present as commensal bacteria in the human microbiome (Verduin et al., 2002). Given the low coverage nature of the data, determining whether the detected bacteria are commensal or pathogenic will be difficult, potentially impossible.

*Mycoplasma pneumoniae* was detected by HOPS in one individual from Iglesia Colonial de Huanchaco (IG_4-136). This infectious agent causes upper respiratory infections and atypical pneumonia (sometimes referred to as “walking pneumonia”) and is today one of the leading causes of community-acquired pneumonia (Hsieh et al., 2022; Kogan Alterman & Maggiolo Massone, 2020). Based on divergence date estimates and modern proportions of lineages in different

geographic regions, researchers have proposed this pathogen emerged in 12th-century Europe and was subsequently spread to the Americas during colonization (Hsieh et al., 2022). While the data we have is currently too low coverage for additional analyses, future research further probing this finding could bring additional clarity to the evolutionary history of this pathogen.

The genus *Neisseria* contains many commensal species found in the microbiome of mucosal surfaces of multiple animals (Liu et al., 2015). Two species are considered pathogenic to humans: Neisseria gonorrheae and Neisseria meningitidis; these cause gonorrhea and bacterial meningitis respectively (Liu et al., 2015). HOPS detected *Neisseria* at the genus level as well as *Neisseria gonorrheae* at the species level in individuals from Kanamarka and Iglesia Colonial de Huanchaco. *Neisseria meningitidis* was detected by HOPS in individuals at all three sites. Given the close relationship with commensal species and the improbability of finding *Neisseria gonorrheae* widespread in tooth samples, the species-level findings must be interpreted with caution and skepticism.

*Plasmodium vivax* is a parasite that is a major cause of malaria worldwide (Tuteja, 2007). *Plasmodium vivax* was detected by HOPS in one individual from Kanamarka, but this will need to be verified through rigorous testing.

*Salmonella enterica* subsp. *enterica* has attracted aDNA researchers' attention in recent years (de-Dios et al., 2021; Haller et al., 2021a; Haller et al., 2021b; Key et al., 2021; Neumann et al., 2022; Vågene et al., 2018; Zhou et al., 2018). HOPS detected this bacteria in individuals from all three sites, but was also detected by

HOPS in a soil sample and a negative control. Mapping statistics did not provide additional clarity on the validity of these findings. Many serovars of this subspecies are widespread and survive for long periods in the environment (Andino & Hanning, 2015), making robust and in-depth examination of any *Salmonella enterica* subsp. *enterica* finding critical if one wants to avoid misrepresenting their findings.

**Future Directions**

Preliminary results from this project provide multiple avenues for potential future research. The pathogen genomes recovered from high-throughput shotgun and capture data as well as previously published genomes of that species (modern and ancient) will be used to reconstruct complex pathogen evolutionary histories. The information regarding divergence dates, diversification events, and relative position on the tree will be examined in conjunction with available contextual information to draw conclusions about the pathogen of interest's origins and its behavior during a time characterized by chaos and upheaval.

Moving forward, I will more deeply investigate our initial findings using the methods detailed earlier. In cases where more than one pathogen is verified in an individual, co-infections may also be considered based on the situation. Verified disease strains will be characterized, placed on a phylogenetic tree, and considered in context when drawing conclusions to answer research questions.

In addition to the encouraging results we've obtained from the sites in conquest-era Peru, we have also begun to expand our sampling to include human

individuals from a broader geographical and temporal range, as well as ancient fauna. Faunal remains are particularly important to include from a One Health perspective, as environmental changes and human-fauna interactions impact the disease ecology landscape as a whole (Myers et al., 2013). This additional data will help contextualize changes seen at this time in the region, which will ultimately provide us with a much richer frame of reference. All of this will contribute to a clearer picture of the pathogen's evolutionary history, which will help us obtain a better understanding of the disease in the present and future.

Another possibility for future research is one that has potential for both beneficial and harmful impacts: examining the impact on human populations. Informative immune markers from the human data produced as a by-product of this research could more directly shed light on pathogens' impacts on the human population itself. As diseases swept through the Americas, they likely enacted a strong selective pressure on immune markers in the Indigenous populations (Lindo et al., 2016). The selective pressure may have been strengthened by a lack of prior exposure to some diseases, but for diseases where it is expected that partial immunity existed (such as tuberculosis), the deaths may have been more random. However, other factors were likely involved as well (such as the contextual factors discussed earlier) that influenced the strength and direction of selection. The consequences of these selective pressures may still be detectable today in modern populations (Lindo et al., 2016), but ancient human data could further fine-tune these findings. This research would require a large amount of good quality data. Ancient data is

notoriously poor quality, but immune marker data could be improved through capture enrichment of HLA (MHC) markers for both the presumed epidemic victims and other ancient humans from the area (regardless of time period). If communities request this work, we could search for selective sweeps using already-published modern data compared to the less well-covered ancient data; it would also possible to simply search through the epidemic victims' data for the presence or absence of known protective genes linked to innate or adaptive immunity that are already known to exist in the population today. This type of research has the power to enhance our understanding of the impacts of colonialism and disease; however, it could also have harmful unexpected consequences. Since this would be identifying how certain diseases impacted (and might still impact) certain groups of people, the potential for this data to advance eugenics-motivated bioterrorism is rightfully a concern. This research should not be pursued without detailed ethics considerations where all possible outcomes are discussed and data restrictions are ensured.

How and why the diseases affected (and continue to affect) populations in Peru and beyond are some of the most important questions to answer if this research is to have a large beneficial impact. Due to the potential for harm upon incorporating human data, we are choosing to focus solely on pathogen data and contextual information unless affected communities explicitly request otherwise.

*Methods for Future Work*

Genomes with visibly uneven coverage are deemed inauthentic and will not be pursued further. Genomes that do pass this initial check will be processed as follows: Pathogens with related environmental relatives will go through a competitive mapping process, environmental microbial background analysis, and soil comparisons when available. Competitive mapping will be performed by concatenating the pathogen reference and non-pathogenic species' reference, then mapping relevant individuals' sequencing data to this concatenated reference using the bwa aln mapping parameters described above. For individuals from Marcajirca, the affiliated soil samples will continue to be treated as negative controls. These will be run through all described screening processes alongside the putatively positive data to determine if the pathogen hits might be due to soil- or laboratory-derived contamination. If proximal soil controls produce genomes comparable to genomes derived from individuals that have passed all of the described screening processes, these individual-derived pathogen genomes will be considered false positives and will not move forward to downstream analyses. If only the grave soil controls produce comparable genomes, this will be reported but it will not be sufficient evidence to consider individual-derived genomes as false positives. Environmental background analysis will be performed using the rma6 files produced while running MALT in the HOPS pipeline. The number of reads assigned to the species node will be compared to the number of reads assigned to the node that leads to both the species of interest and environmental bacteria. For example, the number of reads assigned to

*Mycobacterium tuberculosis* complex bacteria will be compared to the number of reads assigned to the *Mycobacterium* genus node. If the proportion of reads assigned to the pathogenic clade does not meet our threshold (which will be determined on a case-by-case basis), this indicates a capture-enrichment would not be cost-efficient given the high levels of closely-related environmental taxa.

Once pathogen hits have been verified in the future, the corresponding libraries that need higher coverage will undergo capture enrichment. A pathogen-specific hybridization capture for each identified candidate will be generated using customized myBaits probes that correspond to the pathogen of interest's reference genome. The product will be sequenced to exhaustion. De novo reconstruction of the pathogen's genome will be attempted if there is enough data; otherwise, the data will be mapped onto the reference genome using the previously described stringent parameters. Using this data and previously published genomes of related strains (both ancient and modern), maximum-likelihood trees will be built using RAxML (Stamatakis, 2014). Additional trees will be built using BEAST (Suchard et al., 2018) to test the RAxML tree structure as well as to determine divergence times and identify diversification events. If uncertainty in tree structure is suspected to be due to horizontal gene transfer or recombination of some kind, recombination analysis will be performed by cross referencing results from ClonalFrame ML (Didelot & Wilson, 2015) and Gubbins (Croucher et al., 2014) for bacterial pathogens and using RDP5 (Martin et al., 2021) for viral pathogens. To begin to understand the driving forces behind the pathogen's behavior, we will screen

genomes for the presence of known virulence factors and will analyze potential functional differences of ancient variants using SNPeff (Cingolani et al., 2012).

If identified descendant communities and local communities request it, the human data by-product could be combined with existing datasets of ancient and modern human data to investigate the impacts of disease on populations in Peru. By looking at frequencies of known immune-system-related markers (Pittman et al., 2016), future research could identify regions of the genome that were selected upon as a result of the diseases seen in this era. However, given the potential for this research to cause harm, this would require significant additional ethical considerations.

**Discussion and Conclusion**

Those who lived through the COVID-19 pandemic may remember the feelings of fear and uncertainty associated with the early days of this newly emerged disease, where there was no clear answer about its origins, how it spread, or what the future held. We can only imagine that for the individuals studied here, the appearance of new diseases was a similarly terrifying experience. In many cases during the conquest of South America, diseases brought by the Spaniards are purported to have arrived in some regions before the conquistadors themselves (Nunn & Qian, 2010). Too small to see with the naked eye, the arrival of these invisible enemies set the stage for the devastation that followed.

Colonialism has had lasting impacts on societies, including impacts on public health (Mitchell et al., 2019). Many pathogens in the region today are thought to be long-lasting holdovers from the era of colonial conquest, which brought devastation to the level of genocide to the peoples of the Americas, as well as to many other areas of the world (Walker et al., 2015). Many of these pathogens (such as MTBC) are currently wreaking havoc worldwide as they evolve to evade man-made defenses (Kidder, 2009), disproportionately affecting economically struggling countries (often countries still grappling with the aftereffects of European imperialism) and marginalized groups such as Indigenous people (Dehghani et al., 2018; Michaud, 2009; Onozaki et al., 2010). Clearly, this is not due entirely to the characteristics of the pathogens themselves. Colonialism (both past and present) creates unequal power structures and disrupts social systems and community infrastructure, including access to healthcare (Czyzewski, 2011; Wispelwey et al., 2023). Ecosystem dynamics (which we note can *also* be impacted by colonialism) also play a crucial role in the spread and severity of infectious diseases (Engering et al., 2013; Roche & Guégan, 2011).

Here we provide some support for hypotheses previously posed that are based on historical, archaeological and paleopathological research. The three Peruvian sites studied here lie along routes taken by Spanish conquistadors, and the individuals included in our study date to this time period. As seen in our preliminary results, many of the pathogens implicated in the "Columbian Exchange" were likely present at these sites at that time. The tentative detection of pathogens like *Corynebacterium*

*diphtheriae*, *Orthopoxvirus* and *Streptococcus pyogenes*–all thought to be brought to the Americas by Europeans–further supports the widely-accepted idea that the diseases they cause were affecting people during this time period. The detection of pathogens hypothesized to have origins in the Americas (ie *Bartonella bacilliformis*) provides some insight into the impacts of diseases that were there before. Additionally, the detection of pathogens thought to have existed in different forms on each side of the Atlantic prior to 1492 (such as MTBC and *Human gammaherpesvirus 4*) provides opportunities to investigate how and why certain lineages outcompeted others. However, even after we confirm our findings with more in-depth research, we must interpret those results with caution. The presence of a disease after the arrival of Europeans to the Americas does not mean it did not exist in the region before colonization. It also does not mean it was the studied individual's cause of death or even that the infected person was symptomatic. Conversely, absence of evidence for the presence of a pathogen is not evidence of absence; there are multiple other reasons why we may not detect pathogens that were present, such as our study's relatively small sample size, the localization of an infection, and methodological limitations.

As with any paleogenomics research, this project encountered (and continues to face) a number of limitations. Most importantly, the failure rate of ancient samples makes ancient DNA highly opportunistic, and searching for a small subsection of data from microbial organisms further increases that rate of failure (Spyrou et al., 2019). Regardless of effort, retrieving data from the pathogens that affected the studied

individuals is sometimes impossible. Many potential disease agents are also RNA viruses, which would not be detectable using the DNA research methods used here, even if they were to unexpectedly resist degradation over the hundreds of years that have passed since the time of death (Tsangaras & Greenwood, 2018). Similarly, ssDNA viruses can elude detection, as they are suspected to not survive well over time; however, recent research has indicated they actually may survive in ideal conditions by binding to the inside of their capsid (Mühlemann et al., 2018). Additionally, if only using this genomic information, we have no way of knowing how the pathogens we found actually manifested as disease in each individual, what diseases they encountered at earlier points in their lives, or whether a pathogen we recover was their cause of death. It is important to keep in mind that the detection of a pathogen does not equate with the detection of a disease. Pathogens can infect individuals in a variety of ways, including asymptomatically, depending on the environmental conditions, conditions of exposure, and differences in immune responses from person to person (Casadevall & Pirofski, 1999). Producing high-quality and complete data also still proves to be a challenge in the field, but methods continue to improve. This particular project performed much better than expected, providing multiple avenues for future research. While limitations do exist, the preliminary results from this study and the promising contextual background of all three sites merit further investigation. While extensive research may still result in an incomplete picture, it will be more complete than the narrative that currently exists.

This research has proven to be feasible, and has the potential for highly beneficial results.

In the future, data from this research can hopefully be used in initiatives addressing the health-related issues created by settler colonialism. The patterns observed in both novel and semi-novel diseases will be applicable to other diseases of the same types, meaning the information gained from this research can be used to help create predictive models for emerging, reemerging, and already-existing diseases, particularly in contexts of historic and modern colonial violence. By understanding a pathogen's evolutionary history, we can look towards what lies ahead with greater confidence in our predictions. By understanding our own history, we can begin to move towards brighter futures.

**Ethics Statement**

In an effort to reduce any potential this research may have for harm, we attempted to use a collaborative approach in order to appropriately study the short- and long-term health impacts of conquest and colonization. As with any responsible aDNA research, we have been and will continue to enter each stage of research thoughtfully and in collaboration with local groups. Under the direction of the UCSC Human Paleogenomics Lab and the Peruvian Ministry of Culture, in the near future I will work with local Peruvian groups and engage in community outreach in Peru to disseminate results as well as solicit opinions and ideas for future research directions. In addition, final research results will be used by Peru's Ministry of Culture to create

resources such as educational materials and museum exhibits for both local community enrichment and tourism revenue in Peru. All samples from individuals investigated in this study have been exported and analyzed with the Permission of the Peruvian Ministry of Culture (Resolucion Viceministerial No. 000157-2020-VMPCIC/MC, 026-2018-VMPCIC-MC, 144-2016-VMPCIC-MC). All samples and products are loaned by the Ministry of Culture, to be returned on request, and until then reported to the ministry in annual reports. The UCSC-HPL and its collaborators hold long standing collaborative relationships with the communities from which all individuals studied here derived. The research interests, as well as initial results of our analyses, mainly regarding the human DNA results, have been reported to local community representatives, including the Communidad Campecino San Sebastian (Cusco/Kanamarka), Asociación de Pescadores Huanchaco Perú (Huanchaco), and the Ayuntamiento de Huari, Áncash (Macajirca).

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**Figures and Tables**


**Figure 3.1.** Site locations (blue dots) in relation to the rest of South America, with arrows broadly denoting the routes taken by Pizarro. Expedition year labels are color-coded to match the route. Routes derived from Swanston Map Archive.


**Figure 3.2.** Site locations in closer detail, with broad geographic features visible.

Table 1. Sampling information and metadata.

Table 2. HOPS screening results.

Table 3. Mapping statistics.

Table 4. Excluded taxa.

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## Chapter 4: The Wild Warts
## Historical, Biological, and Ecological Context of Treponematoses

### Perspective

In the previous chapter, I discussed diseases brought from Europe to the Americas. Treponematosis is one disease (or rather, one complex of diseases) that may have moved in the other direction. This idea has been the source of intense debate for decades within fields such as paleopathology and history, and has yet to be resolved. In this chapter, I discuss what is currently known about the history and behavior of this disease complex and the microbes that cause the diseases.

### The Trouble with Treponemes and Treponematosis

For centuries, if not millennia, treponemal diseases have persisted as some of the most socially disruptive, stigmatized, and debilitating diseases. Despite their long history as agents of disease in human populations, there is a limited understanding on the evolution and spread of the pathogens known to cause treponematosis. Limitations in our current understanding are due to several factors including challenges in culturing, insufficient high-quality genomic data, and the differences in the disease syndromes despite the remarkable similarity of the treponemes causing them. Recent years have witnessed many of these challenges being overcome through technological advances in laboratory methods and Next Generation Sequencing (Edmondson et al., 2018; Jaiswal et al., 2020). Likewise, such advances have allowed for the first historic *Treponema* genomes (Barquera et al., 2020; Giffin et al., 2020; Majander et al., 2020; Schuenemann et al., 2018, Majander et al., 2024) which have

revealed the presence and movement of *Treponema pallidum* subspecies in historic contexts of Europe and America, thus highlighting the value of paleogenomics to directly address gaps in the understanding of *Treponema* evolutionary history. Ancient genomes provide data points that allow us to map the evolution of *Treponema* species and subspecies in deep time, permitting the evaluation of their relation to each other and their evolutionary history within rich historic and archaeological contexts.

The four *Treponema* known to cause treponematosis in humans cannot be distinguished morphologically or serologically (Fohn et al., 1988), but their geographic distribution and variability in clinical manifestations has permitted the classification of the four treponemal syndromes: pinta, yaws, bejel, and venereal syphilis (Roberts & Buikstra, 2019). These diseases are of clinical concern today and are caused by pathogenic species of *Treponema*: *Treponema pallidum* subsp. *pallidum* (associated with syphilis), *Treponema pallidum* subsp. *pertenue* (associated with yaws), *Treponema pallidum* subsp. *endemicum* (associated with bejel), and *Treponema carateum* (associated with pinta) (Roberts & Buikstra, 2019). The classification of *Treponema carateum* is debatable; its antigenic characteristics suggest it could be another subspecies of *T. pallidum*, but it is currently classified as a separate species (Fohn et al., 1988). This uncertainty stems from the lack of genomic data; no sequencing data for *T. carateum* is available (Giacani & Lukehart, 2014).

Syphilis, considered the most virulent form of treponematosis, is a multi-stage, mostly sexually-transmitted disease with a worldwide distribution.

Syphilis has experienced a recent rapid rise worldwide; there were 8.0 million new infections in 2022, up from 7.1 million new infections in 2020 (World Health Organization, 2021; World Health Organization, 2024). Despite the fact that most cases are currently easily controlled with antibiotics, it still causes deaths in regions and communities with limited access to care (Tao, 2023). After infection, the disease progresses through three stages. The initial stage, also called primary syphilis, is characterized by a chancre on the skin, developing at the site of the infection, most commonly on the genitals, rectum, lips, or tongue. After this initial sore, the pathogen becomes silent and re-emerges weeks later in the form of secondary syphilis. At this stage the pathogen mostly affects the skin with rash and warts covering the entire body. Without antibiotic treatment, the disease progresses to a latent stage where the pathogen becomes undetectable, only to reappear, in a percentage of the cases, years after the initial exposure in what is called tertiary syphilis. At this stage, the pathogen disseminates to infect internal organs including the brain, nervous system, heart and liver eventually leading to the death of the individual (Radolf, 2016).

The other three diseases, yaws, bejel and pinta, are often called endemic treponematosis. In general, these infections tend to be less severe than syphilis (Giacani & Lukehart, 2014). Primary signs in the non-congenital treponematoses are bumps or lesions on the inoculated skin or mucosa that lead to a three-stage progressive disease that parallel the stages of syphilis (Giacani & Lukehart, 2014). The stages of each disease vary in severity, expression, and progression from patient to patient. Yaws and bejel often infect skin, bones and cartilage (Giacani & Lukehart,

2014). Involvement of other internal organs is thought to be rare in yaws and bejel, and pinta is thought to be limited entirely to the skin (Giacani & Lukehart, 2014; Roberts & Buikstra, 2019). While neurological symptoms, cardiovascular symptoms and perinatal congenital transmission have been reported to occasionally occur in infection with yaws and bejel, they are much more common in syphilis infections and appear to never occur in pinta infections (Giacani & Lukehart, 2014; Zoni et al., 2019). In general, these non-syphilis forms of treponematosis are not sexually or congenitally transmitted. Bejel is considered to be a rare disease and the World Health Organization aims to eradicate yaws by 2030, however, gaps in screening and reporting have led some populations to experience an increase in cases (Noda et al., 2018). Pinta used to be endemic in South America, but has not been reported in decades (Baker et al., 2020). However, it has been proposed that its circulation likely continues nowadays but remains unreported (Stamm, 2015).

These four different forms of treponematosis share some similarities in transmission dynamics, but also possess variability that is still not fully understood. For example, the pathogens causing yaws and pinta are believed to be primarily transmitted in childhood through prolonged contact of infected skin, while *T. pallidum subsp. pallidum* is primarily transmitted through sexual contact (causing venereal syphilis) and is thought to be the only treponeme of the four syndromes that can cross the placental barrier to infect the fetus (Baker et al., 2020, Zoni et al., 2019). Bejel is believed to be transmitted primarily through an oral route (i.e., mouth to mouth contact, the sharing of contaminated utensils and food, etc.), but has recently

also been implicated in sexually-transmitted disease (Noda et al., 2018, Shinohara et al., 2022; Vrbová et al., 2022).

Environmental and geographic distributions are also not clear-cut: there are areas where more than one subspecies is found (Giacani & Lukehart, 2014; Hopkins & Flórez, 1977). Today bejel is generally found in the hot and dry eastern Mediterranean and in the Sahel zone (Giacani & Lukehart, 2014). Yaws is mostly found in hot and humid sub-Saharan regions of west and central Africa and in some countries in south-east Asia and Oceania, but it used to have a wider range in the past, including South America, India and Australia (Giacani & Lukehart 2014). Ancient DNA (aDNA) studies have even suggested that its range was extended as far north as Europe during early modern times, further challenging old assumptions made about these diseases (Giffin et al., 2020; Majander et al., 2020).

**Treponemal Disease: Hypotheses and History**

A number of hypotheses have been presented to elucidate the origins and evolutionary history of treponemal diseases. The five most commonly presented are the Unitarian hypothesis, Evolutionary hypothesis, Columbian hypothesis, Pre-Columbian hypothesis, and Modified Columbian hypothesis.

The Unitarian hypothesis suggests all treponematosis, including pinta, is caused by the same organism, *Treponema pallidum*, which is globally distributed and manifests in different ways solely due to sociocultural, climatic, and demographic factors (Hudson, 1946; Hudson, 1965). On the other hand, the Evolutionary

hypothesis states that these diseases are caused by different related species and sub-species that originated from a zoonotic ancestor. Proponents of this hypothesize this ancestor began to infect humans about 20,000 BCE in Africa, Asia, or Europe and eventually diverged into yaws, bejel, pinta, and syphilis (Hackett, 1963).

The Columbian hypothesis asserts sexually-transmitted treponematosis ("syphilis") was inadvertently brought to Europe by Columbus and his crew from South America, where it supposedly originated. This is based on the lack of robust evidence of syphilis infections in Europe before Columbus's voyage and the timing of the large European outbreak (starting in 1495) that is widely regarded as the first recorded epidemic of syphilis in Europe (Baker & Armelagos, 1988). Proponents of this hypothesis argue that this would explain the high death rates recorded during this first European outbreak; Europeans' lack of exposure to syphilis meant the populations would not have had any immunity (Baker & Armelagos, 1988).

The Pre-Columbian hypothesis argues that sexually transmitted and endemic treponematoses originated in Europe, that it was not distinguished in Europe from other diseases like leprosy (Baker et al., 2020), and that the European outbreak starting in 1495 was due to economic and sociocultural factors as well as novel mutations impacting virulence (Baker & Armelagos, 1988; Cockburn, 1961). This hypothesis ignores the Americas, providing a myopic narrative.

The Modified Columbian hypothesis contains aspects of both the Columbian and Pre-Columbian hypotheses: it states non-venereal treponemal disease was brought to Europe from the Americas by Columbus's crew, and the differing social

and environmental factors in late 1400s Europe acted as evolutionary pressure for the disease to adapt to sexual transmission; this became the progenitor of the syphilis we know today (Harper et al., 2008).

Archaeological and historic records do offer some evidence of the treponematoses circulating in populations of the European, Asian, and American continents before Columbus's voyage (Baker et al., 2020). Scholars who believe treponemal diseases existed in Asia and Europe prior to the Columbian Exchange call on historical records from these regions describing symptoms consistent with treponematosis (Hackett, 1963; Holcomb, 1937). There is some paleopathological evidence to support these claims, although they are not strongly supported (Baker & Armelagos, 1988; Baker et al., 2020). From a historical perspective, reports of "venereal leprosy" in Europe before 1492 mention skeletal involvement, sexual transmission, and congenital transmission as characteristics (Baker & Armelagos, 1988; Holcomb, 1941; Hudson, 1972). Hansen's disease (formerly known as leprosy) ironically does not appear to share many similarities with this historic disease in the modern day (Baker & Armelagos, 1988; Holcomb, 1941; Hudson, 1972; Giraldo Herrera, 2018). Some have suggested those descriptions may have instead been cases of treponematosis (Hackett, 1963; Holcomb, 1941; Hudson, 1972).

In contrast to Europe, skeletal evidence of treponemal disease in the Americas is robust, dating back thousands of years, with what are considered diagnoses of higher confidence (Armelagos et al., 2012; Baker & Armelagos, 1988; Harper et al., 2011; Powell & Cook, 2005). Interestingly, the manifestation of treponematosis in the

pre-columbian Americas does not appear to align with modern syphilis *or* the European syphilis outbreak starting in 1495 that is considered by many to be the initial outbreak of syphilis in Europe. Skeletal pathology indicative of treponematosis identified in Indigenous populations from North America from pre-columbian contexts show the skeletal changes were distributed across the skeleton in a unique way, distinct from all of the known treponematoses (Powell, 1988; Reichs, 1989). Demographics of those infected also show a significant proportion of non-congenital childhood infections, indicating the disease was likely not sexually transmitted (Powell & Cook, 2005). This may signal a currently unknown species or subspecies was circulating in the Americas.

