

Tuberculosis and Leprosy in Perspective

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ABSTRACT Two of humankind's most socially and psychologically devastating diseases, tuberculosis and leprosy, have been the subject of intensive paleopathological research due to their antiquity, a presumed association with human settlement and subsistence patterns, and their propensity to leave characteristic lesions on skeletal and mummified remains. Despite a long history of medical research and the development of effective chemotherapy, these diseases remain global health threats even in the 21st century, and as such, their causative agents *Mycobacterium tuberculosis* and *M. leprae*, respectively, have recently been the subject of molecular genetics research. The new genome-level data for several mycobacterial species have informed extensive phylogenetic analyses that call into question previously accepted

theories concerning the origins and antiquity of these diseases. Of special note is the fact that all new models are in broad agreement that human TB predated that in other animals, including cattle and other domesticates, and that this disease originated at least 35,000 years ago and probably closer to 2.6 million years ago. In this work, we review current phylogenetic and biogeographic models derived from molecular biology and explore their implications for the global development of TB and leprosy, past and present. In so doing, we also briefly review the skeletal evidence for TB and leprosy, explore the current status of these pathogens, critically consider current methods for identifying ancient mycobacterial DNA, and evaluate coevolutionary models. *Yrbk Phys Anthropol* 52:66–94, 2009. © 2009 Wiley-Liss, Inc.

At the close of the first decade of the 21st century, a number of factors converge to make a review of mycobacterial disease timely. The first of these is the re-emergence of human tuberculosis (TB) in epidemic proportions on a global scale. As emphasized in the section on Mycobacterial diseases: The Historical, Epidemiological, and Ecological Context Section, by the mid-point of the 20th century the disease was thought to be conquerable and by the 1980s, conquered. How wrong these predictions were! TB, termed “the white plague” during the 19th century, has a very long history as a health burden to humankind, which continues today. A thorough knowledge of the coevolution of humans and pathogenic mycobacteria may inform development of treatment and prevention methods.

A second important factor making a review of mycobacterial disease timely involves the emergence of new techniques in molecular biology. The development of the polymerase chain reaction (PCR) method for amplifying DNA, advancements in sequencing technology, as well as the availability of mycobacterial genome sequences have revolutionized our knowledge of contemporary mycobacterial genetic variation. These advances permit scientists not only to explore global patterning within species, but also to make phylogenetic inferences and understand the evolutionary relationships between mycobacterial strains. In turn, molecular biologists and anthropologists have used this technology to identify ancient pathogens in archaeologically recovered human remains.

To set the stage for evaluating the impact of molecular biology and genomics upon our perspectives on past and present mycobacterial disease, we here first review skeletal evidence for ancient TB and leprosy in the Old and New Worlds (Skeletal Evidence for Mycobacterial Disease in the Old and New Worlds Section). We then turn

to modern genomic and phylogenetic studies of mycobacteria (The Phylogeny of Mycobacteria Section). Our focus is on the *Mycobacterium tuberculosis* complex (MTBC), which includes *M. tuberculosis*, *M. canettii*, *M. microti*, *M. bovis*, *M. caprae*, and *M. africanum*. We also consider leprosy, a related mycobacterial disease caused by *M. leprae*. The phylogenetic relationships of these species are examined here and viewed as a framework on which to contextualize molecular epidemiological data from ancient remains.

Section V reviews ancient DNA (aDNA) methods for studying mycobacterial disease and offers critiques of the less rigorous approaches. Chief among these are the failure to control for contamination and uncritical application of methods appropriate in living groups to archaeological contexts. We then turn (Coevolution of Humans and *M. tuberculosis* Section) to coevolution in the long term, closing with a discussion of the implications of the new genomic and phylogenetic evidence for interpreting the archaeological record (Conclusions Section).

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MYCOBACTERIAL DISEASES: THE HISTORICAL, EPIDEMIOLOGICAL, AND ECOLOGICAL CONTEXT

TB today

In 1988, the medical historian Smith (1988, p 2) stated that, "Tuberculosis is now a conquered disease in the British Isles and the rest of the industrialized world," but this statement turned out to be very wrong. TB is today a major cause of death globally and occurs in both developing and developed countries alike. It is caused by bacteria of the MTBC, with *M. tuberculosis* and *M. bovis* being the most common cause of illness in humans. The former is transmitted by droplet infection via the lungs, and the latter is primarily through the gastrointestinal route from infected animal products (Gordon and Mwandumba, 2008; Ormerod, 2008). Coughing, difficulty in breathing, weakness, lethargy, loss of appetite and weight, night sweats, pallor, and chest pain may occur; if not treated the infection may spread to other organs, including the skeleton, via the lymphatic and blood stream systems.

In 2005, there were an estimated 8.8 million new cases of TB, with 7.4 million occurring in Asia and sub-Saharan Africa (World Health Organization, 2007), and 1.5 million people died of TB. However, the pandemic is believed to be on the threshold of decline with, by 2005, all six World Health Organization (WHO) areas showing a yearly incidence (number of new cases) that was stable or in decline. Nevertheless, the total number of new cases of TB rises slowly, mainly due to population increases in Africa, the Eastern Mediterranean, and the South-East Asia regions. Problems highlighted in dealing with TB include a lack of skilled and highly motivated staff for public health programs, too few laboratories for diagnosis, lack of drug stocks and facilities for screening of human immunodeficiency virus (HIV)-infected people for TB, and lack of resources for diagnosis and treatment of people with extensively drug-resistant TB (EDR-TB or XDR-TB). For example, London accounts for half the national burden of TB in Britain today, and its incidence has doubled in the last 15 years (Anderson et al., 2007). Around 75% of people with TB in London were born abroad; 86% are from ethnic minority groups (one-third from Africa and one third from the Indian subcontinent), and many affected are from homeless, incarcerated, drug- and alcohol-addicted, and immune-suppressed groups. As is suggested, TB diagnosis and control needs to be tailored to address these high incidence groups; similar problems are being experienced worldwide.