By leveraging morphological studies of paleopathology providing evidence of treponemal disease, ancient DNA has attempted to provide resolution to the unknown history of the evolution and migration of these pathogens. Thus far, paleogenomic studies provide evidence that *Treponema* species within the *pallidum* clade existed during the colonial period of the Americas (Barquera et al., 2020; Schuenemann et al., 2018) and in Europe during the late 15th century (Giffin et al., 2020; Majander et al., 2020). While the European individuals studied have the highest probability of dating to the late 15th century, the radiocarbon dates associated with those individuals do span a wide range from both before and after Columbus's voyage (Giffin et al., 2020; Majander et al., 2020), suggesting a diversity of treponemal lineages may have existed in Europe before the 1495 outbreak. While intriguing, these studies did not offer the temporal clarity to identify the geographic source (Europe or America) of

what would become the syphilis pandemic still in existence today. In fact, their archaeological dates of the late 15th century and colonial period, in addition to the identification of infection in Indigenous and African victims of the Trans-Atlantic slave trade, has only increased the intensity of additional investigations.

Much remains unknown about how modern syphilis came to be. What *is* well documented is the sudden 1495 outbreak of aggressive, sexually-transmitted treponematosis. Beginning in Italy among French, Spanish, and Italian soldiers during Charles VIII's attempt to take the Italian peninsula, the disease quickly spread across Europe with the demobilization of the military forces (Quétel, 1990). The disease, reportedly never seen before, was highly transmissible and highly virulent (Tognotti, 2009). The signs and symptoms of the disease were different from those seen in syphilis today: for example, while rare today, alopecia and extremely smelly pus exuding from boils were hallmarks of syphilis in these early years (Tognotti, 2009; Frith, 2012). Furthermore, there was a reported change in disease progression, with virulence declining quickly within the first 10-15 years after the beginning of the European pandemic (Tognotti, 2009).

Regardless of where the disease originated and how it spread, the notion that treponematoses have changed significantly in the past few centuries is supported by recent genomic studies directed at understanding the emergence, evolution, and host specialization of the different *Treponema*. Analysis of modern data suggests that much of the diversity within *T. pallidum subsp. pallidum* strains came into being within the last century, with the deepest split within this subspecies (between the

Nichols and SS14 subclades) likely occurring sometime in the 18th century (Arora et al., 2016). However, Bayesian molecular dating of historic genomes has pushed back an estimated date of divergence for *T. pallidum subsp. pallidum* to approximately 1250 CE (Majander et al., 2020). With additional analysis of modern and new ancient genomes, we can gain a more confident estimation of when and how these clinically concerning *Treponema* emerged to be human pathogens.

**The Wild Warts: Social Implications and Treatments**

Discourse surrounding infectious disease often devolves into problematic narratives, particularly when discussing historically stigmatizing, disfiguring or highly deadly diseases. Syphilis has been especially susceptible to this. One example of this is the French colonization of Arab lands: fabricated and cherry-picked statistics about "exotic syphilis" or "Arab syphilis" prevalence, bolstered by racist and Islamophobic tropes, provided the French with an opportunity to reframe their conquest as a benevolent "civilization" mission until well into the 20th century (Amster, 2016; Amster, 2022). In addition to being used to justify atrocities, syphilis has frequently been used to disparage neighboring societies or further marginalize "othered" groups within societies (Savinetskaya, 2017; Tampa et al, 2014). One of the most prominent ways syphilis has been used for harm in this way is the naming of the disease. While some names, such as "the wild warts" (Moore & Solomon, 1935), were more innocuous, many of the names used to refer to syphilis were explicit accusations blaming specific groups. Beginning with the outbreak in 1495, the

disease was blamed on other nations through naming it– “the French evil”, “the Spanish disease”, “the Chinese pox”, and “the Persian fire” are just a few of many such names (Frith, 2012).

As the horrific disease spread like wildfire through Eurasia, patients desperately sought an effective treatment. At this time in Europe, much of the understanding of disease was based in the miasma and humoral theories of medicine (Giraldo Herrera, 2018; Hankinson, 2017; Plagens-Rotman et al., 2021); the limitations of this school of thought combined with the stigma associated with the disease (many declared syphilis to be punishment for the sin of fornication and were opposed to patients receiving treatment; see Plagens-Rotman et al., 2021) led to some treatments that would be viewed today in most circles as outrageous. A patient could receive treatments such as bloodletting and sweat baths (Frith, 2012; Plagens-Rotman et al., 2021) but some treatments were more extreme. For example, in some regions, patients were stripped naked and buried up to their necks in a pile of dung (Plagens-Rotman et al., 2021). While many of these treatments may appear to demonstrate a complete lack of understanding of the disease, other recommendations suggest otherwise. For example, preventative measures taken such as abstinence, avoiding sexual intercourse with infected persons, and post-intercourse washing of the genitals with water or wine (Plagens-Rotman et al., 2021) indicate there was at least a surface-level understanding of transmission dynamics.

With the belief that the disease came from the Americas, many also turned to treatments reportedly used by Indigenous peoples of the Americas. While some of

these reported treatments (e.g. eating hummingbirds) may not have had a significant healing impact on the infection, use of the guaiacum tree was highly effective (Giraldo Herrera, 2018). Extracts from this tree were a popular treatment, but after a while it appears to have lost efficacy. Some proponents claimed this loss of efficacy was due to the extracts not being fresh, but others wrote off guaiacum as an old wives' tale (Giraldo Herrera, 2018). However, guaiacum extracts have been shown in recent years to have antibacterial properties against other gram-negative bacteria (Castillo-Juárez et al., 2009); this suggests this treatment may truly have been effective initially, but the bacteria developed resistance to the compounds over time (Giraldo Herrera, 2018).

By far the most popular and longest-persisting pre-penicillin treatment (especially after the loss of effectiveness of guaiacum) was treatment with mercury (O'Shea, 1990). Notably, "venereal leprosy" was reportedly also treated effectively with mercury (Hudson, 1972); this could further support the claim that "venereal leprosy" was actually syphilis, but it is also possible that mercury treatment was simply effective in treating a range of diseases. While somewhat effective against syphilis, these treatments were brutal (Frith, 2012; O'Shea, 1990; Plagens-Rotman et al., 2021). With their rise in notoriety, "A night with Venus, a lifetime with Mercury" became a popular warning (Asfiya et al., 2018; Frith, 2012; Tampa et al., 2014), referencing both the sexually transmitted nature of the disease and the long-term nature of mercury treatments. Mercury treatments came in multiple forms; it could be mixed with other compounds or herbs and taken orally, injected, inhaled, applied

topically, and more (Frith, 2012; Tampa et al., 2014; Plagens-Rotman et al., 2021) Some patients had ointment applied to their skin and then had to sit in a steam bath for 20-30 days; this treatment only resulted in a benefit for 1% of patients and was so agonizing that some preferred to die (Plagens-Rotman et al., 2021). Others were shut in fumigation stoves where the mercury was vaporized (Hollingsworth, 1928; Tampa et al., 2014). The side effects of these treatments (including mercury poisoning) were painful, and many patients had to continue treatments for the rest of their lives (Asfiya et al., 2018; Frith, 2012; O'Shea, 1990; Plagens-Rotman, 2021).

**The Broader Picture: Evolutionary Perspectives**

While the context discussed in this chapter so far has been focused on the human experience of treponematosis, an examination of the roles *Treponema* species play in broader ecosystemic contexts is necessary to develop a more holistic understanding of the pathogen. Expanding the focus to examine other host species and more distantly related *Treponema* species can help frame the bigger picture.

The distantly related *Treponema* species *T. pedis, T. medium, and T. phagedenis* are all known to infect pigs and cattle to cause digital dermatitis, a skin infection that can produce large abscesses and leads to lameness (Arrazuria et al., 2020). These species have a broad host range and their genomes are much larger than those of the *T. pallidum* clade.

Another pathogen of interest is *Treponema paraluisleporidarum*. While some *Treponema* species can infect a diverse range of hosts, *T. paraluisleporidarum* is

host-specific to lagomorphs (Jacobsthal, 1920; Smith & Pesetsky, 1967; Strouhal et al., 2007). There are two ecovars, one of which (ecovar lepus) causes disease in both rabbits and hares, while the other (ecovar cuniculi) has even stricter host specificity, only causing disease in rabbits (Lumeij et al., 2013). A lack of human infectivity in this species has been linked to genome decay (Graves et al., 1981; Smajs et al., 2011). Many of the genes affected in this decay are of known function in *T. pallidum subsp. pallidum* and play an active role in the pathogenesis of syphilis in humans, giving rise to the hypothesis that both organisms evolved from a common *pallidum*-like ancestor that was infectious to humans and nonhuman animals (Smajs et al., 2011).

Modern-day *T. pallidum* subspecies also impact animals other than humans. Reports of infections of the *Treponema pallidum* subspecies in wild and captive animals have signaled the need for non-human animal screening and a greater understanding of *Treponema* evolution and host species transmission dynamics (Gogarten et al., 2019). In fact, there are numerous examples of cross-species infections of pathogenic *Treponema* species, with reports of *T. pallidum* infections in non-human primates, rabbits and guinea pigs (Chuma et al., 2018; Wicher et al., 1999). *T. pallidum* subspecies have been shown to be carried (and potentially transmitted) by flies that land on infectious skin lesions (Jahan et al., 2022; Knauf et al., 2016; Stamm, 2016), which could explain disease spread across species barriers. However, these diseases do appear to behave and present differently in non-human animals. In the wild, *T. pallidum* subsp. *pertenue*, most commonly associated with yaws in humans, commonly affects the anogenital regions of non-human primates

(Chuma et al., 2018), and when passaged through rabbits it also eventually changes to present more like syphilis (Wicher et al., 2000). This highlights the influence of selective pressure introduced by hosts on pathogens, particularly for *Treponema pallidum* and related species.

**Conclusion and Relation to My Work**

*T. pallidum* subspecies remain enigmatic due to challenges with research. Aside from causing serious illness and prompting agonizing treatments for a large portion of history, the diseases they cause in humans are highly stigmatized and have prompted intense debates about who is to blame for their existence and distribution. A more inclusive vision of *Treponema* infections in other species and in other time periods may provide more clarity.

For diseases such as syphilis that persist as a global burden of health, understanding when and how they evolved will offer a better understanding of how pathogens develop and rise to prominence. When paired with traditional knowledge, archaeological data and ecological context, paleogenomics is a useful tool in the pursuit of understanding these evolutionary processes. An interdisciplinary approach incorporating those fields can provide key context to reveal how infectious diseases affected ecosystems of people, animals, and the microbes themselves. To better understand *Treponema* presence and evolution in the past, additional ancient DNA data is necessary.

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## Chapter 5: Ancient Pathogen, Novel Genome
## Analysis of a 5,500-year-old *Treponema pallidum*-like genome from Sabana de Bogotá, Colombia

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### Abstract

*Treponema pallidum* subspecies, the pathogenic bacteria responsible for treponematoses, have affected humans worldwide for centuries. The origin, evolution and spread of treponemal diseases are subjects of ongoing debate in infectious disease history. Despite abundant paleopathological evidence and the growing collection of ancient genomic data, the evolutionary history of these pathogens remains ambiguous. This is in part due to the lack of data from a common ancestor or genomes that extend beyond the current documented diversity of *T. pallidum*. Here we present the oldest genomic evidence to date of treponematosis, dating back approximately 5,500 years and recovered from the skeleton of a hunter-gatherer from the Tequendama I archaeological site in Colombia. Phylogenetic analyses show this pathogen to be basal to all known *Treponema pallidum* subspecies. Its unique phylogenetic position indicates this genome does not belong to any of the currently genetically characterized subspecies, providing a novel perspective on the evolutionary history of treponematosis and revealing previously unrecognized diversity among ancient treponemal species. By examining this finding within the archaeological context of hunter-gatherer subsistence, we discuss the long history of

treponematosis in South America and potential role of human-animal interactions in their spread.

**Main**

The origin and evolution of treponemal diseases have been the subject of intense scholarly debates across centuries, involving fields ranging from medical history to microbiology. Historically speaking, this debate has focused on the geographic origin of treponemal pathogens and the spread of treponemal infection to different continents and regions during major historical events such as the apparent epidemics of venereal syphilis across Europe at the turn of the 16$^{th}$ century (Baker, 2022; Cook & Powell, 2012). However, the non-specificity of skeletal changes caused by these diseases and historical reports across the European and American continents have led to several competing hypothetical scenarios focused on explaining the origin and spread of treponemal disease. Unfortunately, much of the research has been constrained by a hyperfocus on the origin of syphilis, which has obscured a deeper understanding of *Treponema* evolution. This narrow focus has often overlooked the broader evolutionary history of *Treponema* species, limiting our comprehension of their full diversity and adaptation. To gain a clearer picture of *Treponema* evolution and the broader historical context of these pathogens, it is essential to expand research efforts beyond the syphilis-centric paradigm and explore deeper evolutionary histories and patterns of treponemal diseases.

Four *Treponema pallidum* subspecies are known to cause four clinically delineated treponemal diseases known as bejel (associated with *Treponema pallidum*

subsp. *endemicum*), yaws (associated with *Treponema pallidum* subsp. *pertenue*), pinta (associated with *Treponema carateum*), and syphilis (associated with *Treponema pallidum* subsp. *pallidum*). Generally, these diseases are transmitted by different forms of direct contact between infected and uninfected persons. While *Treponema pallidum* subsp. *endemicum* (TEN), *Treponema pallidum* subsp. *pertenue* (TPE), and *T. pallidum* subsp. *pallidum* (TPA) have been genetically characterized, no genetic data is presently available for the proposed etiological agent of pinta. As such, the relationship of *Treponema carateum* with the described *T. pallidum* subspecies remains unresolved. The morphological, antigenic, and genetic similarity (Giacani & Lukehart, 2014) of *Treponema pallidum* subspecies often challenged clinical distinction between the different pathogens. Despite these similarities, today *Treponema pallidum* subspecies differ in their ecological/geographic distribution and pathological invasiveness with TPA being the most invasive, causing severe pathological lesions in several tissues of the body, whereas TEN and TPE demonstrate a less invasive pathological response (Šmajs & Strouhal, 2015; Šmajs et al., 2012). Although genomic-level data has provided greater resolution to the behavior and evolutionary relationships of these bacteria, this has historically been hindered by their uncultivable nature, making genomic data challenging to acquire until recent advances in such methods (Edmonson et al., 2018). Application of these advances to archaeological contexts has demonstrated the value and potential of ancient genomes of treponemal pathogens to help untangle unresolved questions about origins, evolution, and the relationships between closely related pathogenic

*Treponema* species and subspecies that remain enigmatic (Barquera et al., 2020; Giffin et al., 2020; Majander et al, 2020; Majander et al., 2024; Schuenemann et al., 2018).

Ancient DNA (aDNA) studies have reported ancient genomes belonging to all three modern *T. pallidum* subspecies across multiple continents (Barquera et al., 2020; Giffin et al., 2020; Majander et al, 2020; Majander et al., 2024; Schuenemann et al., 2018). These findings have enriched the historical narrative of treponematosis and disproved previous assumptions on the ecology of *Treponema pallidum* subsp. in the past. For example, while *T. pallidum* subsp. *pertenue* now seems to be restricted to tropical regions, two aDNA studies detected human infection of this pathogen at northern latitudes in early modern Europe (15th -17th ce.) (Majander et al., 2020; Giffin et al., 2020). Ancient DNA has also elucidated on the historical intercontinental dissemination of TPE through the detection of a West African strain of this pathogen in an individual from 16th-century Colonial Mexico (Barquera et al., 2020). Furthermore, one of the most remarkable findings is the recent detection of *T. pallidum* subsp. *endemicum*, believed to be primarily found in dry and arid climates, in 2000-year-old individuals from archaeological contexts of Brazil (Majander et al., 2024), where it has not been detected in modern populations. These studies may indicate that the geographic distribution of the *T. pallidum* subspecies in the past differed from what we observe today. Alternatively, these findings may point to what is currently an incorrect understanding on the phylogeographic distribution of the *T. pallidum* subspecies– when combined with recent clinical reports, these findings

suggest persistence of TEN in the western hemisphere (Vrbová et al., 2022). Ancient DNA research investigating the treponematoses in the past offers contextualized genomic evidence towards a better understanding of the evolutionary history, geographic origin and persistence of *T. pallidum* subspecies.

Paleopathological assessments have revealed an abundance of human cases of treponematosis circulating in past populations of the European, Asian, and American continents (Baker & Armelagos, 1988; Cook and Powell, 2012). Skeletal evidence of severe infectious disease throughout the pre-Columbian Americas led medical doctors and paleopathologists to identify treponemal disease across broad temporal and regional contexts (Castro et al., 2020; Hutchinson et al., 2006; Jones, 1876; Kaye, 2008; Mansilla et al., 2000). Those ancient cases of treponematosis from pre-European contexts of the Americas have been used as support for the Columbian hypothesis, which posits that Columbus and his crew brought syphilis to Europe in their return from the Americas. This hypothesis possesses greater support from both historical and paleopathological evidence (Baker & Armelagos, 1988; Tampa et al., 2014) and is thereby more popular than the countering pre-Columbian hypothesis which states treponemal disease, including syphilis, already existed throughout Europe, Africa, Asia, and the Americas (Cook and Powell, 2012; Hackett, 1963; Holcomb, 1937). Skeletal evidence of treponemal infection from the Americas appears to be more numerous, dating back thousands of years, and are generally considered to be of higher confidence than those pre-Columbian cases of Europe and Asia (Armelagos et al., 2012; Baker & Armelagos, 1988; Harper et al., 2011).

However, the demographic patterns of treponemal disease in the pre-Columbian era of the Americas demonstrate a young age of treponemal infections with the majority of treponematosis predominantly reported in younger individuals suggesting a non-venereal variant (Armelagos et al., 2012). Paleopathological evidence with the recent contributions by ancient DNA research have shed light on the global presence and spread of treponemal pathogens in the past. However, the majority of paleogenomic data come from contexts dating to and around the colonial era and more recent history. The fixation on the competing hypotheses in both these fields has obscured deeper discussions on the evolution of systemic treponemal pathogens (Baker et al., 2020). As a result, the complex evolutionary and ecological history of treponemal pathogens in deep time remains underexplored.

The opportunity to address the gap in current knowledge on the history of evolution and diversity of treponematosis-causing pathogens was offered in the pathogen screening of ancient metagenomic data from individuals from the archaeological site of Tequendama in what is today Colombia, South America. As the individual was part of a study originally examining human population histories, the identification of *Treponema* DNA was an unexpected find but permitted the genomic reconstruction of a previously unknown 5,500-year-old *Treponema pallidum*-like pathogen discovered in the Sabana de Bogotá, Colombia. This *Treponema* genome predates the existing genomic evidence by 3,500 years and is positioned basal to all known *Treponema pallidum* genomes.

**Bioarchaeological Context**

Situated close to the Bogotá River within the Sabana de Bogotá, the Colombian site of Tequendama (Figure 5.1) was occupied by humans starting in the late Pleistocene through the late Holocene (radiocarbon dates spanning 13,263 to 2,063 cal yBP). The site consists of four caves and rock shelters (named Tequendama I to IV) which were used as semipermanent and permanent habitation throughout this time. The individual discussed in this study in whom we identified the presence of treponematosis is an individual from Tequendama I.

Excavations of Tequendama I recovered burials spanning the period of occupation ca. 10,000 - 2,300 cal yr BP (Correal Urrego & van der Hammen, 1977). In addition to material culture (e.g. lithic tools) associated with the inhabitants of the site and other indicators of cave use (e.g. rock art), archaeologists also recovered a diverse array of faunal remains, most likely representing species hunted for consumption (Correal and van der Hammen, 1977). Besides larger mammals like deer, which seem to date mostly to the early occupation phase, the range of animals in the occupants' diets seems to have consisted mainly of small animals, including armadillos, rodents (e.g. guinea pigs) and lagomorphs (e.g. cottontail rabbits).

Osteological analysis of age-at-death and estimations of biological sex reports the infected individual is an adult male. Genetic analysis supported identification of the individual as biologically male (Delgado et al, 2021). Radiocarbon dates obtained directly from the individual for this study reveal they are associated with the Middle Holocene occupation period (4675 ± 20 14C yr BP [5464 – 5309 cal yr BP 2σ])

(Figure 5.S1, Table 5.S1).

While treponematosis has been identified in other ancient individuals in the region (Correal Urrego, 2012), the surviving skeletal elements of this individual do not appear to present the pathological changes associated with the later stages of a treponemal infection.

**Detection of Ancient Treponemal DNA**

Ancient DNA was recovered from a fragment of the individual's tibia, and human mapping reads were analyzed to determine the genetic ancestry of the individual (Delgado et al., 2024). Full coverage of the mitochondrial genome was obtained revealing that the individual belonged to the Native American haplogroup B2 (Delgado et al., 2021). In parallel with human genomic analyses, all non-human sequencing reads recovered from the individual were screened for the presence of pathogens using the HOPS pipeline with a custom database (Hübler et al., 2019). This permitted the detection of *Treponema* DNA with 4,099 reads assigned to the *T. pallidum* subsp. *pallidum* node. Additionally, 9,948 reads were assigned to *Treponema*, with *Treponema paraluisleporidarum* cuniculi A, a pathogen that is host-specific to lagomorphs, listed as the closest matching reference from our database.

**Identifying a Distinct *Treponema* Pathogen**

To investigate the taxonomic classification of sequencing reads attributed to *Treponema pallidum* and *T. paraluisleporidarum* by HOPS, we performed

competitive mapping using three reference genomes: *T. pallidum subsp. pallidum* (NC_021490.2), *T. paraluisleporidarum* (NC_015714.1) and *T. phagedenis* (NZ_CP042818.1) (Table 5.S2). This analysis demonstrated that the majority of reads aligned with the T. pallidum subsp. pallidum reference, yielding 2,318 unique reads, compared to 344 and 400 reads mapping to *T. paraluisleporidarum* and *T. phagedenis*, respectively.

This was further evaluated through additional stringent mapping to four reference genomes, representing each of the genomically characterized *T. pallidum* subspecies and *T. paraluisleporidarum*. This approach resulted in reconstructed genomes with an average coverage of 1.65 to 1.70-fold, covering 78.5% to 78.7% of each *T. pallidum* subspecies reference (Table 5.1, Figure 5.2, Figure 5.S2). In contrast, mapping to the *T. paraluisleporidarum* reference (NC_015714.1) resulted in slightly lower coverage, averaging 1.65-fold with 77.0% of the genome covered. While the results of mapping to the different *T. pallidum* subspecies references were generally consistent, we observed marginally higher genomic coverage (1x to 3x) when mapping against the *T. pallidum* subsp. *endemicum* reference genome (NZ_CP007548.1) (Table 5.1). Additionally, each reconstructed genome exhibited variations in the number of single nucleotide polymorphisms (SNPs) identified across the three *T. pallidum* subspecies. Specifically, 535 SNPs were identified when aligned to *T. pallidum* subsp. *pertenue* (NC_016842.1), with slightly higher numbers observed in *T. pallidum* subsp. *pallidum* (546 SNPs) and *T. pallidum* subsp.

*endemicum* (542 SNPs). A comprehensive list of positions where TE13 differs from each reference is provided in the Supplementary Materials (Table 5.S3).

Visual inspection of read distribution across these genomes indicated similarity in the regions covered and in the stacking of reads. It is likely the observed minor differences in mapping metrics are artifacts arising from variations in base pair counts among the reference genomes. For instance, the *T. pallidum* subsp. *endemicum* genome appears to have higher coverage due to its smaller size compared to other reference genomes. Specifically, the *T. pallidum* subsp. *pallidum* reference genome contains approximately 2,000 bp more than the *T. pallidum* subsp. *endemicum* assembly and 300 bp more than the *T. pallidum* subsp. *pertenue* genome.

Considering the comparable coverage metrics, the slight differences in variant counts across the three subspecies, and the discrepancies in reference genome sizes, we selected the genomic reconstruction based on the *T. pallidum* subsp. *pallidum* reference (NC_021490.2) for all subsequent analyses. The finalized genome, designated as TE13, encompasses 78.5% of the *T. pallidum* subsp. *pallidum* reference with a mean coverage of 1.7-fold. Furthermore, TE13 exhibits 3% and 10% damage on the 5' and 3' terminal bases, respectively (Figure 5.S3), with an average fragment length of 66 base pairs (Figure 5.S4), consistent with ancient DNA from partially UDG-treated, single-stranded DNA libraries.

**The Genome is Basal to All Modern Clades**

The phylogenetic position of TE13 was investigated using a curated dataset

composed of 100 additional genomes, including 89 modern genomes, 9 previously published ancient *Treponema pallidum* subsp. genomes and 2 *T. paraluisleporidarum* genomes (Table 5.S4, Table 5.S5). Phylogenetic analyses were constructed using variant positions (10,499 positions) called at a minimum threshold of 3x coverage. Recombinant, repetitive, and tRNA regions were removed from the SNP alignment (see Methods, Table 5.S6) for the final phylogeny, resulting in 8,583 positions being included (Figure 5.3). The maximum-likelihood phylogenies (Figure 5.3, Figure 5.S5) positioned TE13 as basal to all ancient and modern published *T. pallidum* subspecies. Maximum parsimony trees built using the same alignment (Figure 5.S6) support the topology.

The variant positions responsible for the topology of the TE13 branch were identified using the MultiVCF Analyzer output (see Methods) which revealed TE13's branch is constituted by 231 unique variants against the Nichols reference (NC_021490.2). Furthermore, the shared branch of TE13 and *T. paraluisleporidarum* is made up of 216 variant sites.

The confidence of the phylogenetic position of TE13 and the overall topology of the tree were tested by constructing multiple phylogenies against the four TPA (NC_021490.2), TPE (NC_016842.1), TEN (NZ_CP007548.1), and *T. paraluisleporidarum* (NC_015714.1) references. This ruled out the potential influence of reference bias and confirmed the basal position of TE13 across the multiple phylogenetic reconstructions (Figure 5.S7). A maximum likelihood

phylogeny constructed with assemblies and a whole-genome multiple sequence alignment (Figure 5.S8) is highly consistent with the phylogeny built with the mapping-based approach, indicating the reference-mapping itself was not impacting the topology. Furthermore, the positioning remained robust despite the inclusion or exclusion of recombinant, repetitive, and tRNA regions (Table 5.S6, Figure 5.S9). As shown, TE13 is basal to the *T. pallidum* subsp. clades, constituting a novel phylogenetic branch.

A subsampling experiment, where other modern and ancient high coverage genomes were sub-sampled to a similar coverage as TE13 demonstrate that none of the subsampled genomes is wrongly classified to a different subspecies or is placed in a basal position similar to TE13 (Figure 5.S10). Interestingly, reducing the coverage does not affect the phylogenetic placement of the ancient and modern genomes. However, some of them, especially ZH1540, display an increased branch length as observed for other low coverage ancient genomes. This provides further support for the phylogenetic placement of TE13, indicating its basal positioning is not due to its low coverage.

*T. paraluisleporidarum,* a pathogen closely related to *T. pallidum* that is host-specific to lagomorphs, was identified as the outgroup through an orthology-based analysis that took advantage of orthologous sites in *Treponema phagedenis* to resolve the evolutionary relationship of *T. pallidum*, *T. paraluisleporidarum* and the ancient genome TE13 (Figure 5.S11). This analysis

identified 316 and 314 conserved single copy Orthogroups (OGs) when TE13 was mapped to Nichols and Cuniculi A references respectively. These genes were aligned, concatenated and used to infer maximum likelihood (ML) phylogenetic trees (see Methods). The resolved tree shows *T. paraluisleporidarum* diverging from the modern and previously published ancient *T. pallidum* genomes before TE13, positioning TE13 as a basal sister branch of the human-associated clade. This placement was observed even when reads were mapped to the *T. paraluisleporidarum* reference (Figure 5.S11b), indicating that the reference-attraction bias is not playing a major role in this analysis. The inclusion of more outgroups in the analysis did not change this observation, leading to similar results (Figure 5.S11 c and d). In these cases, Orthofinder identified 291 conserved single copy OGs. The bootstrap support for the node of interest and for the other nodes defining the sub-species clades is 100 in all the inferred phylogenies.

To ensure this finding of a putative pathogen with a long branch length was not due to the inclusion of environmental reads, we performed a BLAST analysis on the mapped reads (Figure 5.S12). This confirmed our stringent mapping parameters were sufficient to remove contaminating reads from the genome that would impact branch length.

**Virulence and Functional Variants**

To assess the virulence of this ancient *Treponema* genome in comparison to contemporary strains and previously published ancient genomes, we investigated the

presence of genes associated with pathogen virulence. We compiled a comprehensive list of 59 virulence genes specific to *T. pallidum*, based on the works of Šmajs et al. (2012), Radolf et al. (2016), and Majander et al. (2020) (Table 5.S7). The proportion of covered bases for each of these genes (breath of coverage) was determined using both the stringent mapping parameters used for the phylogenetic analysis (Figure 5.4a) and while deactivating the mapping-quality filtering, to allow reads to map to multiple locations in the genome (Figure 5.4b) (see Methods). The latter approach allows reads to map to paralogous genes that would otherwise compete for the same reads yielding an apparent lower coverage. Despite the low genomic coverage, this analysis demonstrates TE13 likely possessed all known virulence factors associated with modern *T. pallidum* subspecies. The *tpr* paralogous genes yield low coverage across all genomes when stringent mapping parameters are applied (i.e. when reads mapping equally well to more than one location are discarded), but if reads are allowed to map to multiple locations, all the paralogous genes are detected (Figure 5.4b). Since this change in coverage is systematic and affects both modern and ancient genomes we believe it represents a mapping artifact, suggesting that all the *tpr* paralogous genes were already present in TE13. For the remaining genes, the change in parameter has minimal effect. When mapping without quality filtering, the minimum breath of coverage for the considered virulence genes in TE13 is 48% (Figure 5.4b). While this can only be confirmed with functional tests, the genome of TE13 likely represents a virulent and infectious pathogen, similar to what is observed with modern *Treponema pallidum* subspecies.

Virulence analysis was also performed at the SNP level using snpEff v3.1i (build 2012-12-12) (Cingolani et al., 2012) to investigate functional changes within the phylogenetic dataset against the *T. pallidum* subsp. *pallidum* Nichols reference (NC_021490.2). To increase the confidence of variant positions called in this dataset, a minimum mean coverage of 3-fold was applied. TE13 specifically has 208 variants that are expected to result in a protein different from that in the reference; 83 of these are only seen in TE13. One of these falls into the "high impact" category–the loss of a stop in the gene mazG, a putative nucleoside-triphosphate diphosphatase (Table 5.S8). Additionally, 16 of the variants seen in TE13 are located in genetic regions associated with virulence, spread across 11 different virulence genes. While these results suggest TE13 may possess a different protein profile in comparison to *T. pallidum* subsp. *pallidum*, caution should be taken when evaluating these results due to the low coverage at these informative sites.

**Discussion**

An abundance of paleopathological evidence of treponematosis has been identified in pre-European contexts of the Americas. Some of these cases have yielded genomic evidence of historic treponemal disease (Barquera et al., 2020; Giffin et al., 2020; Majander et al., 2020; Schuenemann et al., 2018). However, the genome presented here, TE13, predates all others by at least 3,500 years revealing a long history of treponematosis as a human disease in the Americas. This finding was made possible through the exerted effort and exhaustive sequencing of paleogenomic

data to be included in both ancient human and ancient pathogen research. Despite the abundance of skeletal evidence for treponematosis in the archaeological record, the variability in bacterial load across different stages of infection challenges the recovery of *Treponema* DNA from disease-altered bone. This is due to the developmental timing of skeletal pathology from treponemal infection during an infection phase with low pathogen load in the body. As such, identifying cases of treponematosis for ancient DNA research is highly challenging  and causes genomic data from ancient *Treponema* to be particularly difficult to obtain.

Most investigations on the history of treponematosis focus on syphilis and its origin and spread in relation to a devastating syphilis epidemic that occurred in Europe soon after Columbus's 1492 expedition. However, our results call attention to a complex history of pathogen evolution that goes beyond 15th-century syphilis epidemics. Genomic data provides a wealth of information, but there are limitations to what it can tell us. In modern treponematoses, there is evidence of overlap between the subspecies in symptoms, skeletal signs, severity, geographic range and transmission dynamics (Giacani & Lukehart, 2014; Shinohara et al., 2022; Vrbová et al., 2022). This suggests inference of disease phenotype from genomic data alone may be over-simplistic; host factors and other environmental conditions likely also influence these aspects of disease manifestation (Giacani & Lukehart, 2014). This is further complicated by the limited understanding of the genetic basis of *T. pallidum* pathogenicity. Further research into these topics is necessary, but there are other sources of information that can inform our understanding of our findings in the

meantime. The inclusion of paleopathological data, archaeological context, and a broad understanding of the ecological dynamics of these diseases enrich our interpretation.

**One Health Approaches**

With TE13, the genomic identification of treponematosis shifts our understanding of the ecology of treponematosis as it was identified in what can be considered an atypical context of *Treponema pallidum* subsp. epidemics today, i.e., hunter-gatherer, small community, and ancient context. While modern epidemics of *Treponema pallidum* subspecies are driven by different social and economic factors, particularly with differences between venereal and nonvenereal types, the identification of treponematosis here beckons One Health approaches to perform a deeper examination of the ecology and evolution of treponemal pathogens. The circulation of treponematosis-causing pathogens at this time and context suggests the influence of environment and human-animal interaction on infectious disease histories. “One Health” is the concept that the health of humans, wildlife, and the environment are interconnected and directly influencing the overall health of one another (Zinsstag et al., 2005). Reports of non-human infection of the *Treponema pallidum* subspecies in wild animals have signaled the need for animal screening and a greater understanding of *Treponema* evolution and host species transmission dynamics (Gogarten et al., 2019). In fact, there are numerous examples of cross-species infection in *Treponema*, with reports of *T. pallidum* infections in wild non-human primates, and laboratory-induced infections in rabbits, hamsters, and

guinea pigs (Chuma et al., 2018; Wicher et al., 1999). While these *Treponema* species are considered risks for wild and domesticated animal health, the true diversity of possible hosts of these pathogens in the past is not known. Skeletal evidence of treponematosis has been documented in contemporaneous and nearby contexts which supports the possibility of human-to-human transmission. However, given the small population size of Tequendama and surrounding sites, animal to human transmission through activities like hunting cannot be discounted. As *Treponema pallidum* subspecies demonstrate a broader host tropism relative to the closely related *Treponema paraluisleporidarum,* known to be species specific to rabbits and hares (Šmajs et al., 2012), observations of the genetic composition of TE13 suggest this pathogen may possibly be spread between animals and humans.

While genetic bases of host tropism and transmission dynamics of *Treponema pallidum* have not yet been determined and therefore are impossible to infer from our data, recent evidence of continued genetic accumulation (a feature associated with microbial adaptability to multiple environments) of the *T. pallidum* subspecies (Jaiswal et al. 2020) and the presence of *T. pallidum* infections in non-human hosts today support the plausibility of a broad host range for ancient forms of *T. pallidum*. Further support can be given with the observation of pathogen adaptation to a specific host or environment being accompanied by genome size reduction and reduced horizontal gene transfer (Šmajs et al., 2012). Like modern *Treponema pallidum* subspecies, it may be that TE13 represents a pathogen that could have been transmitted person-to-person, and/or between species by the casual skin contact that

occurs while hunting, eating, or handling infected animals. Notably, the categories of small animals frequently consumed at Tequendama I at this time period are all known vectors of infectious diseases impacting humans. Additionally, the Middle Holocene heralded warming temperatures (and the environmental changes associated with that), along with the arrival of new groups of people to the area (Delgado, 2012; Delado 2017). Regardless of the route of transmission, when considering the small human population sizes and interactions of humans and animals in this cultural and ecological context, future pathogen screening of zooarchaeological remains is prudent to investigate potential ancient animal reservoirs.

**High Value of Low Coverage aDNA Data**

Despite the low coverage of this genome (1.7 mean fold coverage), TE13 offers the oldest genomic evidence of a *Treponema* infection without introducing the bias of targeted hybridization. This provides a genomic baseline for evolutionary studies of treponemal disease in human populations, complementing the large body of archaeological and paleopathological evidence for treponematosis in the Americas prior to European arrival. Its divergence predates the MRCA of all the genomically described subspecies circulating today. These results are consistent with the radiocarbon dates of the infected individual, dated at ~5,500 years BP. Phylogenetic analysis identifies the ancient genome as a pathogen possessing a unique evolutionary history with no known representatives today, adding to the known diversity of *Treponema pallidum*-like pathogens. While the identification and characterization of TE13 confirms that during the mid-Holocene, in northern South America, an ancient

treponemal pathogen was infecting human individuals, higher coverage data of the pathogen will allow for analyses to produce molecular dating estimates of the divergence of this pathogen and increase the resolution and confidence of genome characterization. Despite limitations in coverage, the rigorous testing of phylogenetic models and downsampling experiments demonstrate a confident phylogenetic position of TE13 as closely related yet basal to all known *Treponema pallidum* genomes.

The interrogation of the sequencing data leading to the genomic reconstruction and phylogenetic analysis presented in this work demonstrates the benefits of pursuing methods of validation in low coverage data in conjunction with contextual background before investing in capture enrichment. The preparation of DNA libraries and process of targeted enrichment of *Treponema spp.* DNA inherently and inevitably biases the DNA recovery and genomic reconstruction (Avila-Arcos et al., 2011; Hayden et al., 2022). This measure is often unavoidable in ancient DNA and has been used in every publication examining the evolution of *Treponema pallidum* evolutionary histories through ancient DNA to date (Barquera et al., 2020; Giffin et al., 2020; Majander et al., 2020; Majander et al., 2024; Schuenemann et al., 2018). Likewise, targeted capture of *Treponema pallidum* DNA is frequently used in modern studies and the level of bias this method introduces has not yet been adequately quantified (Beale et al., 2022). However, with this approach, we were able to confirm the ancient pathogen we report is *Treponema pallidum*-like being basal yet closely related to *Treponema pallidum* and rigorously examine the reconstructed

genome prior to treatment with targeted capture. This property is essential for the examination of ancient pathogens for which a representative genome may not currently exist or for which the pathogen may now be extinct. The age of TE13 as approximately 5,500 years old suggests this pathogen is unlikely to be identical to those references currently in use for genomic research of *Treponema* species. A deep examination of the data as demonstrated here provides valuable information that is typically lost in ancient DNA research. In such studies, capture can, and often does, fail to yield the desired coverage. Here, rigorous examination of the data and testing of methods offered valuable insight to treponematosis of the past, particularly in connection with the archaeological and paleopathological context.

**Treponematosis in the Ancient Americas**

For nearly a century, paleopathologists have presented evidence that a form of treponematosis infected humans in the Americas at least as far back as 7,000 years ago (Standen & Arriaza, 2000). Comparisons of skeletal changes identified in Indigenous populations from pre-columbian contexts of the Americas show pathological signs shared across treponemal disease, but the distribution of skeletal pathology differs from what is seen with treponemal infections from more recent history (Powell, 1988; Powell & Cook, 2005; Reichs, 1989). While it is possible these differences are due to environmental and/or host factors, the unique distribution in skeletal pathology found in the pre-European Americas may also signal currently unknown species or *T. pallidum* subspecies endemic to the continent. Given the discovered genome's basal sister branch positioning, it is possible this ancient

pathogen was contributing to the phenotypic variant of skeletal treponematosis that paleopathologists have observed in the pre-Columbian Americas. However, the relationship of TE13 to *T. carateum* remains unknown due to the lack of molecular data associated with the latter. As such, we cannot exclude the possibility that the genome we report is ancestral to a clade that causes diseases clinically grouped with pinta caused by *T. carateum*. These two hypotheses are not mutually exclusive, as it is possible an ancestral lineage of *T. carateum* was responsible (or one of multiple species responsible) for the treponematosis observed in the pre-columbian Americas. While there are still many unresolved questions about the emergence and evolution of *T. pallidum* as a human pathogen, this data point is a significant finding that advances our understanding of this clade's ancient diversity.

**Ethics Statement**

We acknowledge the Indigenous people of Colombia and the present day community and descendants who supported this work. The analysis of aDNA and specifically here, ancient pathogen DNA, can have strong impacts on the community and people related to this research. Discourse surrounding infectious disease often devolves into narratives of blame, particularly regarding historically stigmatizing, disfiguring or highly deadly diseases. Research of treponematosis has been particularly susceptible to this. Our team continues to work to involve the communities that could be affected by this work and not only seek permission but also guidance in how we proceed towards publications and presentations to the broader public. Through an international team of researchers, we sought to move

beyond this limited scope and to examine a deeper history of not only the experience of this ancient individual, but also human history and the microbes that influence our lives. Given this individual lived thousands of years ago during the Middle Holocene, our first approach was to reach out to public spaces in which this work is relevant and seek out communities residing in the area today. During this work we met and consulted with local community members in Colombia. MD led public outreach efforts, connecting with these stakeholders by establishing a presentation and interviews with the Instituto Colombiano de Antropología e Historia (ICANH) in Bogotá on November 28, 2023 and a presentation and meetings with the local communities affiliated with La Corporatión Universitaria Minuto de Dios (Uniminuto) in Bogotá on December 1, 2023. This research was made possible through the formal permits for exportation and study (ICANH archaeological intervention license number 8270, issued to MD 20/08/2019) and with the support of local academic and general community members.

## Figures & Tables

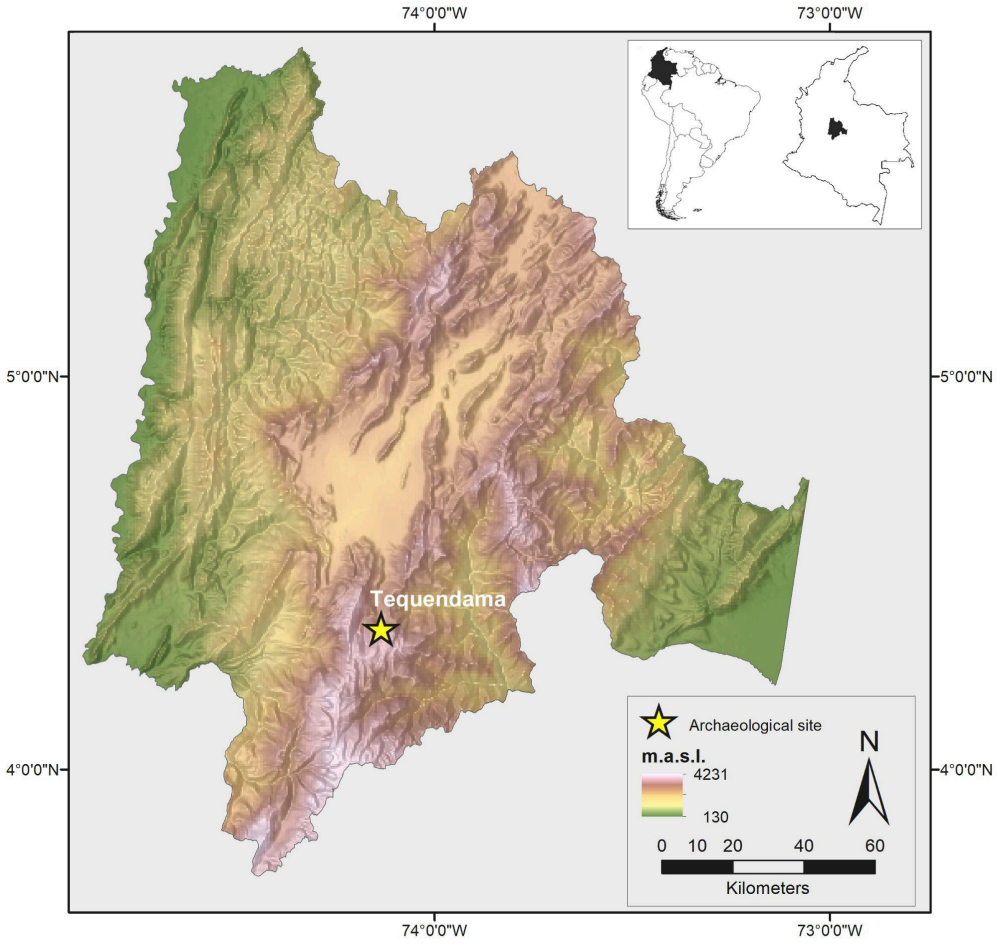


**Figure 5.1.** Map showing the location of Tequendama I in Sabana de Bogotá region, Colombia, South America.

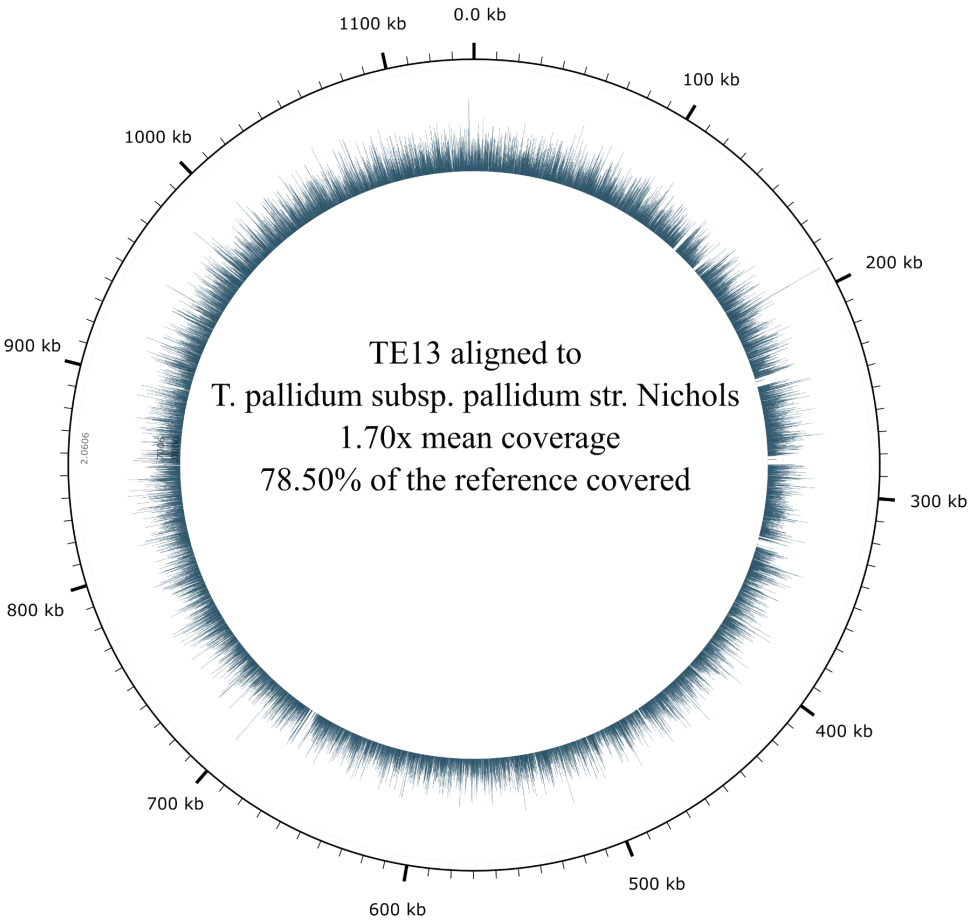


**Figure 5.2.** Circleator plot displaying the coverage of the ancient genome when mapped with stringent parameters. Coverage values are in log scale and a window size of 66 was used. The reference genome used is *T. pallidum subsp. pallidum* (Nichols).

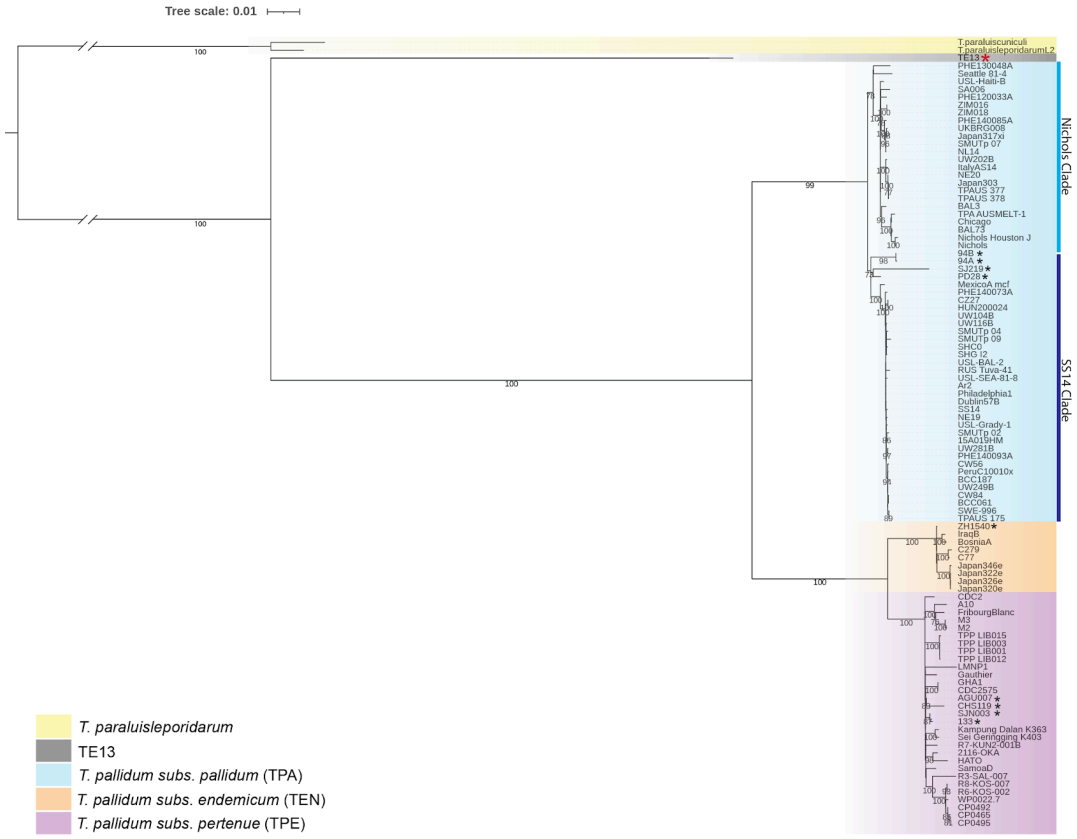


**Figure 5.3.** Maximum likelihood phylogeny of the curated dataset (Table 5.S4, Table 5.S5) of 101 genomes constructed using 8,582 variant positions against the TPA Nichols reference (NC_021490.2). Regions flagged as potentially recombinant by Gubbins and ClonalFrameML (listed in Table 5.S6) were excluded. Established *T. pallidum* subspecies clades are highlighted in different colors and within TPA subclades are noted with bar labels. Ancient genomes are indicated with an asterisk "*" with TE13 indicated with a red asterisk. The tree was computed with RAxML-NG using a GTR-G substitution model. Bootstrap support values were computed using 1000 iterations.

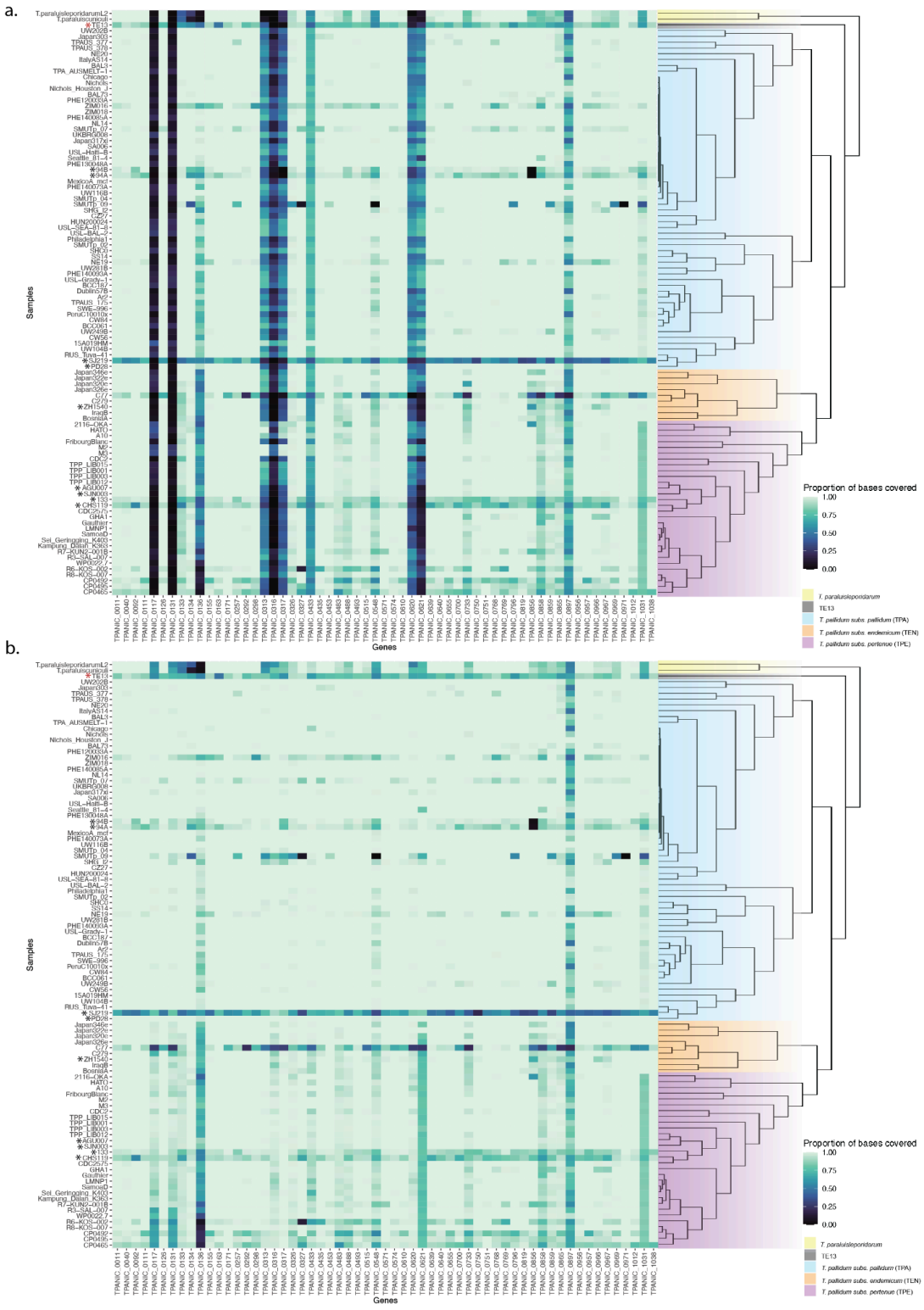


**Figure 5.4.** Presence of virulence genes by coverage proportions. Heatmap showing

the proportion of bases covered at least once during the process of mapping to the Nichols reference for 59 genes previously associated to virulence (x axis), for all the genomes included in the mapping-based phylogenetic analysis (y axis). **a)** Gene coverage when using the stringent mapping parameters employed throughout the study and **b)** gene coverage when mapping parameters are changed to avoid discarding reads that would map equally well to more than one location. Samples are sorted according to the topology of the inferred phylogenetic tree (Figure 5.3) depicted as a cladogram on the right. For labels and color coding see Figure 5.3 and the corresponding legend. Ancient genomes are indicated with an asterisk "*" next to their label. TE13 is marked with a red asterisk.

| **Subspecies Reference** | **Quality filtered deduplicated Mapped reads** | **Mean coverage** | **% of reference covered >= 1X** | **% of reference covered >= 2X** | **% of reference covered >= 3X** | **SNPs in relation to ref (3x)** |
|---|---|---|---|---|---|---|
| *pallidum* (Nichols) NC_021490.2 | 29815 | 1.70 | 78.50 | 49.50 | 25.16 | 546 |
| *paraluisleporidarum* (Cuniculi A) NC_015714.1 | 28665 | 1.65 | 76.96 | 47.92 | 24.09 | 1202 |
| *pertenue* (Samoa D) NC_016842.1 | 29777 | 1.70 | 78.51 | 49.45 | 25.12 | 535 |
| *endemicum* (Bosnia A) NZ_CP007548.1 | 29817 | 1.70 | 78.71 | 49.61 | 25.17 | 542 |

**Table 5.1.** Mapping statistics of sequencing data to multiple references. Adapter removed and trimmed reads (1,572,365,899 reads in total) were mapped with a maximum mismatch allowance of 0.1, quality filtered with a threshold of 37 and duplicates were removed.

## Methods

### Generating Sequencing Data

All data generation was performed in a dedicated ancient DNA laboratory at UC Santa Cruz. A total of 180 mg of bone powder was collected from the individual's right tibia using an electric saw and rotary blade and a ball mill. A total of 120 mg of bone powder was used for the initial DNA extraction following a protocol optimized for the recovery of short fragments (Dabney et al., 2013). Powder was incubated overnight (16 hrs) at 37° C in EDTA lysis buffer treated with Proteinase K. Purification of DNA extracts was performed using a GuHCl binding buffer with silica based purification columns and followed by a final elution of 100 µl of Tris-EDTA-Tween buffer.

Extracts were prepared into single-stranded DNA (ssDNA) libraries (Kapp et al., 2021) with initial treatment of partial uracil DNA glycosylase treatment (UDG, modified from Rohland et al., 2015) for Illumina sequencing. All libraries were dual-indexed for downstream identification (Kircher et al., 2012). Libraries were sequenced on an Illumina NovaSeq resulting in a total of 1,572,365,899 paired-end reads. Prior mitochondrial data published from this individual (Delgado et al., 2021) was generated from this same library we retrieved pathogen data from, and the tibia fragment used to generate this library was also what was sampled for radiocarbon dating. Detailed information on the preparation of ssDNA libraries, quality control measures, and preparation for sequencing can be found in supplemental information.

**Detection and Genomic Reconstruction of Ancient *Treponema***

Sequencing reads were adapter-trimmed and merged using AdapterRemoval with a minimum adapter overlap of 1, minimum length cutoff of 30, minimum quality cutoff of 20, and consecutive Ns and low-quality bases trimmed from the 5' and 3' ends (Schubert et al., 2016). All reads were then screened for the presence of pathogen DNA using the HOPS pipeline (Hübler et al., 2019) that incorporates MALT v.0.04 (Vågene et al., 2018) using a custom RefSeq Genome database (constructed on November 3, 2017) consisting of representative viral, bacterial, and eukaryotic genomes. Screening was performed using a minimum 90% identity for pathogen assignment. Applying hierarchical filtering in its evaluation, HOPS assesses assigned reads for edit distance against a reference of a detected pathogen, evaluates the presence of DNA damage consistent with ancient DNA and finally, investigates the decline in edit distance in only those reads possessing ancient DNA damage patterns. The results revealed several sequencing reads assigned to *Treponema pallidum* subsp. *pallidum (*NC_021490.2) with the additional assignment of reads to the broader *Treponema* node (strongest reference identity *Treponema paraluisleporidarum*).

To authenticate the detection of *Treponema* the data were mapped with bwa aln (Li and Durbin, 2009) to the *Treponema pallidum pallidum* Nichols reference genome (NC_021490.2), as well as to the *Treponema paraluisleporidarum* cuniculi A reference (NC_015714.1). Additional mapping was performed to genomic references of the closely related *Treponema pallidum* subspecies *Treponema pallidum*

*endemicum* Bosnia A (NZ_CP007548.1) and *Treponema pallidum pertenue* Samoa D (NC_016842.1). Mapping parameters were specified for strict mapping to reduce mismatches (-n 0.1) and quality filtered (-q 37) with a seed length of 32. Mapped reads were then quality filtered using samtools (Danecek et al., 2021) with a threshold of 37. Duplicate reads were removed using the Mark Duplicates function of Picard GATK (DePristo et al., 2011). DamageProfiler (Neukamm et al., 2020) was used to generate damage profiles of mapped data (Figure 5.S3) and fragment length distribution plots (Figure 5.S4). Bam files were then trimmed one base on the left and three bases on the right of each read using bamUtil (Jun et al., 2015) for downstream analyses. Mapping statistics were recorded (Table 5.1), and bam files were visualized in Geneious (Geneious v. 9.1.8, Biomatters Ltd., https://www.geneious.com; Figure 5.S2). A circular plot (Figure 5.2) was created using Circleator (Crabtree et al., 2014).

**Competitive Mapping**

To further assess the categorization of the ancient genome as closely related to *Treponema pallidum* subspecies, we moved forward with a competitive mapping approach. As the subspecies of *T. pallidum* share over 99.78% sequence identity (Štaudová et al., 2014), the *T. pallidum* subsp. *pallidum* Nichols genome (NC_021490.2) was chosen to act as the representative genome for these *T. pallidum* subspecies. The aforementioned Nichols reference was concatenated in one multifasta file with the *Treponema paraluisleporidarum* Cuniculi A reference (NC_015714.1) and a reference genome for *Treponema phagedenis* (NZ_CP042818.1), the next most closely related outgroup. Adapter-trimmed sequencing data from individual TE13

was mapped to this concatenated reference with bwa aln (Li & Durbin, 2009), using the strict mapping parameters described earlier (maximum edit distance of 0.1, -q of 37, a seed length of 32, samtools mapping quality threshold of 37 and duplicate removal using MarkDuplicates). Mapping statistics are reported in Table 5.S2.

**Dataset Preparation**

A dataset of 100 other genomes (Table 5.S4) was selected to include all previously published ancient genomes with coverage higher than 1x using our mapping parameters and to provide an accurate representation of the modern diversity of *Treponema pallidum* and *Treponema paraluisleporidarum*. For all ancient genomes and modern genomes with good-quality sequencing data available, we downloaded the fastq files and ran them through the adapter trimming and mapping process described above to produce final bams. For modern genomes without easily-accessible sequencing data or with lower-quality or lower coverage sequencing data, we used their assemblies to produce fastq files of 100,000 reads using the randomreads feature of bbmap (Bushnell, 2014), specifying a minimum length of 50, a maximum length of 125, and quality score of 40 to mimic ancient read lengths. These were then mapped using the same parameters described above to produce final bams.

**SNP Calling, Heterozygosity Estimates, and Functional Analysis of Variant Sites**

After mapping to each of the four references (NC_021490.2, NC_016842.1, NZ_CP007548.1 and NC_015714.1) we performed genotyping and variant calling on

TE13 using Unified Genotyper in the Genome Analysis ToolKit (GATK) v. 3.5 (DePristo et al., 2011) selecting for the emission of all sites. This produced a call for every position and was then compared to the relevant reference using MultiVCFAnalyzer v 0.85 (Bos et al., 2014) to determine the number of variant sites (Table 5.S9).

Bam preparation and genotypes were prepared as described above for the dataset of 100 other genomes (Table 5.S4) and compared to TE13 and the four selected references (NC_021490.2, NC_016842.1, NZ_CP007548.1 and NC_015714.1) using MultiVCFAnalyzer v. 0.85 (Bos et al., 2014). This analysis was run selecting for a minimal genotype quality of 30. Positions were called only with 90% read support (ensuring only homozygous sites are called), and data required a minimum coverage of 3 fold. This was repeated with selected regions removed (see Table 5.S6 for regions removed). A third run exclusively used for heterozygosity plots was set up with the same original parameters except a 10% read support threshold in order to call heterozygous sites. These reported heterozygous sites of TE13 are shown in a bar plot form (Figure 5.S13).

**A Closer Look at Shared SNPs**

Using the SNP table output from the MultiVCFAnalyzer (Bos et al., 2014) run using all data aligned to the Nichols reference (NC_021490.2) and called with a 3-fold coverage minimum (Table 5.S9), variants found in TE13 were identified by filtering out any sites where TE13 was ambiguous or matched the reference. The

remaining sites were organized in a table (Table 5.S10) by what other groups of genomes (defined as “ancient genomes”, “TPA”, “TPE”, “TEN”, and “T. paraluisleporidarum”) shared those variants. Variants were considered present in a group if at least one genome shared that variant.

**SnpEff**

Using the table of variant sites (snpTableForSnpEff.tsv) produced by the MultiVCFAnalyzer run (Bos et al., 2014) that includes data aligned to the Nichols reference (NC_021490.2) and called with a 3-fold coverage minimum, analysis of all SNPs called in TE13 was performed using snpEff v3.1i (build 2012-12-12) (Cingolani et al., 2012) and the annotation of the same Nichols reference (NC_021490.2). The resulting output was input into another MultiVCFAnalyzer run with otherwise identical parameters to produce an annotated SNP table (Table 5.S8). Using the vlookup function in Microsoft Excel, gene functions were copied from the reference annotation into this table for non-synonomous changes impacting gene products.

**Recombinant and Other Removed Regions**

A list of tRNA regions, repetitive regions, and recombinant regions (Table 5.S6) was compiled using the annotation of the Nichols reference (NC_021490.2), prior literature (Beale et al., 2021; Giffin et al., 2018; Lieberman et al., 2021; Pla-Díaz et al., 2022; Taouk et al., 2022), and two recombination analyses. The recombination analyses were performed using ClonalFrameML (Didelot & Wilson,

2015) and Gubbins (Croucher et al., 2015). The Nichols-aligned dataset called at 3-fold minimum coverage was used for both analyses. Outputs from both analyses were curated manually. Where Gubbins and ClonalFrameML output segments overlapped with a difference of less than 12 bp, the longer segment of these was used and the other removed from the list. In situations where the overlap was less precise, ClonalFrameML output coordinates were used.

**Virulence Factor and Selective Gene Analysis**

An analysis to detect the presence of candidate *T. pallidum* virulence factors in the ancient genomes was performed following an approach already used for ancient *T. pallidum* genomes (Majander et al., 2020). Here, TE13 was evaluated for the presence of a set of 59 different putative virulence factors (Table 5.S7), identified in previous studies (Radolf et al., 2016; Schuenemann et al., 2018; Šmajs et al., 2012; Weinstock et al., 1998). Based on the mapping to the Nichols reference (NC_021490.2), for each target gene we computed the proportion of bases covered at least once. We did this for all the ancient and modern genomes included in the analysis. We repeated this analysis twice, once with the mapping parameters used throughout the study and once without quality filtering (MAPQ = 0). This second approach preserves reads that map to multiple locations, allowing us to inspect the presence of closely related paralogous genes (such at the *tpr* genes) that would otherwise apparently look as absent. Results were plotted in R in a color coded heatmap with genomes sorted according to their phylogenetic relationships (Figure 5.4).

**Phylogenomic Analysis**

To evaluate the phylogenetic position of TE13, two alignments were constructed: one with all data from the selected dataset of 101 genomes (Table 5.S4) and one with specific regions masked (Table 5.S6). These were created using MultiVCFAnalyzer v 0.85 (Bos et al., 2014) as described in the prior section. These alignments were used to construct maximum parsimony phylogenies (Figure 5.S6) with MEGA X (Kumar et al., 2018). For each phylogeny all variant sites were used and phylogenies were constructed using a subtree pruning regrafting model (SPR), 10 initial trees, and 200 bootstrap replications. Maximum likelihood trees (Figure 5.S5, Figure 5.S7) were generated from these same SNP alignments with RAxML-ng (Kozlov et al., 2019). A GTR-G model was utilized and all variant sites were included in the construction of the tree. Consensus trees were created with 1000 FBP bootstraps using a seed length of 23. Phylogenies were repeated with recombinant sites (identified in ClonalFrameML, Gubbins, and in previous literature) removed along with repetitive regions, the tpr gene family, and tRNA regions (Figure 5.3; Table 5.S6).

**Whole Genome Multiple Sequence Alignment**

As an alternative to the mapping-based approach, given the highly conserved genomic structure of *T. pallidum*, we investigated the possibility of inferring a ML phylogeny starting from a whole genome multiple sequence alignment (MSA). We focused on a subset of our dataset. Assemblies for the modern genomes were

downloaded and integrated with the ancient samples. Chromosome level assemblies were downloaded from NCBI RefSeq for a subset of the genomes used in the mapping-based phylogenetic analysis (see Table 5.S11 for list of genomes used). All the ancient genomes were mapped to their closest reference (see related methods section) and a consensus sequence was derived with samtools consensus (v1.15.1) (Danecek et al., 2021) using simple mode and imposing a minimum mapping quality of 30, a minimum proportion of agreeing bases of 0.75 and a minimum coverage of 3. Both insertions and deletions were allowed to be included in the consensus sequence. Whole genome sequences were then aligned with MAFFT (v7.481) (Katoh & Standley, 2013) (default settings) and IQTREE (v2.2.0.3) (Minh et al., 2020) was used for ML phylogenetic inference as described above.

**Low-Coverage Genome Simulations**

To assess the potential effect of having a low coverage genome on the phylogenetic reconstruction and on its placement on a ML tree we decided to simulate other low coverage genomes for each *T. pallidum subspecies*. Specifically, we down-sampled two high coverage genomes per subspecies (one modern and one ancient) to the average depth of TE13, for a total of 6 down-sampled genomes. Based on their estimated coverage, we selected the following genomes for the down-sampling experiment: C279 and ZH1540 (TEN), Gauthier and AGU007 (TPE), SS14 and PD28 (TPA). For each genome we estimated the fraction of the reads that needed to be sub-sampled to reach a coverage comparable to TE13. Then we used samtools (v1.15.1) to down-sample the bam files (Danecek et al., 2021). For this

analysis each genome was mapped to its closest reference and a phylogeny was built as described in the Whole Genome Multiple Sequence Alignment Methods section above. Results are provided in Figure 5.S10. The command used was “samtools view -bu --subsample 0.FRAC --subsample-seed INT in.bam > out.bam**”,** with 0.FRAC specifying the fraction of reads to sub-sample to achieve the desired coverage.

**Rooting the Phylogenetic Tree**

To assess the relationship of TE13 to *T. pallidum* and *T. paraluisleporidarum* and its positioning in respect of the root of the phylogenetic tree we inferred phylogenetic trees while including one or more outgroups. The closest species *T. pallidum* and *T. paraluisleporidarum* for which a sequenced genome is available is *Treponema phagedenis*. This bacterium is very divergent, and its genome is heavily re-arranged compared to the one of *T. pallidum* and *T. paraluisleporidarum*. As such, we could not rely on the usual approaches of reference-mapping or whole-genome multiple sequence alignment (MSA). Instead, we decided to identify conserved orthologous genes, found across the genomes in single copy and to infer the phylogeny from their concatenated MSA. For this analysis only proteomes from chromosome-level assemblies were downloaded from NCBI RefSeq. We repeated this analysis including a single outgroup (*T. phagedenis*) or more outgroups (*T. phagedenis*, *T medium* and *T. vincentii*). Twenty-nine *T. pallidum,* three *T. paraluisleporidarum*, one *T. phagedenis*, one *T. medium* and one *T. vincentii proteomes* from chromosome-level assemblies were downloaded from NCBI (18

April 2024). The list of proteomes included in these analyses is provided in Table 5.S11. For TE13, reads mapped to the relevant reference (see related Methods section) were used to generate a consensus whole-genome sequence for the ancient genome. To exclude potential biases induced by the choice of the reference, we repeated all the analyses twice, once mapping to the *T. pallidum reference* (Nichols) and once mapping to the *T. paraluisleporidarum* reference (Cuniculi A). The consensus sequences were obtained with the samtools consensus (v1.15.1) command (Bayesian mode) (Danecek et al., 2021) imposing a minimum mapping quality of 30. We then used bedtools getfasta (v2.30.0) (Quinlan & Hall, 2010) to extract the coding regions from the sequence based on the coordinates of the reference genome used. Deletions (denoted as "*") were converted to missing data "N", and inferred insertions in the ancient sequence were disregarded, preserving the reading frame of the reference and its gene coordinates. The sequence was then converted into amino acids with EMBOSS Transeq (v6.6.0.0) (Madeira et al., 2022).

Conserved single-copy orthologous sequences were identified with Orthofinder (v2.5.5) (Emms & Kelly, 2019) (default settings) and genes were aligned with MAFFT (v7.481) (Katoh & Standley, 2013) (default settings). The aligned genes were then concatenated with seqkit (v2.2.0) concat command (Shen et al., 2016) and used for phylogenetic tree inference with IQTREE (v2.2.0.3) (Minh et al., 2020), activating ModelFinder to detect and select the best-fit substitution model and with a thousand bootstrap replicates. Trees were visualized with the aid of iTOL (v6) (Letunic & Bork, 2021).

**Data Availability**

Data will be made available upon publication in a journal.

**Acknowledgements**

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**Author Contributions**

Community engagement: organized by MD, presentations by MD, LFS, and NB, discussions and participation from MD, LFS, NB, EAN, JB, and DB.

Wetlab work: LFS and NB

Project conception and lead: MD, EAN, LFS, and ASM

Primary writing and figures: NB, DB, MD, and EAN

Analyses: NB, DB, and EAN

Revisions and ideas: all authors

**Competing Interests Declaration**

The authors declare no competing interests.

## Supplementary Text

**Supplementary Discussion**

When paired with paleopathological data and archaeological context, paleogenomics is a useful tool in the pursuit of understanding the evolutionary dynamics of human pathogens. An interdisciplinary approach incorporating these fields can provide key context to reveal how infectious diseases affected ecosystems of people, animals, and the microbes themselves.

**Archaeological and Bioarchaeological Context**

The Sabana de Bogotá (SB) in the eastern highlands of Colombia is a well-known archaeological region that preserves evidence about the initial human expansion into South America during the late Pleistocene. In addition, abundant

archaeological materials including lithic tools (unifacial and bifacial), botanical (squash, potato, beans) and animal remains (deer, guinea pig) along with burials with well-preserved human skeletal remains have been recovered from contexts that encompass the entire Holocene period (~12,000 - 300 cal years BP) (Archila et al., 2020; Ardila, 1984; Botiva, 1989; Correal, 1990; Correal and van der Hammen, 1977; Groot, 1992; Orrantia, 1997; Triana, 2019). The Sabana de Bogotá is one of the few regions in South America that possesses a detailed archaeological and bioarchaeological record, reliable chronological contexts, and paleoenvironmental reconstructions useful to reconstruct the Holocene human history.

**Tequendama I Site**

Tequendama I (latitude 4.358104; longitude -74.134561) is a large rock shelter located at 2566 masl in Soacha municipality (Cundinamarca) south of Bogotá city, 1 km from the Bogotá river. Tequendama is a multicomponent site occupied continuously by humans since the late Pleistocene 10,920 ± 250 14C yr BP (*GrN-6539*) (13,263 – 12,072 cal yr BP 2σ) until the final late Holocene 2225 ± 35 14C yr BP (*GrN-6536*) (2329 – 2063 cal yr BP 2σ). This site contains evidence of rock art, two well-differentiated lithic techno-complexes spanning the entire cultural sequence Abriense (expedite) and Tequendamiense (more elaborate) along with skeletal remains of highly diverse Holocene faunas (mainly deer and small mammals like guinea pig, rabbit and armadillo) (Correal and van der Hammen, 1977).

A total of 22 burials, widely distributed across the rock shelter, were

excavated in Tequendama I containing diverse skeletal elements with variable preservation representing adults and sub-adults of different ages and sexes (Correal and van der Hammen, 1977; Triana, 2019). The burial patterns of adult individuals were performed by placing the body in lateral or dorsal decubitus position with flexed limbs. Some skeletons were stained with red ochre or cremated. Variable grave offerings were also found, including lithic and bone artifacts, shells, horns, etc (Correal and van der Hammen, 1977; Triana, 2019). Burials of adults were most commonly oval and elongated while sub-adult burials were small and circular. Tequendama I radiocarbon chronology and stratigraphy indicate that human burials are restricted to strata 7a and 7b (~ 10,200– 9900 cal yr BP 2σ), 8a and 8b (*GrN-6729* 7090 ± 75 14C yr BP [8011 – 7710 cal yr BP 2σ]; *GrN-6728* 6990 ± 110 14C yr BP [7970 – 7588 cal yr BP 2σ] and *GrN-6537* 6395 ± 70 14C yr BP [7427 – 7076 cal yr BP 2σ]) and 9 (2225 ± 35 14C yr BP [2329 – 2063 cal yr BP 2σ]) (Correal and van der Hammen, 1977). Previous direct betacounting and AMS dates obtained from human skeletal remains revealed the following dates (*GrN-7477*) 7235 ± 60 [8167 – 7877 cal yr BP 2σ], (*Col-AAA*) 6080 ± 40 [7001 – 6750 cal yr BP 2σ], (*GrN-7478*) 6020 ± 45 and [6707 – 6679 cal yr BP 2σ] (*GrN-7476*) 5850 ± 50 14C yr BP [6739 – 6481 cal yr BP 2σ] for individuals 12, R6I1, 13 and 7 respectively, confirming the stratigraphic sequence (Correal and van der Hammen, 1977; Triana, 2019). Human skeletal remains from Tequendama have been systematically investigated including morphological, isotopic and paleogenetic assessments (Delgado, 2012; Delgado, 2017; Delgado, 2018; Delgado, 2021; Delgado et al., 2021; Neves et al., 2007).

Likewise, coincident with the findings of the present study, previous studies reported skeletal lesions related to treponemal and other infectious diseases in early and middle Holocene hunter-gatherers from Sabana de Bogotá (Correal, 2012).

**Individual No. 13 Tequendama I**

Osteological and genetic analyses of the individual studied here indicate the individual was male, and died at a middle adult age with an estimated stature of 158,5 ± 3.2 cm (Delgado et al, 2021). Craniodental analysis suggest generalized morphology characteristic of early/middle Holocene Sabana de Bogota hunter-gatherer populations (Delgado, 2017). A new AMS radiocarbon date obtained from the individual 13 revealed a middle Holocene age (*UCIAMS* 288522 4675 ± 20 14C yr BP [5464 – 5309 cal yr BP 2σ]). Stable isotope measurements ($\delta^{13}C_{col}$ -20.5‰, $\delta^{15}N$ 6.0, C:N atomic 3.2, C:N wt%/wt% 2.7) indicate an individual consuming $C_3$ resources, mainly plants and to a lesser extent animals similar to other early and middle Holocene individuals from Tequendama and other archaeological sites (Delgado, 2021). 14C date calibration was done with the R package Rcarbon (Crema & Bevan, 2021) using the SHCal20 curve (Hogg et al., 2020). DNA was recovered from a tibial fragment and analyzed for human population genomics (Delgado et al., 2024) and screened for pathogens, leading to the identification of *Treponema* DNA. The skeleton of this individual does not present the pathological lesions associated with the later stages of a treponemal infection. However, paleopathological evidence of similar disease manifestation at Tequendama I and surrounding sites (e.g., Aguazuque) suggests the pathogen genome reported here may correspond to that

reported ancient form of treponemal disease.

**Paleopathology, Hypotheses, and Treponematosis**

Since the late 1800s, paleopathologists have reported evidence of these ancient infections in the Americas (Baker & Armelagos, 1988). Due to inconsistent diagnostic criteria, these early diagnoses were later disputed, but many have stood the test of time (and the test of new, standardized diagnostic criteria) (Baker & Armelagos, 1988). Some of these confirmed diagnoses of treponematosis are in individuals dating back at least 7,000 years (Allison et al., 1982; Eggers et al., 2008; Filippini et al., 2019; Pechenkina et al., 2007; Standen & Arriaza, 2000; Vento Canosa & González Rodríguez, 1996).

The sudden onset of a pandemic of virulent and obvious (yet apparently novel) disease throughout Europe following the invasion of French soldiers in Spain in 1495 led to blame of multiple groups and nations for the spread of syphilis. It is believed many first blamed the Franks, some blamed the Christians or the Polish or the Spaniards. The emergence of the pandemic following the expeditions and conquest of the Americas by Europeans led to the popular Columbian hypothesis. This posits that treponemal disease was circulating in the Americas prior to European invasion and that Europeans brought *Treponema*, specifically *Treponema pallidum subsp. pallidum* back to Europe upon their return from the Americas. This is supported by reports of rapidly declining virulence that was observed shortly after, but countered by the Pre-Columbian hypothesis which argues syphilis existed in

Europe prior to 1492 and was brought to the Americas by Europeans. Since both of these arguments have been put forth and each arguably has archaeological and historical support, additional arguments adapted from the hypotheses have been developed to acknowledge the complexity of human-pathogen evolutionary histories. The Unitarian hypothesis suggests all treponematoses, including pinta, are caused by the same organism, *Treponema pallidum*, which is globally distributed and manifests in different ways solely due to sociocultural, climatic, and demographic factors (Hudson 1946; Hudson 1965). Alternatively, the Evolutionary hypothesis states that these diseases are caused by different related species and sub-species originating from a zoonotic ancestor emerging from Afro-Asia (Hackett 1963). Proponents hypothesize this ancestor began to infect humans about 10,000 BCE in Africa and/or Asia and eventually diverged into yaws, bejel, pinta, and syphilis (Hackett 1963). There are also hypotheses focused on historical context. For example, the Modified Columbian hypothesis states that non-venereal treponemal disease existed in the Americas before Columbus's voyage. The existence of a non-venereal variant is supported by paleopathological evidence of a high prevalence of treponemal disease yet low age of infection along with the unique distribution of skeletal pathology observed in pre-European contexts. This Modified Columbian hypothesis suggests it was brought to Europe by the return crew of the initial expeditions where under different selective pressures the pathogen evolved towards new transmission strategies through sexual contact (Armelagos et al., 2005; Harper et al., 2008).

**Animal Hosts and One Health**

*T. pallidum* is not only restricted to humans. Molecular studies have identified the presence of *Treponema pallidum subsp. pertenue* in different species of African primates (Chuma et al., 2019; Knauf et al., 2018; Mubemba et al., 2020; Zobanikova et al., 2013). These studies have shown that the strains currently circulating in wild primate populations are the results of one or more spillover events from humans to monkeys. Interestingly, some studies have suggested that geography, rather than host species, seems to be responsible for the pathogen phylogeny, indicating the existence of interspecies transmission (Chuma et al., 2019; Mubemba et al., 2020). Some researchers have hypothesized a fly-mediated spread of the pathogen in this context (Gogarten et al., 2019; Jahan et al., 2022; Knauf et al., 2016; Stamm, 2016; Stoffolano 2022; Thomson & Lamborn, 1934). The disease in non-human primates species is similar to what is commonly observed in humans, causing in some cases yaws-like orofacial and limb lesions but also anogenital lesions, potentially suggesting a variety of transmission routes (Knauf et al., 2018).

The species most closely related to *T. pallidum* is *Treponema paraluisleporidarum*, a pathogen causing venereal spirochetosis in lagomorphs (i.e. domestic rabbits and wild hares). Numerous studies have shown the extensive circulation of *Treponema paraluisleporidarum* in wild populations using serological tests (Hisgen et al., 2020, Hisgen et al., 2021; Lumeij et al., 1994; Nováková et al., 2019; Verin et al., 2012) indicating this is a widespread infectious disease in the *Leporidae* family. What was formerly described as two species are now considered

two ecovars. Ecovar Lepus (formerly known as *Treponema paraluisleporis*) can cause disease in both rabbits and wild hares, but ecovar Cuniculi (formerly known as *Treponema paraluiscuniculi*) is only capable of causing disease in rabbits (Lumeij et al., 2013). With new whole genomes sequences available we could corroborate previous reports that suggested the two bacteria belonged to the same species based on 16S rRNA gene phylogenetic trees (Lumeij et al 2013). Genome-wide ANI values show a similarity exceeding 99.9%, supporting their classification as a single species.

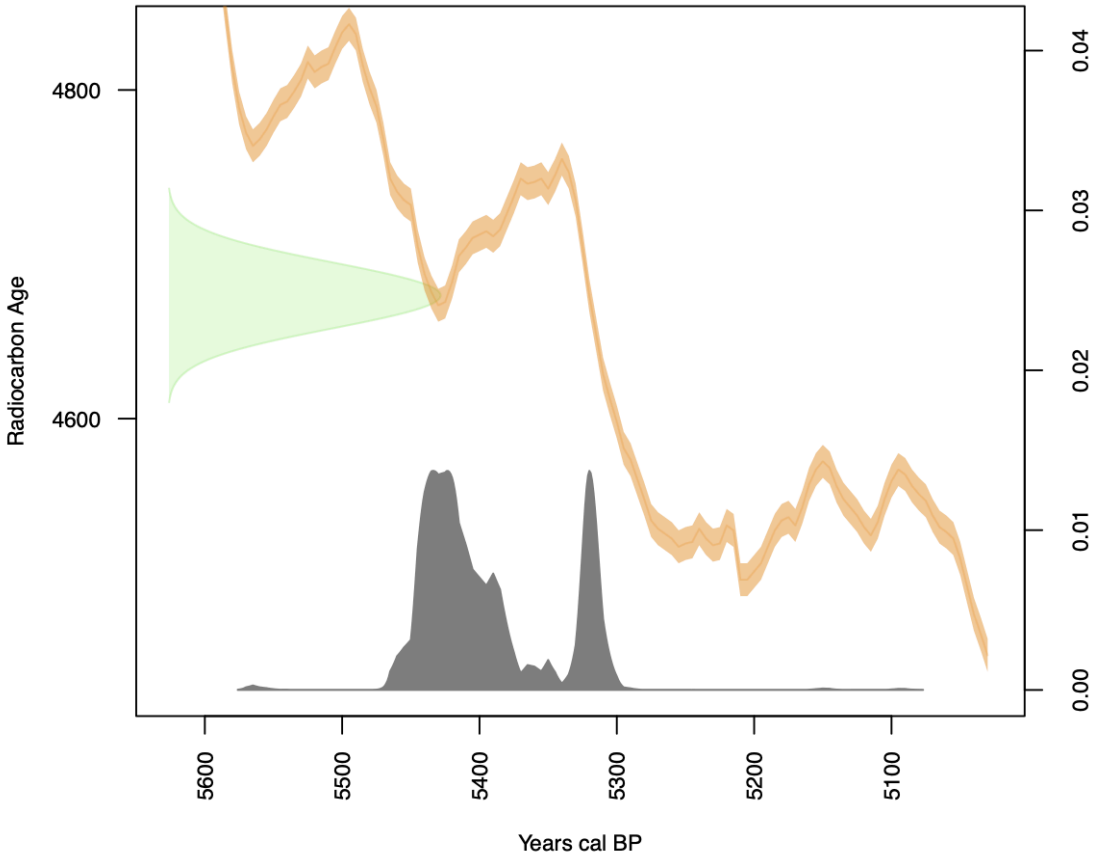
Previous studies have suggested a scenario in which *Treponema paraluisleporidarum* evolved from a *T. pallidum*-like ancestor based on its pseudogenization rate and the resulting genome decay (Šmajs et al., 2011). While *T. pallidum* is capable of infecting rabbits and other animals, *Treponema paraluisleporidarum* is host-specific to lagomorphs (Jacobsthal, 1920; Smith and Pesetsky, 1967; Strouhal et al., 2007). This host-specificity is thought to be due to genome decay (Graves & Downes, 1981; Šmajs et al., 2011). Many of the genes affected in this decay are of known function in *T. pallidum subsp. pallidum* and play an active role in the pathogenesis of syphilis in humans, suggesting that both organisms evolved from a common *pallidum*-like ancestor that was infectious to humans and nonhuman animals (Šmajs et al., 2011). The phylogenetic position of TE13 in addition to the identification in TE13 of shared variants between both clades in TE13 (Table 5.S10) supports this idea and the existence of the MRCA being T.pallidum-like, with a divergence date of more than 5.5 kya.

"One Health" foundations have increasingly been integrated into recent paleopathological research; in accordance with this "One Paleopathology" approach (Buikstra & Uhl, 2023), it is critical to consider the ecological context of ancient animals and their associated infections. The evidence of *Treponema* infections in non-human animals in the Americas prior to European arrival is scant. A report of an infection in a Pleistocene bear bone exists (Rothschild, 1988; Rothschild & Turnbull, 1987), although this report garnered significant critique and may not be reliable (Neiburger, 1988). To our knowledge, no other zooarchaeological treponemal infections have been reported (Thomas, 2019). However, evidence of these infections might have been missed due to sampling and preservation biases. Faunal remains from archaeological contexts are not always recovered or cataloged (Peres, 2010), and remains of small animals like rabbits are also less likely to be preserved in or recovered from the archaeological record (Behrensmeyer et al., 1979). Furthermore, the cooking and consumption of animals (as well as laboratory processing practices of the past) can complicate the detection of pathology, which itself is not well-defined as a diagnostic tool in modern faunal analysis (Thomas, 2019). Another compounding factor would be the unknown pathophysiology and pathology manifestation of *Treponema pallidum* (or closely related species) in animals of the past. With these issues in mind, a recommendation is made for those researchers working with animal remains in these contexts should to be sure to include pathogen screening as part of the analysis in genomic studies.

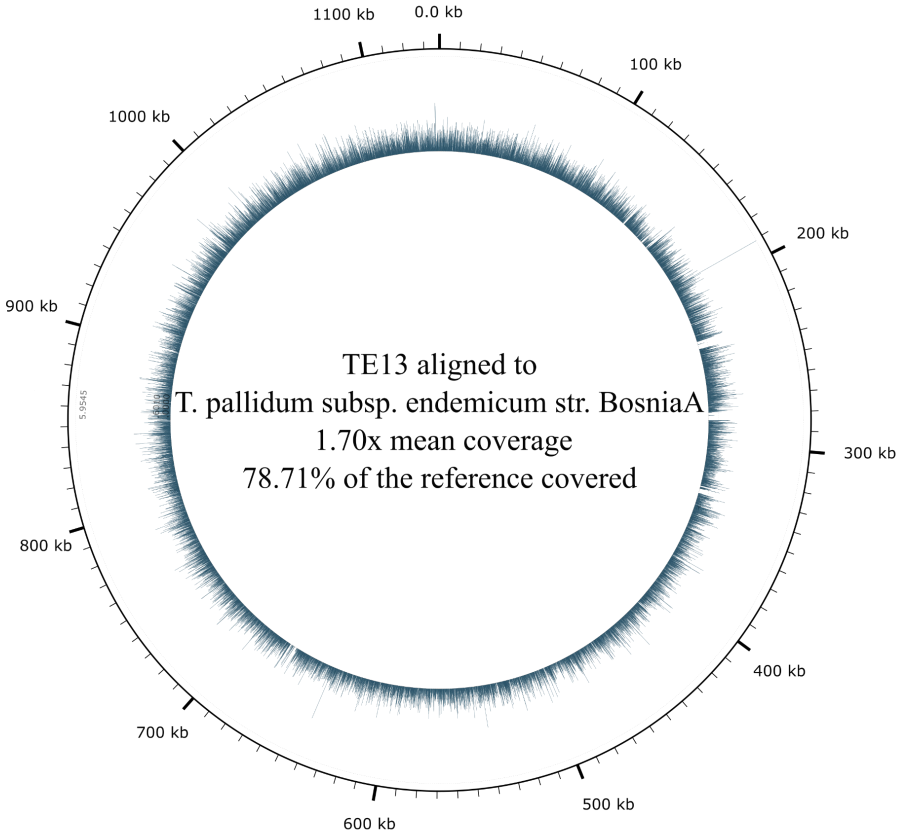
If the individual was infected by a nonhuman animal host, archaeological

evidence suggests a mode of transmission: the hunting and consumption of rabbits at Tequendama. *Treponema pallidum* subspecies have also been shown to be carried (and potentially transmitted) by flies (Knauf et al. 2016; Stamm 2016; Jahan et al. 2022), so it is also possible that the *Treponema* species found here was transmitted by an insect vector.

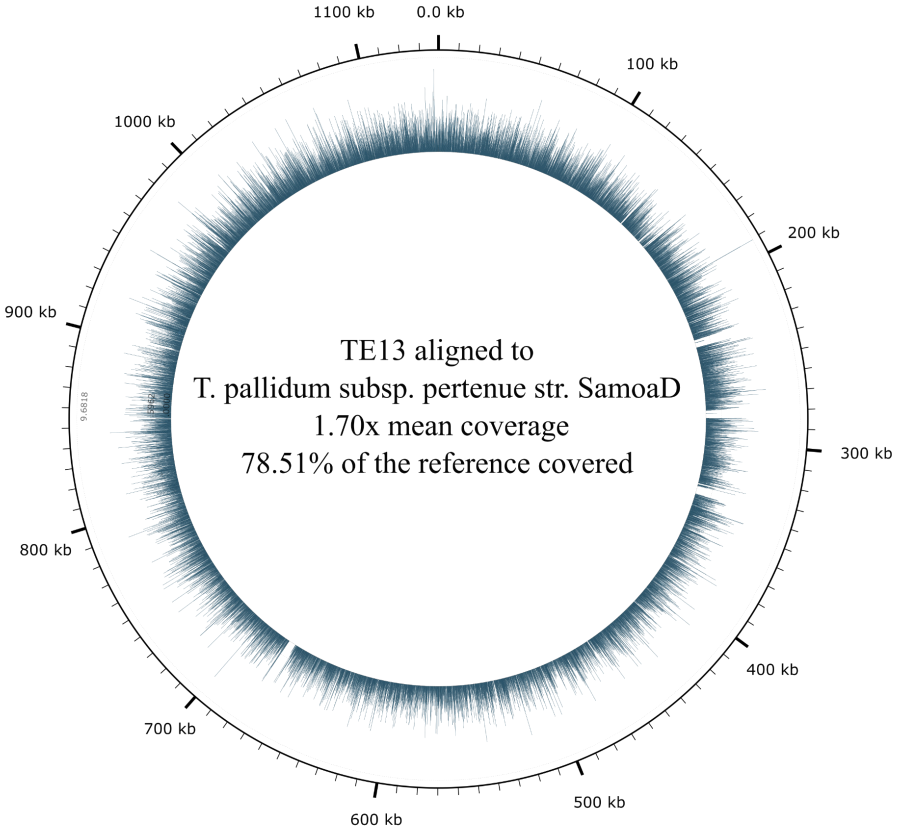
**Supplementary Figures and Tables**



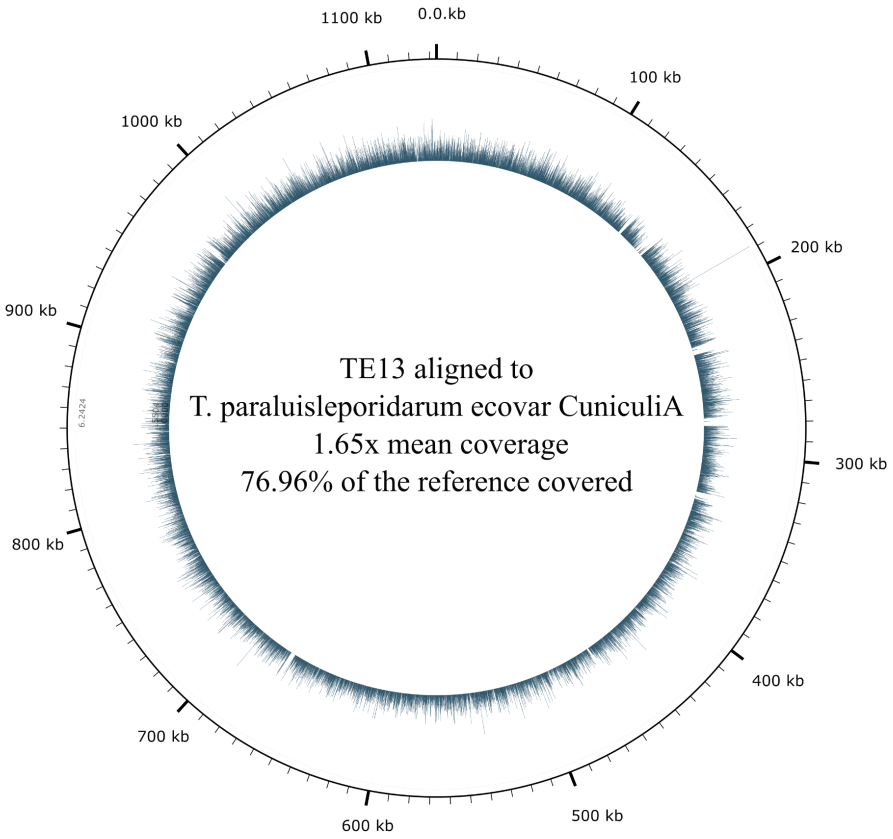

**Supplementary Figure 5.S1.** Radiocarbon calibration curve.


0.0 kb
100 kb
200 kb
300 kb
400 kb
500 kb
600 kb
700 kb
800 kb
900 kb
1000 kb
1100 kb
TE13 aligned to
T. pallidum subsp. endemicum str. BosniaA
1.70x mean coverage
78.71% of the reference covered

**5.S2a)**

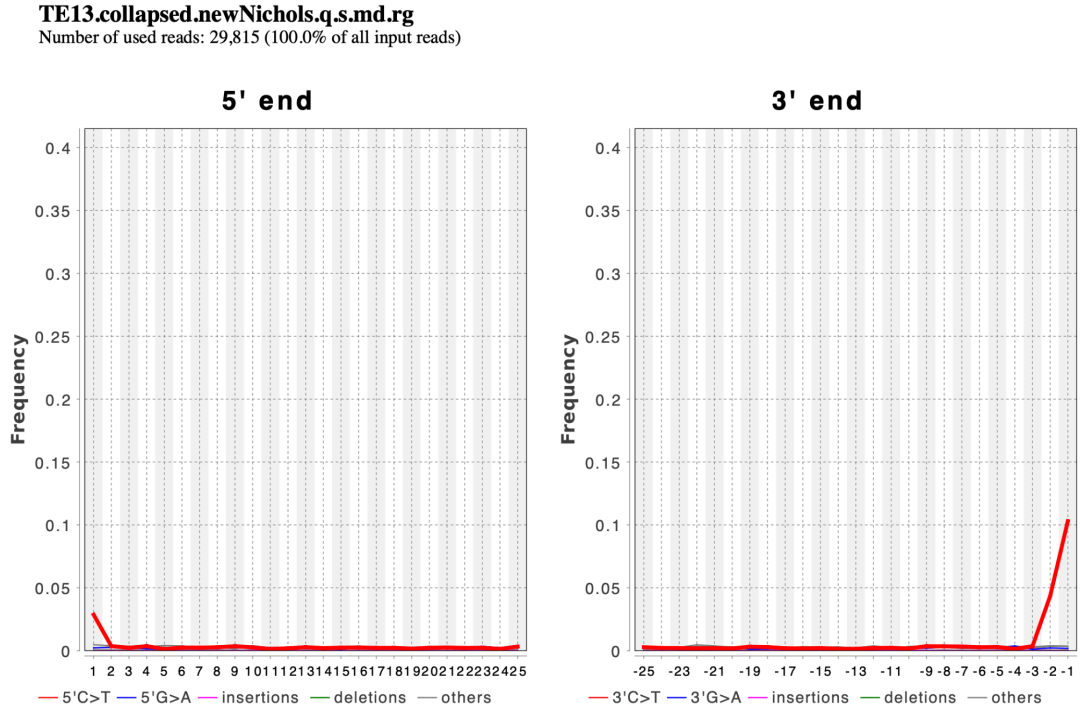

1100 kb
0.0 kb
100 kb
200 kb
300 kb
400 kb
500 kb
600 kb
700 kb
800 kb
900 kb
1000 kb
TE13 aligned to
T. pallidum subsp. pertenue str. SamoaD
1.70x mean coverage
78.51% of the reference covered

**5.S2b)**

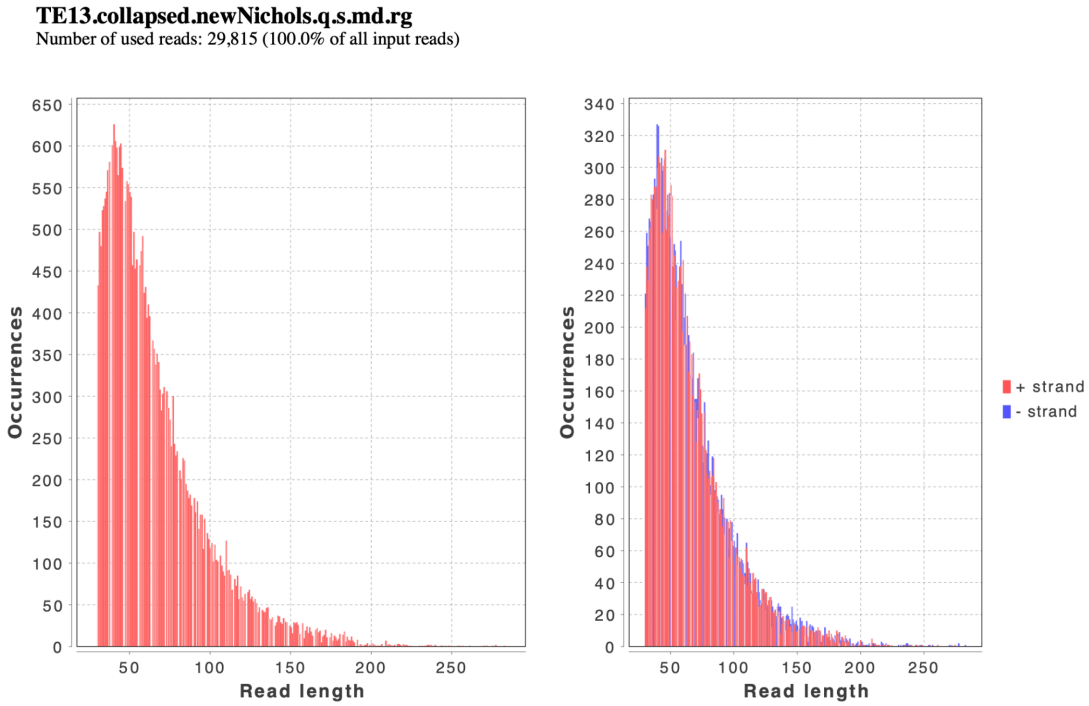



**5.S2c)**

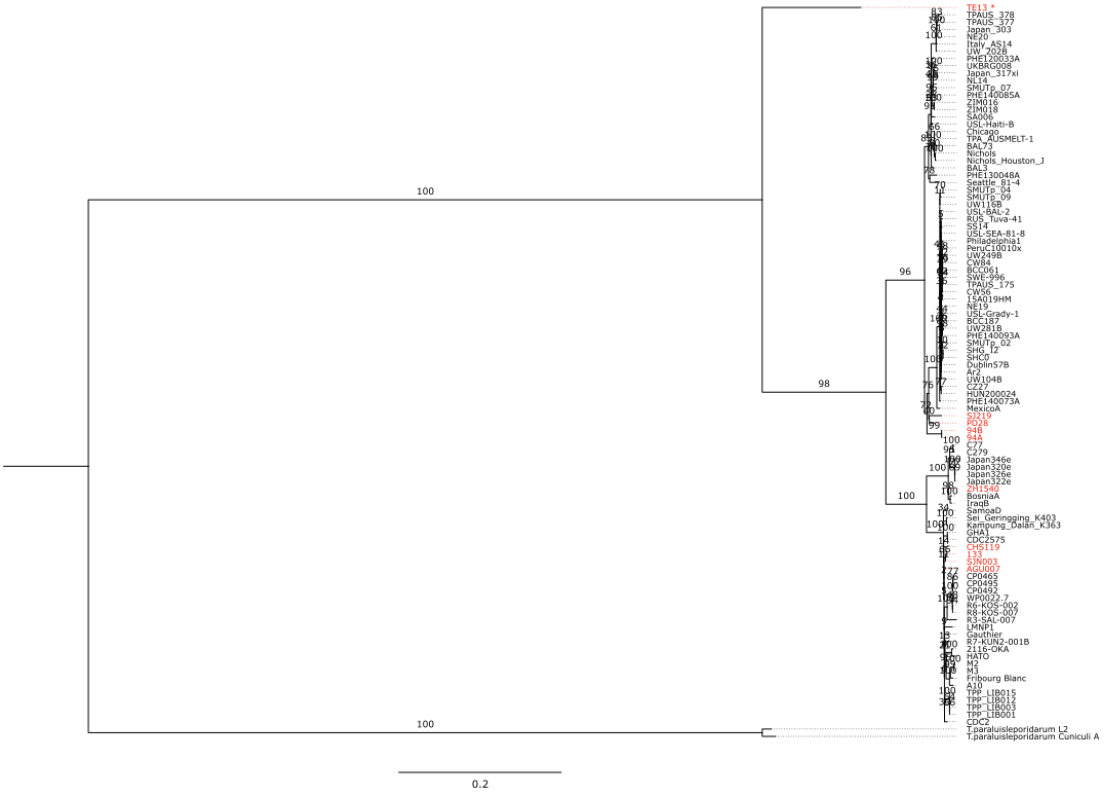
**Supplementary Figure 5.S2.** Comparison of reads aligning to various references. Circleator plots (window size of 66) of coverage and depth of the ancient partial genome (log scale) when mapped to **a)** *T.pallidum* subsp. *endemicum* str. Bosnia A (NZ_CP007548.1) **b)** *T. pallidum* subsp. *pertenue* str. SamoaD (NC_016842.1) and **c)** *T. paraluisleporidarum* str. cuniculi A (NC_015714.1). Alignments shown are of bam files produced with quality filtered reads and strict mapping parameters. Most of the genome is covered, with few regions stacking significantly more than the average.




**Supplementary Figure 5.S3.** DamageProfiler damage profile for TE13 data mapped to *T. pallidum* subsp. *pallidum* str. Nichols (NC_021490.2)




**Supplementary Figure 5.S4.** Fragment length distribution plots generated with DamageProfiler for TE13 data mapped to *T. pallidum* subsp. *pallidum* str. Nichols (NC_021490.2).

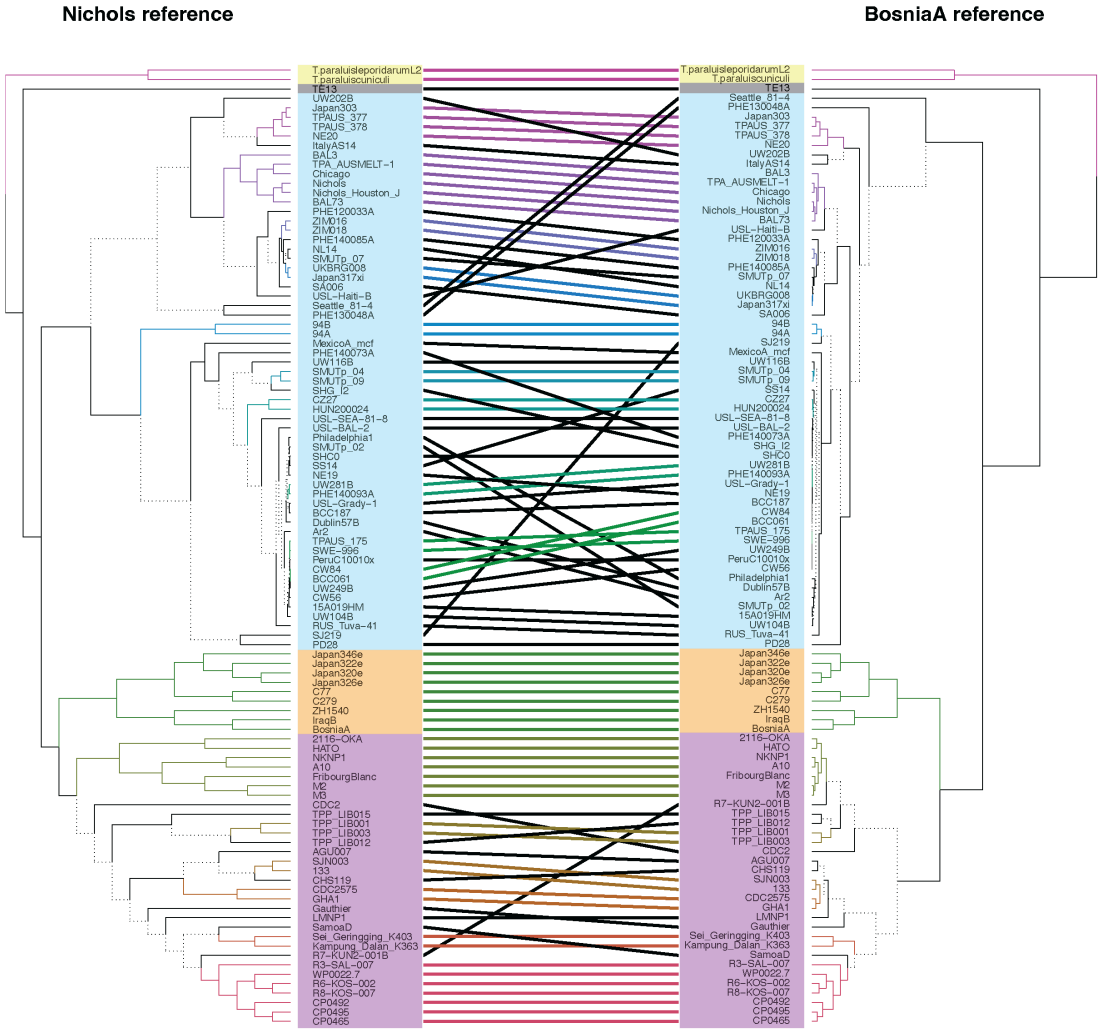



**Supplementary Figure 5.S5.** Maximum likelihood phylogeny using all variants. Maximum likelihood phylogeny of the curated dataset (Table 5.S4, Table 5.S5) of 101 genomes constructed using 10,499 variant positions against the TPA Nichols reference (NC_021490.2). Ancient genomes are written in red, TE13 is denoted with a *. The tree was computed with RAxML-NG using a GTR-G substitution model. Bootstrap support values were computed using 1000 iterations.

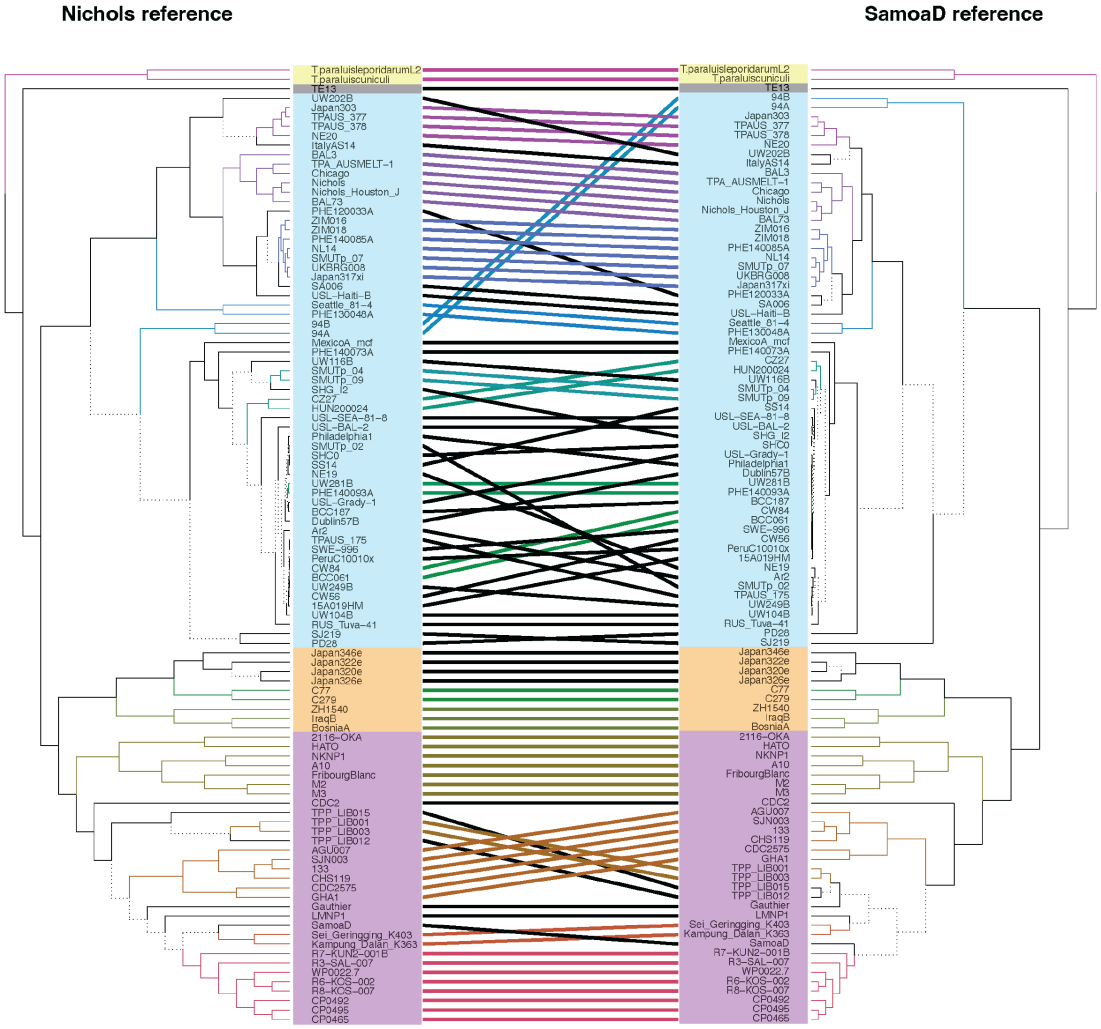

**5.S6a)** **5.S6b)**

**Supplementary Figure 5.S6.** Maximum Parsimony trees with and without regions removed. Both trees were obtained using the Subtree-Pruning-Regrafting algorithm in MEGA X with search level 1 in which initial trees were obtained by the random addition of sequences (10 replicates). Branch lengths were calculated using the average pathway method. **a)** The dataset using all sites (called at 3x coverage) was used. The most parsimonious tree is shown (length= 11311). There were a total of 10,499 sites used. **b)** The dataset with specified regions removed (Table 5.S6) and

called at 3x coverage was used. The most parsimonious tree is shown (length= 8768). There were a total of 8,582 sites used.




**5.S7a)**


Nichols reference
SamoaD reference
T.paraluisleporidarumL2
T.paraluiscuniculi
TE13
UW202B
Japan303
TPAUS_377
TPAUS_378
NE20
ItalyAS14
BAL3
TPA_AUSMELT-1
Chicago
Nichols
Nichols_Houston_J
BAL73
PHE120033A
ZIM016
ZIM018
PHE140085A
NL14
SMUTp_07
UKBRG008
Japan317xi
SA006
USL-Haiti-B
Seattle_81-4
PHE130046A
94B
94A
MexicoA_mcf
PHE140073A
UW116B
SMUTp_04
SMUTp_09
SHG_I2
CZ27
HUN200024
USL-SEA-81-8
USL-BAL-2
Philadelphia1
SMUTp_02
SHC0
SS14
NE19
UW281B
PHE140093A
USL-Grady-1
BCC187
Dublin57B
Af2
TPAUS_175
SWE-996
PeruC10010x
CW84
BCC061
UW249B
CW56
15A019HM
UW104B
RUS_Tuva-41
SJ219
PD28
Japan346e
Japan322e
Japan320e
Japan326e
C77
C279
ZH1540
IraqB
BosniaA
2116-OKA
HATO
NKNP1
A10
FribourgBlanc
M2
M3
CDC2
TPP_LIB015
TPP_LIB001
TPP_LIB003
TPP_LIB012
AGU007
SJN003
133
CHS119
CDC2575
GHA1
Gauthier
LMNP1
SamoaD
Sei_Geringging_K403
Kampung_Dalan_K363
R7-KUN2-001B
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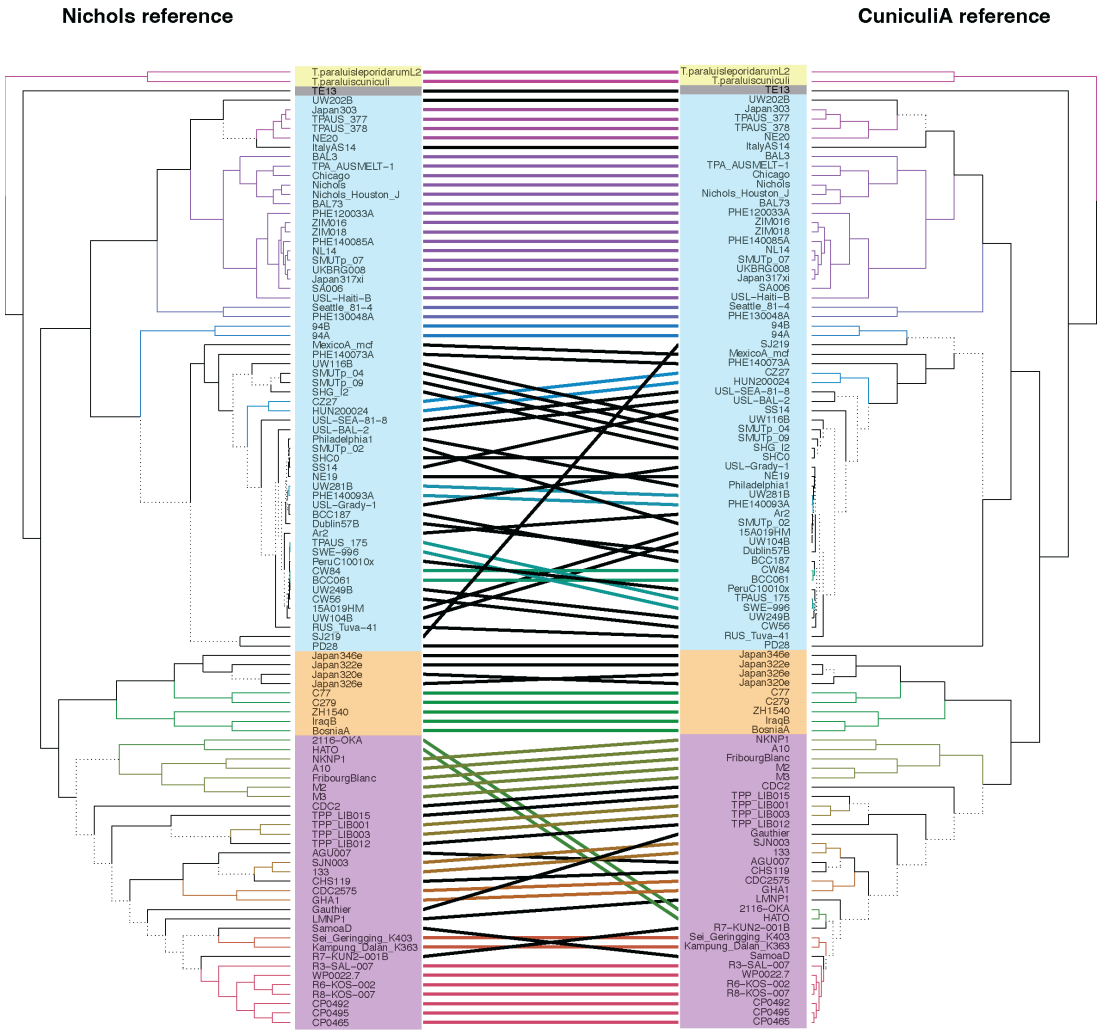
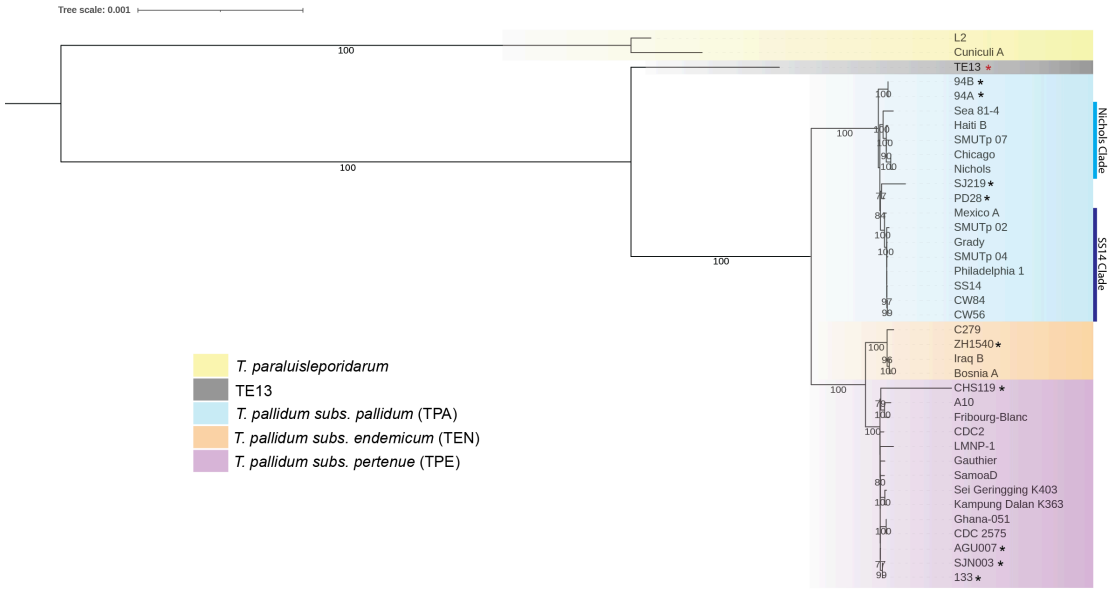
R6-KOS-002
R8-KOS-007
CP0492
CP0496
CP0465

**5.S7b)**

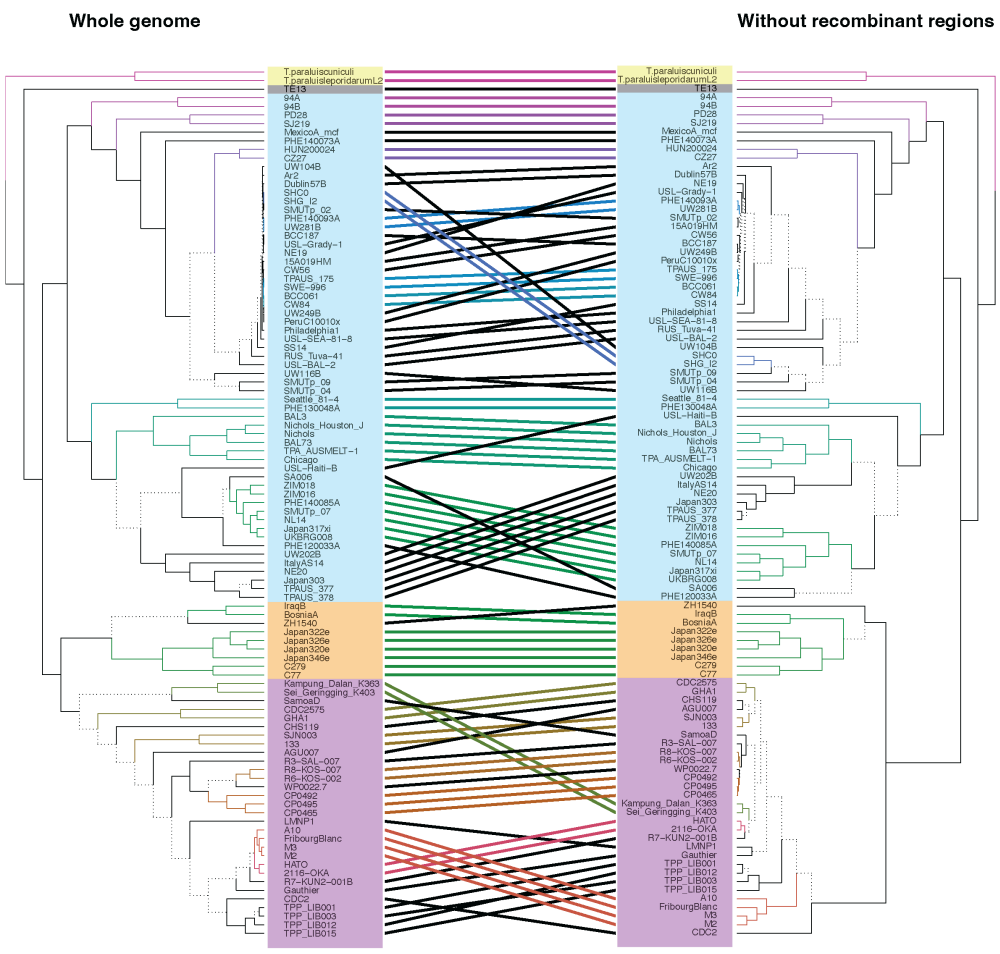



**5.S7c)**

**Supplementary Figure 5.S7.** Maximum likelihood phylogenies: effects of different references. The effects of aligning to different references are compared. For each phylogeny, the entire dataset was mapped to the reference listed. **a)** SNP-based maximum likelihood phylogenies using the Nichols (NC_021490.2) and BosniaA (NZ_CP007548.1) references are compared. **b)** SNP-based maximum likelihood phylogenies using the Nichols (NC_021490.2) and SamoaD (NC_016842.1) references are compared. **c)** SNP-based maximum likelihood phylogenies using the Nichols (NC_021490.2) and *T. paraluisleporidarum* str Cuniculi A (NC_015714.1) references are compared.




**Supplementary Figure 5.S8.** Whole genomeMSA-based maximum likelihood phylogenetic tree. Maximum likelihood phylogenetic tree built from a whole-genome multiple sequence alignment (MSA). Assemblies for the modern genomes were retrieved from NCBI. Ancient genomes were mapped to their closest modern reference. A consensus sequence was then inferred and aligned with the modern ones (see methods for details). Regions identified as recombinant were removed from the alignment. Established T. pallidum subspecies clades are highlighted in different colors. Within-TPA subspecies clades are reported as side bars. Ancient genomes are indicated with an asterisk "*" next to their label. TE13 is marked with a red asterisk. Bootstrap support values within the range (71-100) are displayed. This phylogenetic tree is mostly consistent with the one inferred with the reads mapping-based approach (see Figure 5.3, Figure 5.S5, and Figure 5.S7). Each genome is assigned to the expected subspecies and TE13 is positioned basal to the T. pallidum clade with 100 bootstrap value. Some differences can be observed in the topology of the subspecies clades. Specifically, we observe that the ancient genomes 94A and 94B are in a more basal position compared to the SNP-based tree, diverging before the split of the Nichols and the SS14 clade instead of being in a basal position to the SS14 clade only (see Figure 5.3). We also observe changes in the placement of other ancient genomes as ZH1540 (found in a less basal position) and CHS119. We note that some of the ancient genomes, specifically the ones with lower coverage (SJ219: ~1.1X and CHS119: ~2.4X), exhibit longer branch lengths.


Whole genome
Without recombinant regions
T.paraluiscuniculi
T.paraluisleporidarumL2
TE13
94A
94B
PD28
SJ219
MexicoA_mcf
PHE140073A
HUN200024
CZ27
UW104B
Ar2
Dublin57B
SHC0
SHG_I2
SMUTp_02
PHE140093A
UW281B
BCC187
USL-Grady-1
NE19
15A019HM
CW56
TPAUS_175
SWE-996
BCC061
CW84
UW249B
PeruC10010x
Philadelphia1
USL-SEA-81-8
SS14
RUS_Tuva-41
USL-BAL-2
UW116B
SMUTp_09
SMUTp_04
Seattle_81-4
PHE130048A
BAL3
Nichols_Houston_J
Nichols
BAL73
TPA_AUSMELT-1
Chicago
USL-Haiti-B
SA006
ZIM018
ZIM016
PHE140085A
SMUTp_07
NL14
Japan317xi
UKBRG008
PHE120033A
UW202B
ItalyAS14
NE20
Japan303
TPAUS_377
TPAUS_378
IraqB
BosniaA
ZH1540
Japan322e
Japan326e
Japan320e
Japan346e
C279
C77
Kampung_Dalan_K363
Sei_Geringging_K403
SamoaD
CDC2575
GHA1
CHS119
SJN003
133
AGU007
R3-SAL-007
R8-KOS-007
R6-KOS-002
WP0022.7
CP0492
CP0495
CP0465
LMNP1
A10
FribourgBlanc
M3
M2
HATO
2116-OKA
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CDC2
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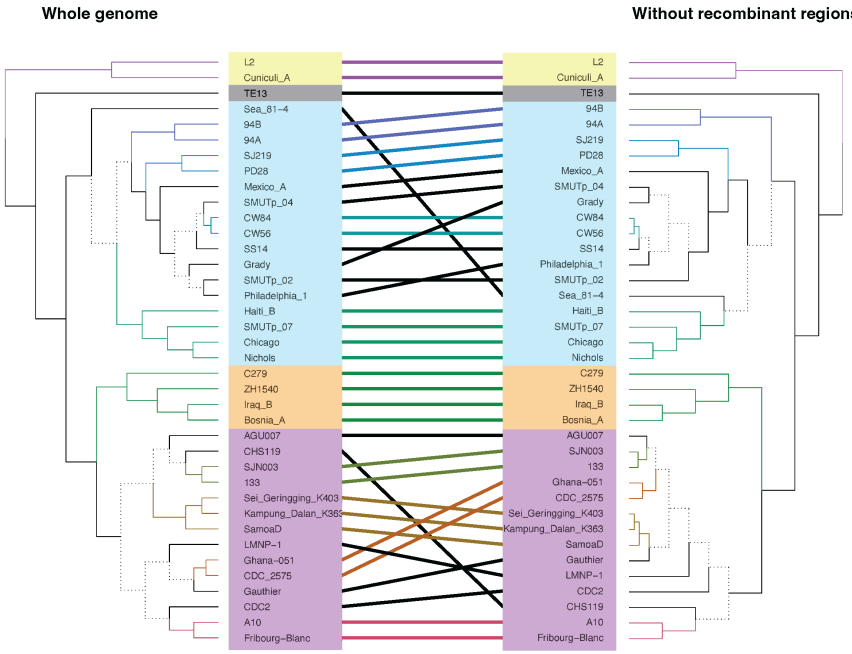
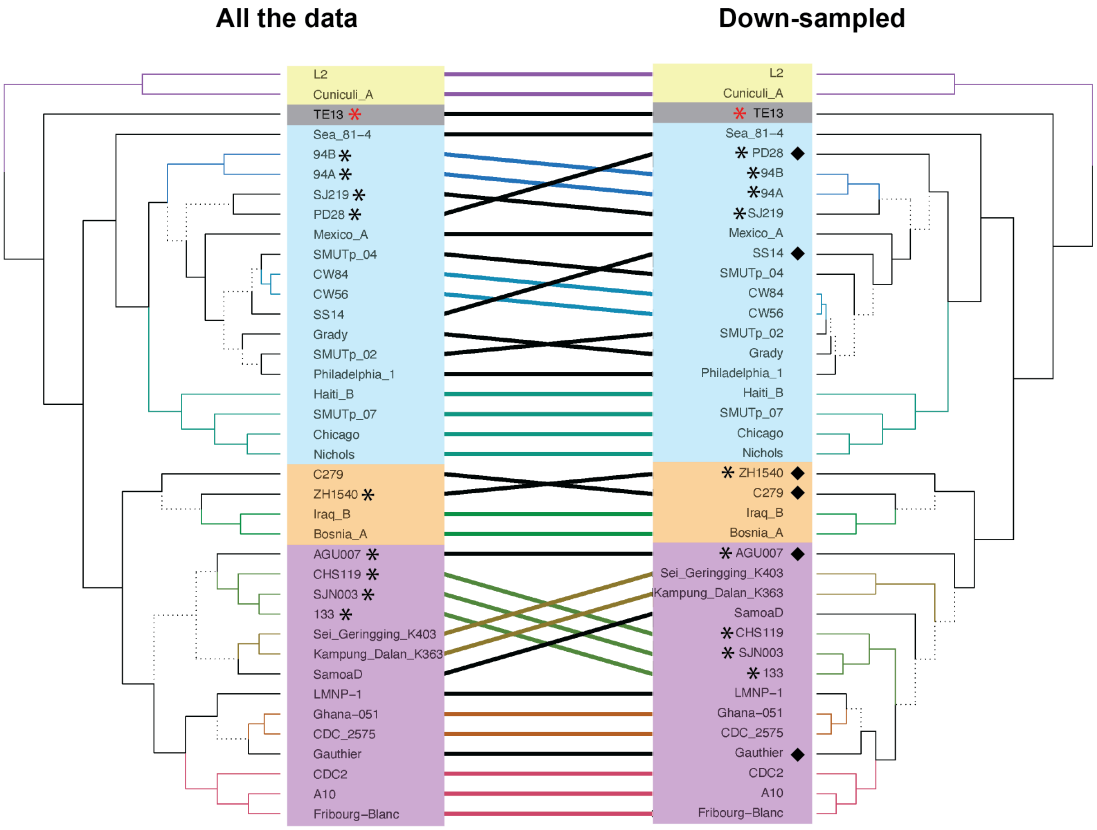
TPP_LIB003
TPP_LIB012
TPP_LIB015

**5.S9a)**

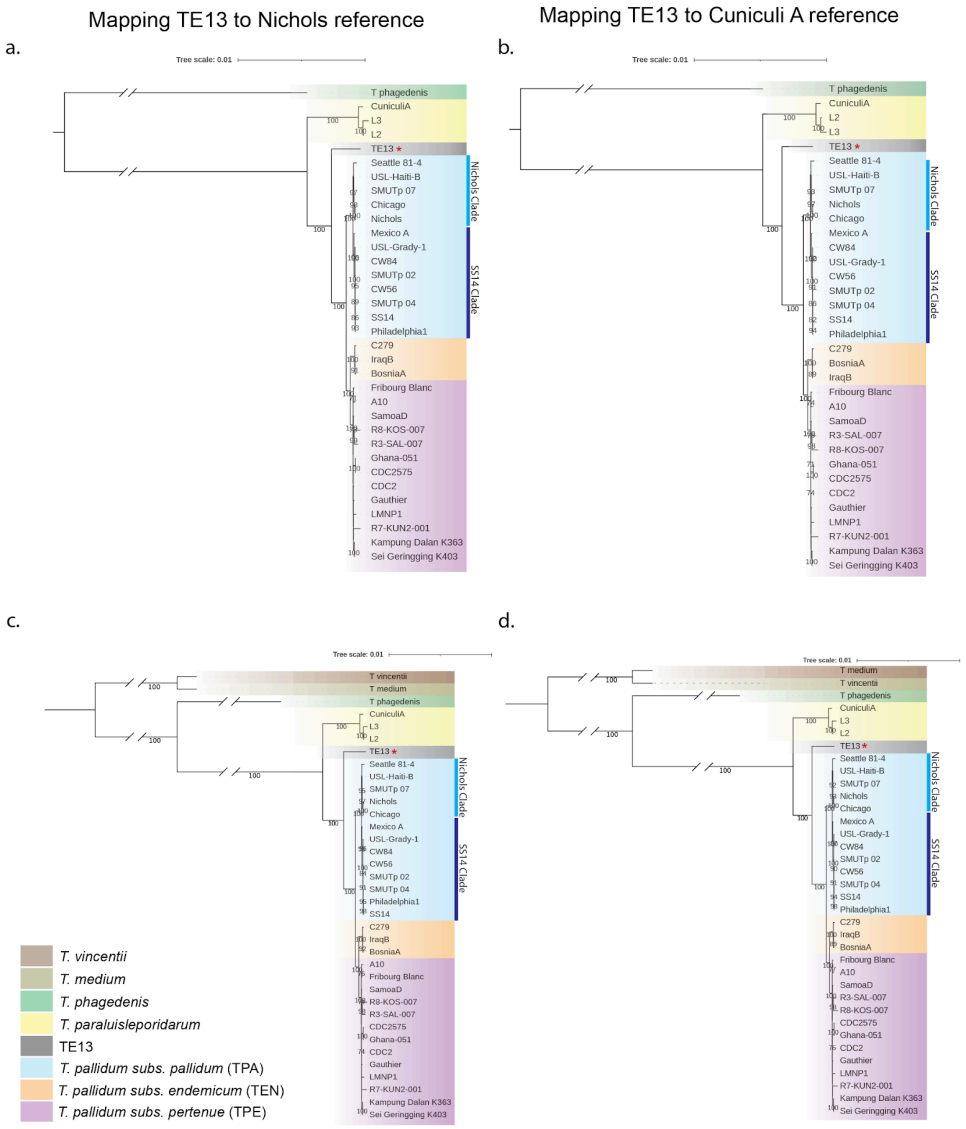



**5.S9b)**

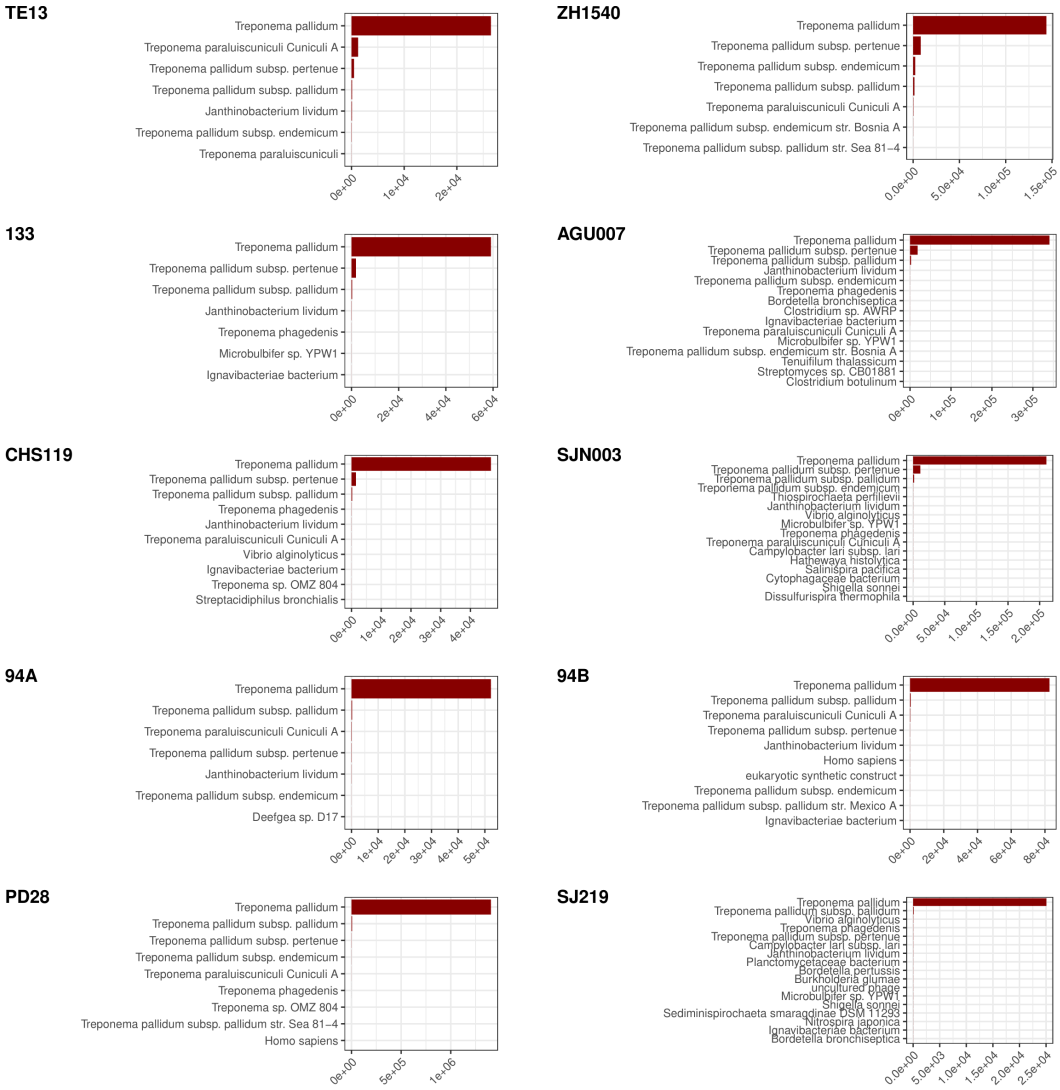
**Supplementary Figure 5.S9.** Tree topology comparison: the effect of recombination. Comparison of the tree topology when keeping (left) or removing (right) the recombinant regions in the genome. Phylogenies computed from **a)** the mapping-based phylogeny (Nichols reference) and **b)** the whole-genome MSA. The phylogenetic trees are represented as cladograms with the clades conserved between the two phylogenies without rearrangements highlighted with a color-code. Nodes that change in the two topologies are plotted as dashed lines in the cladograms. The horizontal lines link each specific genome between the two topologies. As for the cladogram clades, colored horizontal lines identify the conserved clades between the compared trees. Black lines identify genomes whose position changes in the compared trees or single-genome clades (e.g. TE13). Genomes labels are color-coded depending on their species or sub-species with the same color-coding used in the phylogenetic tree (see Figure 5.3).



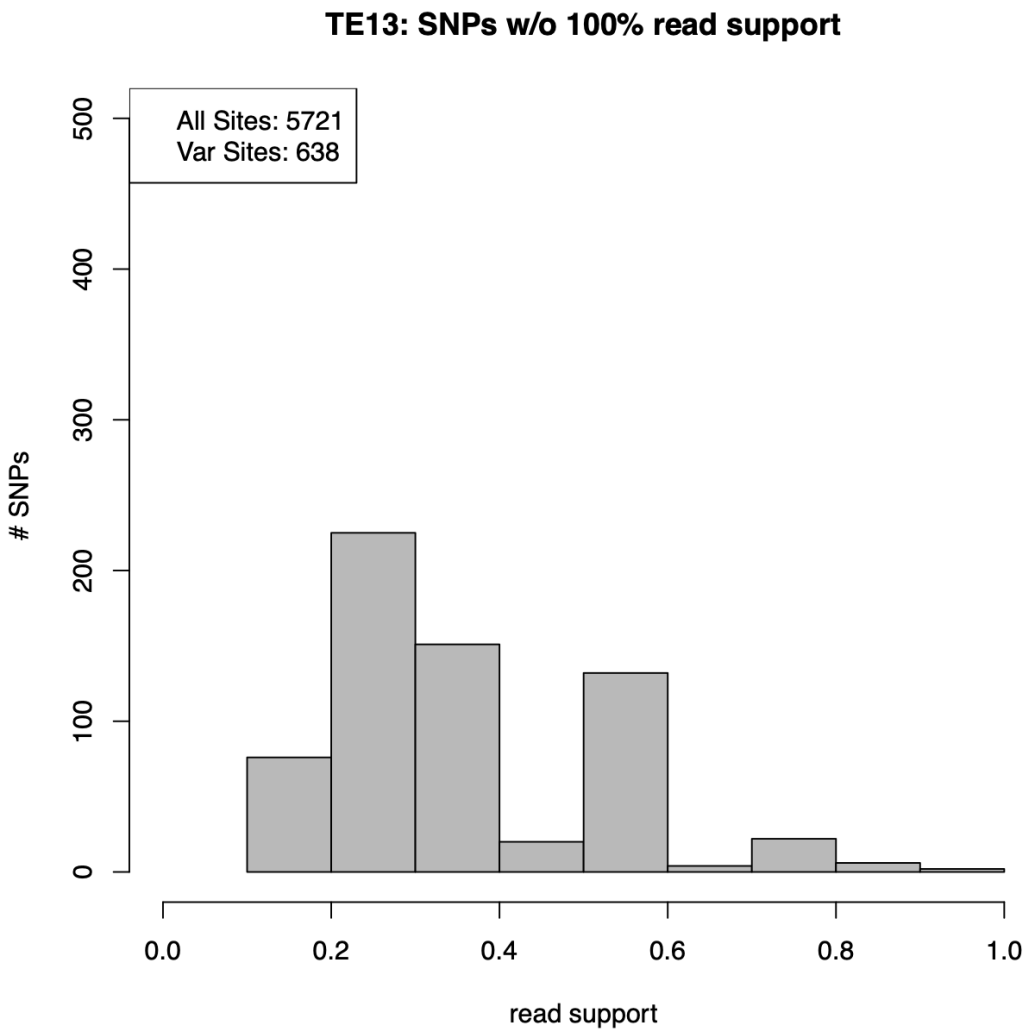

**Supplementary Figure 5.S10.** Tree topology comparison: the effect of low coverage. Comparison of the tree topology when down-sampling other modern and ancient genomes to the same depth observed in TE13. Genomes marked with a black diamond asterisk on the cladogram on the right were down sampled to ~1.7X coverage before mapping. Two high-coverage (>9X) genomes per subspecies (one modern and one ancient) were selected for the experiment. The phylogenies were computed from the whole-genome MSA. The phylogenetic trees are represented as cladograms with the clades conserved between the two phylogenies without rearrangements highlighted with a color-code. Nodes that change in the two topologies are plotted as dashed lines in the cladograms. The horizontal lines link each specific genome between the two topologies. As for the cladogram clades, colored horizontal lines identify the conserved clades between the compared trees. Black lines identify genomes whose position changes in the compared trees or single-genome clades (e.g. TE13). Genomes labels are color-coded depending on their species or sub-species with the same color-coding used in the phylogenetic tree (see Figure 5.3). Ancient genomes are marked with asterisk "*".




**Supplementary Figure 5.S11.** Maximum likelihood phylogeny based on single copy orthologous genes. Phylogenetic trees obtained from the concatenate multiple sequence alignments of single copy orthologous genes when mapping TE13 to the T. pallidum Nichols reference **(a and c)** or to the T. paraluisleporidarium Cuniculi A reference **(b and d)**, when including one **(a and b)** or more outgroups **(c and d)**. TE13 has the same position within the phylogeny in all the analyses, being closer to T. pallidum rather than T. paraluisleporidarium and representing an outgroup to the entire T. pallidum clade. This positioning is supported with high bootstrap support independently of the reference used for the mapping or the number of outgroup species included. Bootstrap support values within the range (71-100) are displayed.




**Supplementary Figure 5.S12.** BLAST confirmation of mapped reads. BLASTn results for the mapped reads for each ancient sample included in the analyses. For each ancient genome included in the study we BLASTed all the mapped reads against the nt database. For each read the best-hit was retrieved and the total number of reads assigned to a given taxa were plotted as a histogram. Only taxa with more than 10 reads are visualized.




**Supplementary Figure 5.S13.** Bar chart of heterozygous sites.

**Supplementary Table 5.S1**. Radiocarbon dating data.

**Supplementary Table 5.S2**. Competitive mapping results, displaying the number of unique, quality filtered reads assigned to each genome, the percent of the corresponding reference these reads cover, and the depths of coverage.

**Supplementary Table 5.S3**. Variants present in TE13 when compared to various references.

**Supplementary Table 5.S4**. Dataset metadata.

**Supplementary Table 5.S5**. Accession numbers for the sequencing data files of other published ancient genomes that were merged together.

**Supplementary Table 5.S6**. Regions removed in specified analyses.

**Supplementary Table 5.S7**. A comprehensive list of virulence genes specific to T. pallidum, compiled from literature.

**Supplementary Table 5.S8**. SnpEff results.

**Supplementary Table 5.S9**. SNP table of variant sites across the whole dataset when mapped to the *T. pallidum* subsp. *pallidum* Nichols reference.

**Supplementary Table 5.S10**. Variants shared between clades.

**Supplementary Table 5.S11**. List of assemblies used in MSA phylogeny and orthology-based rooting analysis.

## References

Allison, M. J., Aste, G. F., Gerszten, E., Fouant, M., & Cebelin, M. (1982). La sífilis ¿ una enfermedad americana? *Chungara: Revista de Antropología Chilena, 9*, 275–283. https://www.jstor.org/stable/27801756

Archila, S., Groot, A. M., Ospina, J. P., Mejía, M., & Zorro, C. (2020). Dwelling the hill: Traces of increasing sedentism in hunter-gatherers societies at Checua site, Colombia (9500-5052 cal BP). *Quaternary International, 578*, 102–119.

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As a coauthor of the manuscript "Analysis of a 5,500-year-old *Treponema pallidum*-like genome from Colombia", I hereby grant permission for this current version of the manuscript to be included in Nasreen Broomandkhoshbacht's dissertation.

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## Chapter 6: Of Shoes and Ships and Sealing Wax
## Advances in the Retrieval of Ancient DNA from Non-Traditional DNA Sources

### Introduction

Recent research trends in the ancient DNA field have moved towards method innovation and optimization. In the past, much of the research was PCR-based, generally focused on short regions of mitochondrial genomes (Hagelberg et al., 2015). It was necessary to destroy large amounts of bone powder to obtain data from ancient bones, and it was highly prone to failure (Atmore et al., 2023; Hagelberg et al., 2015; Jeffreys, 1984). If the segment of the genome specified by primers is not preserved in an extract (or if there is no intact molecule spanning the entire length from primer to primer), the experiment will yield no data. Data from PCR-based methods is limited in its ability to answer large-scale questions, and obtaining this data can be laborious. While DNA immortalized as a library can be continually re-amplified, PCR methods generally use DNA extracts directly as input; once that extract is used up, researchers have to go back to the original source material to extract more. The advent of next-generation sequencing in the 2000s has seen most ancient DNA researchers move over to high-throughput techniques (Orlando et al., 2021), although the ease and low cost of PCR means some researchers in underfunded labs still rely on those older methods. However, if researchers can afford the larger up-front cost, next-generation methods can be more cost-efficient in the long run due to the lower per-base sequencing cost. Next-generation methods often have a higher success rate in obtaining endogenous DNA (that is, DNA actually from

the sample), can amplify all DNA present rather than one specified region, and make it much easier to validate damage patterns and contamination rates (Orlando et al., 2021). Partial treatment of DNA libraries with uracil-DNA glycosylase to repair characteristic C-to-T damage along the length of the molecules while excluding the bases at the ends is an innovation that allows more accurate base calls while retaining damage on the ends for confirmation that the reads are truly ancient (Rohland et al., 2015). More recently, the move from double-stranded to single-stranded DNA library preparation methods has further improved complexity by making it possible to retain even more DNA molecules than before, as double-stranded methods do not convert single-stranded molecules or overhangs into readable molecules (Gansauge & Meyer, 2013; Kapp et al., 2021). Additionally, extraction buffers optimized for ancient DNA research can now target the short and even ultra-short molecules characteristic of ancient DNA (Dabney et al., 2013; Glocke & Meyer, 2017). Together, these innovations have made the exploration of our field past PCR possible.

In the past decade, researchers have also focused on getting the best-quality data in the least-destructive manner. The discovery that the petrous portion of the temporal bone (particularly the cochlea, ossicles, and semi-circular canals) produced higher quality data with a higher endogenous DNA percentage than even teeth in most cases (which are now considered the next-best source) led to these portions being haphazardly and completely destroyed for analysis (Charlton et al., 2019; Hansen et al., 2017; Pinhasi et al., 2015; Sirak et. al, 2020). In response to this, Sirak et al. (2017) developed a cranial-base drilling method to collect bone powder from the

human petrous, leaving the skull intact and only leaving a small hole underneath, minimizing invasiveness while still maximizing data quality. Other researchers have attempted to further reduce the destruction of tooth roots and bones from drilling or cut-and-mill methods by developing minimally-invasive extraction methods where the sample of interest is dipped into the extraction buffer and allowed to soak for a few hours (Harney et al., 2021; Tejero et al., 2023). This results in usable extracts and no destruction of the sample visible to the naked eye, as only a fraction of a millimeter of the outermost layer of the bone or cementum is dissolved by the extraction buffer (Harney et al., 2021; Tejero et al., 2023). While minimally-invasive methods such as these "dip" extractions (Harney et al., 2021; Tejero et al., 2023) or carefully drilling miniscule amounts of bone powder can be useful, sometimes the use of bones or teeth is not permissible or remains are simply not available. Additionally, there are research questions that may require researchers to search for DNA in unexpected places. In addition to introducing a fraction of relevant research and possibilities this field creates, in this chapter I will explore the benefits, downsides, ethical concerns, and legal considerations of using non-traditional DNA sources for ancient DNA research.

**Relevant Research**

As an undergraduate, my first ancient DNA research experience was assisting Dr. Meradeth Snow with a project involving a non-traditional DNA source. For this project, we attempted to extract DNA from the microcracks of lithic tools from

Bridge River, an archaeological site located in British Columbia dating at least as far back as ~1800 BP (Edwards et al., 2022; Prentiss et al., 2008). Lithic tools were used in the past for a number of things–butchering meat, processing plants, scraping hides and more (Odell, 2004). During that usage, DNA from these plants and animals hypothetically would soak in and become trapped in the microcracks of the lithic tools (Shanks et al., 2001). The DNA is then hypothetically protected in the lithic matrix, and remains there even after archaeologists wash the tools after excavation hundreds or thousands of years later (Green & Speller, 2017; Shanks et al., 2001; Shanks et al., 2005). To extract this DNA, scientists must ultrasonicate the lithic tools in a solution (such as ammonium hydroxide) to coax the DNA out of the microcracks and create a DNA extract (Shanks et al., 2001). At this point, one could use these extracts like any other, for example to target something general like cytochrome B with PCR or to build next-generation sequencing libraries for metabarcoding or shotgun sequencing. Proof-of-concept experiments using both ancient tools (Hardy et al., 1997; Loy, 1993; Loy & Dixon, 1998; Shanks et al., 2005) and experimental archaeological methods (Hardy et al., 1997; Shanks et al., 2001; Shanks et al., 2004) were published in the 90s and early 2000s, but due to debate about the authenticity of results, the method fell into relative obscurity after these publications (Green & Speller, 2017).

The initial experiment I assisted with was not successful in amplifying DNA, which could have been for a number of reasons; for example, the methodological instructions were not clearly described at points in the publications, we did not have

access to all the same machinery, and our experiment was PCR-based. However, when adjustments were made and Dr. Snow and her team repeated the experiment, they successfully amplified mountain lion DNA from a 1300-year-old scraper (Super, 2017). Archaeological and traditional knowledge indicate mountain lion hides may have been highly prized in the community living at Bridge River at the time these lithics were used (Super, 2017), which supports the authenticity of their finding and the validity of the method. Using lithic tools as an alternative to bones expanded the possibilities in my mind of what constitutes a DNA "source" and shifted my paradigm of how ancient DNA research "should" be conducted. In truth, there are so many other possibilities that are often ignored.

In addition to obtaining aDNA data from lithic tools, scientists in this area of research have gleaned a wide variety of data from unexpected sources. Starting on the more "traditional" end of the spectrum, we have non-traditional sources that are closely affiliated with the host of interest's body. Many researchers have used dental calculus and coprolites from ancient humans to retrieve host DNA as well as microbiome, pathogen and host diet DNA for study (Gilbert et al., 2008; Jenkins et al., 2012; Warinner et al., 2014; Weyrich et al., 2017; Witt et al., 2021). The use of dental calculus in particular has become commonplace. Locks of hair have also been used as sources of host and pathogen DNA, such as in the case where multiple locks attributed to Ludvig van Beethoven were used as DNA sources (Begg et al., 2023). The researchers were able to authenticate a number of the locks as actually belonging to Beethoven, but also were able to reconstruct a Hepatitis B Virus genome, providing

an answer to some longstanding questions about his health (Begg et al., 2023). In other cases, researchers have delved even further into unexpected territory. For example, DNA from scabs and dried lymph in an American Civil War era smallpox vaccination kit were sequenced to reveal the genome of the virus used to vaccinate, as well as the genomes of the scab and lymph donors, the people used to propagate the vaccine (Duggan et al., 2020). While the aforementioned DNA sources are primarily affiliated with the human body in most existing research, these types of samples could potentially be affiliated with other animals, and there are also items of material culture used in a similar manner. For example, historic shipwreck timbers have been analyzed in an attempt to determine the geographic origin of the wood (Speirs et al., 2009), and various pieces of parchment have been analyzed to determine what species' skin it was made of (Campana et al., 2010; Pangallo et al., 2010; Teasdale et al., 2017).

There are also DNA sources that are completely unaffiliated with a host, but came into contact with the species of interest. The most commonly used DNA source in this category is soil or sediment. With the rise of environmental DNA research, ancient DNA researchers have pursued similar sources to obtain ancient DNA. Research in this area has grown quickly in popularity for a variety of reasons, such as the ease of finding samples and the hypothesized better preservation of DNA in sediment through adsorption (Kjær et al., 2022; Sand et al., 2024). Investigations of DNA in ancient sediments have led to discoveries about population connections (Pedersen et al., 2021) and even the presence of previously unknown lineages (Kjær

et al., 2022). Data from these sources also often tell us something about the ecology of that place and time (Courtin et al., 2022; Dussex et al., 2021), which is a much broader viewpoint than traditional ancient DNA sources allow. However, sediment is not the only substrate that can harbor information about local ecosystems. A study of DNA in ancient sealing wax suggests beeswax seals may also harbor DNA from plants in the ecosystems they were made in (Kasso et al., 2023). Little pollen was actually present in the seals, but they were able to successfully sequence ancient DNA from multiple plant species. These seals were made of beeswax and pressed into shape by humans, so the authors originally embarked on the project in an attempt to amplify DNA from humans and honeybees. While the authors of that study lamented the lack of honeybee and human DNA amplified from the beeswax seals (Kasso et al., 2023), the presence of over 5k honeybee reads and 13k human reads in their relatively low-coverage sequencing data in fact point to this being a viable source of trace DNA from both species, albeit not the most DNA-rich.

Dental calculus and coprolites have already been mentioned as sources of both human and human-associated microbiome DNA, but it would be a mistake to overlook chewed materials as a DNA source as well. In one study, birch pitch used as chewing gum yielded a human genome and associated oral microbiome data (Jensen et al., 2019), and chewed yucca fibers (known as quids) have also been used as a source of human DNA (Hamilton-Brehm et al., 2018; LeBlanc et al., 2007).

Pottery is another category of items that has significant potential as a DNA source. Pottery, especially un-glazed pottery, has the potential for residues of food and

other substances to be left behind on it. Chemical analysis of residues in pottery from many regions have indicated the presence of medicinal and psychoactive compounds, so DNA analysis could identify which plants specifically were used to create those medicines. In this same manner, blood residue from ritual sacrifice was identified in ancient Mayan pottery (Matheson et al., 2009), which could also potentially be used as a DNA source. However, the more common use of pottery in DNA analysis has been regarding food residue. Residues of food in archaeological pottery, such as amphorae from ancient shipwrecks (Foley et al., 2012; Hansson & Foley, 2008) have yielded DNA, enabling researchers to approach questions regarding diets and trade.

**Unpublished Work**

Two projects using non-traditional DNA sources I undertook during the course of my PhD were the Promontory Caves project and a project using DNA from Peruvian textiles. A more detailed description of the Promontory Caves project and its implications can be found in Chapter 7 of this dissertation, but in short, we demonstrated that it is possible to get ancient trace human DNA out of woven mats, cordage and moccasins. It makes sense: both in the creation and use of these materials, skin cells–which contain DNA– are shed onto it. In this vein, a recently published study retrieved a human genome from an ancient pendant, presumably from the wearer (Essel et al., 2023), further supporting the authenticity of this proposed mechanism of DNA deposition. This presumably also holds true for other high-touch articles, such as other types of footwear and textiles.

While knowing this, the other project, the one investigating Peruvian textiles, was not embarked upon in an attempt to obtain human trace DNA. This project was instead focused on the textile materials themselves–animal hairs and plant fibers. In attempting to create a universal extraction buffer for textiles that works equally well for animal hairs and plant fibers, we ended up with data from multiple ancient Peruvian textiles made of plant fibers, animal hairs, or a mixture of both. This method would enable researchers to sequence materials used in textile production to learn about species usage or even potentially answering larger questions about population genomics of the species used (to research the domestication of cotton, for example) to create textiles while only destroying small amounts of textile instead of faunal or plant remains (which may not have survived the archaeological record). We ran this data through our screening pipeline and found human and microbial reads as well, although the data is such low coverage and poor quality that we are currently unable to tell if these are truly ancient reads, mismapped reads, or from modern contamination (Valle et al., 2023). While the microbial data from our textile project is not high-coverage enough to draw any conclusions, textiles in the future could serve as a source of pathogen DNA to monitor disease presence in a community or even examine the deliberate introduction of diseases, as in the case with so-called "smallpox blankets" (Fenn, 2000).

**The Good, the Bad, and the Ugly**

Digesting the studies listed above, it is clear that DNA retrieval from non-traditional source materials has numerous benefits. It makes ancient DNA

research possible when more traditional ancient DNA sources (such as ancestors' bones or faunal remains) no longer exist, are not available, or when cultural and religious beliefs of research-minded descendant groups do not permit the disturbance or destruction of ancestors. It allows us to ask many targeted questions that cannot be answered using data from traditional ancient DNA sources and broadens the possibilities of research in our minds, allowing researchers and community stakeholders to work together creatively to design research projects of interest to all parties. And of course, sometimes the DNA from these sources could hypothetically even be better preserved, like it has been proposed in the case of ancient DNA from sediment (Kjær et al., 2022; Sand et al., 2024).

While DNA from non-traditional sources can hypothetically be better preserved in some instances, it is often of much worse quality than DNA retrieved from ancient bone, tissue, or plant remains (Green & Speller, 2017). This means there tend to be higher damage rates, shorter reads, and lower percentages of on-target DNA. Other downsides include higher costs (due to specialized extraction methods and the increased amount of sequencing required) and potentially higher rates of contamination. Archaeologists and museum curators often do not take the same level of contamination precautions with objects they do not view as DNA sources. Unfortunately, DNA can obtain damage in as little as a few years, meaning that direct skin contact with a non-traditional DNA source during excavation or in a museum a decade earlier can cause contaminating DNA to contain "ancient" characteristics (Zimmermann et al., 2008). While these issues should be considered, the biggest

potential disadvantage of this type of research is, of course, the sticky ethical situation it can create.

The potential ethical issues with non-traditional DNA sources are concerning. Some researchers may use these methods as a way to skirt consultation or get around things like NAGPRA when in reality, the communities of interest do not want their ancestors' DNA investigated at all. DNA is highly personal information, which is one of many reasons why modern genomics research on live humans requires measures like IRB approval and in-depth consent forms (Matrana & Campbell, 2020). Ancient people cannot sign such consent forms (which is a broader ethical quandary with ancient DNA), but consultation with (or better yet, doing research in service of) appropriate descendant communities often can serve as an acceptable proxy. When researchers aim to obtain ancestors' DNA from non-bone sources in an attempt to evade consultation requirements, they are engaging in not only colonial practices, but practices that should arguably be considered criminal. DNA is also shared between relatives, so sharing your own genetic data means you are also sharing portions of your relatives' data as well. This has been a topic of ethics discussions recently in the wake of forensic cases like the Golden State Killer, which was solved using trace DNA and direct-to-consumer DNA testing data (Lund, 2020; Maher, 2023). While humans in ancient DNA studies are generally not close relatives to living people, they're still relatives both in the senses of kinship and sociocultural traditions, and the use of their data affects the descendant community at-large (Tsosie et al., 2020). If the data can be used to say something about a community's ancestors, the appropriate

community should be fully informed and hold the power to make decisions about the research. The use of non-human DNA also does not exempt researchers from engagement and collaboration with Indigenous communities of the region–there are many animals, plants, and fungi, and ecosystems as a whole that hold an important space in many worldviews, and "progress" should not supersede consideration of those voices and values. In addition to purely ethical concerns, there are also legal implications to consider. For example, the Navajo Nation has banned any and all genetics research regarding them (Claw et al., 2021)–if researchers were to obtain Navajo ancestors' DNA from something like high-touch artifacts, it could open them up to a lawsuit. In short, the impacts of potential results and the research itself should always be carefully considered by all stakeholders before starting.

**Moving Forward**

Non-traditional sources of ancient DNA have many advantages, but even more caveats to take into consideration, especially when it comes to questions of ethics. Because these non-traditional sources are not usually thought of as sources of DNA, they may be overlooked when it comes to research approval, so it is the responsibility of the researcher to seek out the appropriate community stakeholders, even when not legally required. If ancient DNA researchers are serious about moving towards community-led research, they need to start listening to communities and providing them with clear and complete information about their options. When communities are interested in genomics research but do not want to use human

remains, these non-traditional sources are options that could be presented to them. However, it is important to also be clear about the limitations that accompany the use of those sources. Research questions are important to consider when determining if a DNA source is actually appropriate, and all possible outcomes should be discussed. The reasoning behind decisions is also an important thing to keep in mind. For example, suppose a descendant group rejects a proposed project using their ancestors' remains because they oppose genomics research as a whole. In that case, researchers should not pivot to testing soil or sediment cores for human DNA–the reasoning behind the refusal is more important than whether or not something is technically legal. Descendant communities should be advised not to approve these types of research if all possible outcomes are not thought through and researchers should be careful to communicate this type of information to descendant groups as well. Consent from all stakeholders should be specific, unambiguously positive, and freely given, not coerced.

With these nontraditional DNA sources, scientists can get creative. That creativity opens doors to questions we never even thought to ask. We no longer need to disturb burials or destroy human remains to answer questions, and we now have the ability to tackle questions that bones could not answer in the first place. Say you're interested in the genetic disorders of a royal family, but you do not have access to the remains of anyone from the family. Or perhaps you're interested in the domestication-affiliated changes in Brassica species consumed in different time periods, but plant matter does not preserve well in the archaeological record at the site

of interest. In these cases, non-traditional DNA sources such as preserved royal shoes or wood from barrels used to ferment sauerkraut could come in handy. To poorly co-opt a Lewis Carroll poem: DNA, the scientists said, can come from many things: from shoes and ships and sealing wax– cabbages and kings not required.

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**Pages 239-281 redacted due to the sensitive nature of the data.**
**Chapter may be released following tribal approval.**

## Chapter 8: Conclusion

This dissertation touches on topics of colonialism, diseases, and the way these interact. The topics of the chapters alone may initially seem disjointed, but we see these themes tie them together. These issues are interconnected, and there are other related facets that have yet to be fully explored.

While readers may not be able to read Chapter 7 due to the embargo, the important takeaway is that the method of retrieving authentic ancient DNA from high-touch items is sound–we were able to detect human DNA with damage characteristics and fragment length that indicate it is ancient. While it is possible some or all of the DNA is contamination from excavators handling the artifacts a decade prior, in all likelihood at least some of it is authentic ancient DNA from the people who used, wore, or made the moccasins, cordage, and mat. As discussed in Chapter 6, these and other high-touch materials should be considered as sources of trace ancient DNA in the future and presented to descendant communities as an alternative to the destruction of ancestors' bones. However, it is also important to convey that the data produced from these types of alternative sources will likely be of lesser quality, and therefore may not be able to answer all of the questions they may have. Offering this as an alternative method provides descendant groups with options; this can fit into a decolonial research paradigm (as discussed in Chapter 2) that centers the agency of descendants and redistributes power.

In Chapters 3 and 5, I used contextual information to enrich our understanding of certain pathogens. Chapter 5 focuses on one specific ancient pathogen, a

*Treponema pallidum*-like bacterial lineage. This finding shows that *Treponema pallidum*-like pathogens were infecting humans in South America at least as early as 5,500 years ago. Falling outside of the range of genomic diversity of all previously described modern and ancient *Treponema pallidum* subspecies, the pathogen could be a form of *T. carateum* (the proposed causative agent of pinta that has not been sequenced before) or a previously-unknown extinct lineage. This could be the pathogen (or perhaps one of many) responsible for the pre-columbian American variation of treponematosis recognized by paleopathologists (Powell & Cook, 2005). This does not resolve the questions on the origins or evolution of modern *Treponema* pathogens, but provides exciting new questions to investigate. Given the antiquity of the individual, the subject of this chapter seemingly has nothing to do with colonialism. However, as discussed in Chapter 4, narratives about treponematosis as a whole intersect with colonialism. Much of the discourse surrounds the Columbian exchange from European perspectives, and treponematosis has been used as a tool of colonialism in the past, such as in the case of Morocco (Amster, 2016; Amster 2022). An argument could also be made about how much of the related scientific and historical research is Eurocentric and engages in colonial research methods that we reject, as touched on in Chapter 2.

Chapter 3, while also about pathogens, deals explicitly with the topic of colonialism. We found genomic evidence to support the presence of multiple diseases thought to be brought to the Americas by European colonizers, but we also found genomic material of pathogens known to have been present in Peru before European

colonization. Historical, archaeological and ecological context are crucial to consider when interpreting this data in order to accurately reconstruct the impact and evolution of these pathogens.

Historical colonialism continues to influence the disease landscape. While the completely 'new' pathogens brought from Europe to the Americas by colonizers provide clear-cut examples, the impacts from other pathogens can be less obvious. One example of a more complex story is that of tuberculosis. *Mycobacterium tuberculosis* complex (MTBC), one group of pathogens detected in the preliminary results of Chapter 3, has links to European colonization and still is a major public health concern in Peru today (World Health Organization, 2023a). As discussed in Chapter 3, tuberculosis existed in Peru before the arrival of Europeans, but was caused by a member of the *Mycobacterium tuberculosis* complex most closely related to *Mycobacterium pinnipedii* (Bos et al., 2014). The Spanish then arrived in the 1500s, causing social upheaval along with ecological and environmental changes. In 2022, Peru had an estimated tuberculosis incidence rate of 151 cases /100,000 people, one of the highest in the world; for comparison, the US had an incidence of 3/100,000 that same year (World Health Organization, 2023a). However, modern tuberculosis cases in Peru are predominantly caused by *Mycobacterium tuberculosis* lineage 4, a lineage of European origin (Gagneux et al., 2006; Santos-Lazaro et al., 2021). While the details of how this shift in causative agents occurred are unknown, this is an example of a direct impact of historical colonialism that has had a lasting impact on communities.

While it is clear we must not ignore historical colonialism, it is also inappropriate to discuss colonialism as something that exists exclusively in the past–it may have evolved, but it has not been eliminated (Bacon & Norton, 2019; Blume, 2022; Moufawad-Paul, 2013); the psychological and cultural undergirding remains the same (Blume, 2022). This has a direct impact on public health. COVID-19 is a case that demonstrates this well. The Palestinian territories of the West Bank and Gaza are occupied by Israel (Albanese, 2023); this has an impact on Palestinians' health, as Israel controls what and who goes in or out, frequently preventing Palestinians living in these areas from accessing life-saving care (B'Tselem, 2023; Medecins Sans Frontieres, 2024b; World Health Organization, 2023b). Additionally, Israel's assault on Gaza (the one beginning in October 2023) targeted hospitals, cut off clean water, destroyed sanitation systems, and displaced massive numbers of people (Hall et al., 2024; Vinall & El Chamaa, 2024); a wide variety of infectious diseases increased as a result, including large numbers of respiratory infections (International Rescue Committee, 2024; Medecins Sans Frontieres, 2024a). While the healthcare system–which includes COVID-19 testing and reporting capacity– has collapsed (Kekatos, 2023; Medecins Sans Frontieres, 2024a), when considering what was seen during the 2021 bombing campaign (Ducharme, 2021), this rise in respiratory infections likely includes a rise in COVID-19 infections (World Health Organization, 2023c). While undoubtedly connected issues, some may argue this specific instance is due to "war" and is a separate argument from the effects of colonialism. However, there are pieces of the COVID-19 story in Palestine that

unambiguously are a direct result of colonialism and are separate from times of intensified bombing campaigns. Imports to Gaza and the West Bank are controlled by Israel; “dual use” bans of critical items to Gaza and hold-ups at West Bank checkpoints have led to delivery delays and shortages of medical supplies, medications, and vaccines (Rosenbloom & Leff, 2022; Viñoles, 2024). As the occupying power, Israel also has a legal obligation under international law to provide equal and fair vaccine access to Palestinians (United Nations, 2021). However, the Israeli administration consistently and repeatedly ignored the calls to include people in the West Bank and Gaza in their vaccination plans (Howard & Schneider, 2022). After international outcry, the Israeli government eventually did offer to provide the Palestinian Authority’s health system with vaccines (as a trade for a later shipment of vaccines the Palestinian Authority was set to receive from a manufacturer, not as aid), but the batches Israel delivered were too close to their expiration date for the Palestinians to effectively distribute, rendering them useless (“Palestinian Authority”, 2021). In February of 2021, Israel also actively prevented the delivery of vaccines from the West Bank to Gaza, (Hendrix & Rubin, 2021). At this point in time, approximately 40% of Israel’s population was fully vaccinated, and Gaza had yet to receive a single vaccine (Hendrix & Rubin, 2021). While the vaccines in question only required refrigeration, if they had not been returned to Ramallah during this two-day delay, the vaccines likely would have been damaged by the time they finally were allowed to enter Gaza. In September of the same year, the Ministry of Health in Gaza was forced to destroy 50,000 vaccine doses upon arrival– the Israeli military

removed them for long periods from the cold chain multiple times at checkpoints, damaging them beyond the point of use ("Israel's obstruction", 2021).

In contrast to Palestinians, Israelis–including Israelis living in illegal settlements in the occupied West Bank– were among the first in the world to be vaccinated at high rates (Estrin, 2021). While vaccines were in short supply worldwide, the Israeli government was able to secure large numbers of vaccines for their citizens in a campaign widely viewed as a public health success story (Muhsen & Cohen, 2021). Much of the criticism of this campaign stemmed from the general issue of unequal access to vaccines during a time of scarcity; however, this critique is not exclusive to Israel–many high income countries hoarded vaccines early on and were rebuked for doing so (Binagwaho et al., 2022; Smith, 2020). However, Israel's refusal to provide vaccines to people it occupies provided an additional source of outrage. Palestinian Arab citizens of Israel also were vaccinated at lower percentages, although the reasons for this may stem more from Palestinians' distrust in the Israeli government (which is arguably justified given the history and current system of apartheid [Albanese, 2023; International Court of Justice, 2024]) than an intentional action carried out by the administration (Green et al., 2021; Muhsen et al., 2021). While this example is stark, disproportionate impacts from COVID-19 are not exclusively found in settler-colonial contexts. In an example from a different form of modern colonialism, lower and middle income countries (whose resources are often exploited by "western" companies and governments) also had higher disease burdens

and more limited access to vaccines during the pandemic than high-income countries (Blume, 2022; Levin et al., 2022).

Historical colonialism also played a hand in how the COVID-19 pandemic played out. Formerly colonized countries struggled with the limited capacity for vaccine distribution and COVID-19 response (Blume, 2022; Smith, 2020). Even in the United States, a country responsible for much of the historical and modern colonialism worldwide (Bacon & Norton, 2019), the impact of colonialism on the country's COVID-19 response was evident. To understand this, one must understand colonialism's connection between socioeconomic status–people belonging to groups impacted by historical colonialism and white supremacy often have low socioeconomic status as a direct result (Blume, 2022; Emerson & Montoya, 2021). Many in this economic bracket have jobs that are deemed "essential"; these positions forced them to interact with others during the COVID-19 lockdowns in the US, which resulted in a higher rate of COVID-19 exposure for people in these roles (Blume, 2022). Additionally, due to the lack of socialized healthcare in the United States, many of these people could not access or afford the appropriate PPE, tests, vaccines, or treatment (Emerson & Montoya, 2021; Galvani et al., 2022; Kashyap, 2020). The interconnected issue of environmental racism also leads to these groups being concentrated in specific areas contaminated with pollutants that have known health impacts, leading to these groups having a higher prevalence of pre-existing conditions known to increase the danger of COVID-19 infection (Bonhomme, 2020; Emerson & Montoya, 2021; Kashyap, 2020). Similarly, current physical, mental, and

environmental stressors stemming from oppression have also been linked to health disparities and differences in health outcomes (Blume, 2022; Kashyap, 2020). Clearly, current and historic colonialism intersect through human health; whether people were colonized in the past or present, it can put them at a disadvantage and disrupt their access to care.

This dissertation is not an endpoint, but the beginning. In the future, I will follow up on the findings from the preliminary work described here, expand existing projects, and search for pathogen DNA in underexplored areas such as textiles and faunal remains. This type of transdisciplinary pathogen paleogenomics research is impactful, as more pathogen genomes allow us to view the evolutionary story of the pathogens as well as their impacts in higher definition. Additional focus on this area of research can contribute both to research fields battling diseases and to fields in the humanities studying the past and present impact of European colonial conquest. However, the manner in which this research is designed and carried out has the potential to cause harm and perpetuate colonialism, so care must be taken to avoid this. When carried out in partnership with those it is meant to serve, this field of research promises breakthroughs. In learning more about our past, we can better understand our present. Looking towards the future, we can use this information to better prepare for what comes next.

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## Addendum

In the summer of 2023, I spent some time traveling. I visited Dr. Nelson in Paris while she was working at the Pasteur Institute, I attended the ISBA conference in Tartu, Estonia, and I traveled back to my father's homeland to visit relatives and landscapes I hadn't seen in a long time.

I lived in Iran for a few years as an adolescent, but after moving back to the US I never looked back. This trip was the first time I had been back in 18 years. Whether out of internalized racism, running from intergenerational trauma, or the realities of life and politics, I had been running from Iran–the land, my people, and everything the name represents. But the tether to my ancestors grew tighter the more forcefully I tugged against it. Facing our past can be painful, but we cannot outrun it or erase who we are. After years of distancing myself from my heritage, I was finally able to visit the graves of my paternal grandparents and my recently deceased aunt to pay my respects. The guilt of feeling like I had abandoned people who helped raise me hit like a wave, but the reunions were joyful. Family members were constantly offering me food, going out of their way to accommodate my dietary restrictions. The mountains greeted me like an old friend. I experienced many powerful emotions during that trip, which were punctuated by an event that occurred earlier in my travels.

While visiting Dr. Nelson in Paris, I took a day trip to London. I spent most of that morning in the British Museum. In this museum, there are large exhibits on Iran and Nineveh (modern-day Mosul, Iraq). These are two of the three places where I can

trace my lineages to (ironically, the third place my ancestry comes from is England–I could write a whole book about the experience of being mixed, especially between groups of people who historically have had animosity between them, the feeling of never fully belonging anywhere and the internal conflicts, but that is a story for another time). Looking at these connections to my ancestors raised many emotions; knowing they were stolen from us raised even more. I lived as a child in Iran. Traveled all over the country. Visited archaeological sites. In fact, visiting places like Persepolis was one of the early sparks of interest for me in archaeology. However, these items that hold so much meaning to us, that are emblematic of our heritage and what we think of when we talk about our culture? I didn't get to see them until I was 31, traveling thousands of miles away from the land they were taken from.

While not the topic of my dissertation, I spent a significant amount of my time in grad school involved in repatriation efforts. I was drawn to this work because it provided an opportunity to use my skills to take action against injustice. My experience doing this work has influenced many of my opinions and decisions in both my daily life and my "unrelated" research. As someone also belonging to groups directly impacted by the theft of our material and cultural heritage, I have issues with colonial museum collections on not just an academic level, but a personal one as well. My involvement in NAGPRA work has only deepened my resolve to advocate loudly for repatriation and the transformation of the ways museums operate.

The tradition of poetry runs deep through the cultures of my Persian, Kurdish, and Arab ancestors, and I carry on this tradition. This is, after all, an Anthropology

degree, so it felt appropriate to include a creative form of artistic expression that relates so deeply to my lived experience, my time in grad school, and the theme of decolonization that underlies the work I've done and hope to do in the future.

**Why is this in the British Museum**

Those marvels and treasures
From far-off lands
Do not belong to you.
Those sacred objects
You stole from them
Do not belong to you.
Human remains
Do not.
Belong.
To you.

You say you “can’t change what happened”.
But it’s still ongoing.
Now you see it occurring
But you say your “hands are tied”.
But bound hands can’t accept statues
Closed minds won’t accept statutes
You ask for reimbursement
But it was paid for in blood.

Why is this in the British Museum?
My land’s precious stones and my ancestors’ bones
Why is this in the British Museum?
Our pride and our history, so far from home
Why is this in the British Museum?
If we weren’t so “barbaric” they’d consider a loan
Why did I have to fly thousands of miles
To see my own birthright, my own history?
I’ve walked on the soil my bloodlines run down through
But the British are far more deserving than me
Because the creators of civilizations
are “too uncivilized”
to trust with their own belongings.

It’s a terrible look
Desperate for control
Spending your dying breath
Defending what you stole
As you gasp for air
Drowning in your spite
Remember
Just because you **can**
Doesn’t make it **right.**
You’re frenzied and desperate
Like you’re under attack
There’s an easy solution:
Give it back.