The many factors, which have maintained TB in the world today, include (often in combination) poverty (Bhatti et al., 1995), poor diet, TB-contaminated foods [e.g., see Harris et al. (2007), overcrowding (Elender et al., 1998), specific occupations such as working with animals (Thoen and Steele, 1995), being in prison (Balinska, 2000), increased mobility through travel (business and pleasure—Driver et al., 1994) and migration (from troubled parts of the world—Bhatia et al., 2001)], the presence of the HIV, age (Johnston, 1995), sex (Hudelson, 1996), and ethnicity (Newport and Levin, 1999), concepts of TB occurrence and access to health education and care (e.g. see Foster, 1999), treatment compliance (Farmer, 1999), and multiple drug resistance. For example, according to Ravigione et al. (1995), HIV was the most important single risk factor for the progression of dormant TB into clinical disease, as the virus compro-

mises the immune response. The WHO (2007) also notes this to be a significant factor in TB today. Additionally, concepts of the causation of TB and associated stigma can vary considerably in different cultures, which then affects what treatments are provided, the opportunity for access to, and success of, available treatments, and the implementation of preventive measures [see Long et al. (1999) for an example]. The combination of poverty, HIV, and drug resistance makes for a challenging and terrifying situation for many people in the world.

Unfortunately, as Walt (1999) indicates, politics often determines who is treated, when, and how, in different countries of the world. We also have to consider that males, females, and children may be treated differently, not only with TB but with any health problem. Hudelson (1996), for example, notes that women in sub-Saharan Africa are at greater risk for contracting both the HIV and TB. Clearly, TB remains a major morbidity and mortality problem in the world, and there are multiple variables that will affect the frequency and success of treatment, but there are small signs that control measures are starting to work. Treatment with a multiple antibiotic regime remains the usual therapy, and treatment is monitored through DOTS (Directly Observed Therapy Short Course) whereby the taking of medication is monitored directly by a health care worker, thus ensuring that the patient completes the course of treatment. It is now estimated that 89% of the world's population live in areas where DOTS has been implemented by public health services (WHO, 2007); the signs therefore look very promising, but attending to poverty and the HIV remains a great challenge. TB, like leprosy, is essentially a disease of poverty.

Leprosy today

Compared to TB, leprosy is seen as very much a declining infectious disease. However, "Leprosy occupies a special position among communicable diseases because of the long duration of the disease, the frequency of disabilities and the social and economic consequences it endangers" (Dayal et al., 1990, p 170). In 2005, the Centers for Disease Control and Prevention (CDC; CDC, 2005) reported that 1–2 million people worldwide were permanently disabled as a result of Hansen's disease, and, as reported by the WHO (2002), it has been estimated that 177,000 disability adjusted life years were lost due to leprosy in 2001.

Caused by the *Mycobacterium leprae*, leprosy is known mainly as a disease of humans. At our present stage of knowledge, it is thought that leprosy is transmitted by nasal or oral droplets, but in truth, this has yet to be fully elucidated [see Girdhar (2005) for a review]. The nasal mucosa, and possibly skin lesions, of leprosy patients may have significant numbers of bacilli, and several possible modes of transmission have been investigated. Some of these are skin-to-skin contact (Dayal et al., 1990), mother to fetus transmission in utero (e.g. Melsom et al., 1980), breast milk (Pedley, 1967), via sexual intercourse (Duncan, 1985), tattooing (Singh et al., 1985), insect bites (Sreevatasana, 1993), and even environmental reservoirs (Lavania et al., 2008). However, it is generally agreed that droplet infection is likely the key transmission mode and that the disease is neither inherited nor transmitted venereally. There is a long incubation period, and the main risk factors are poverty and a poor diet, along with overcrowding, lack of under-

standing about leprosy transmission (Barkataki et al., 2006), and access to health education and care, along with treatment compliance and drug resistance and stigma (Mehta, 2002). The peripheral nerves (sensory, motor, and autonomic) are primarily affected, leading to the paralysis of muscle groups in the limbs and loss of sensation in the hands and feet especially; there can also be involvement of the skin, eyes, skeleton, kidneys, liver, adrenal glands, and testes. Depending on the patient's cell-mediated immunity, there could be oedema of the lower legs (due to increased permeability of skin capillaries due to bacilli in them, damage to autonomic fibers in the skin nerves and gravity), thinning of the eyebrows, broadening of the nose, deepening of the forehead lines, possible loss of eyelashes, nasal collapse, loss of the incisors, voice hoarseness, hardness and ulceration of the legs, plantar ulcers, and shortening of the fingers and toes due to bone damage (Jopling, 1982).

Leprosy has a wide range of clinical and histopathological manifestations (Scollard et al., 2006), which are incorporated into a practical classification scheme (Ridley and Jopling, 1966). At one end of the immune spectrum is lepromatous leprosy where there is no resistance to *M. leprae*, and at the other is tuberculoid leprosy, where there is cell-mediated immunity and delayed hypersensitivity. However, most people with leprosy are in the borderline category (borderline, mid-borderline, and borderline tuberculoid). Unlike TB, leprosy is not more likely in people with HIV (Lucas, 1993), possibly because *M. leprae* has a relatively low virulence or that individuals with the HIV die before leprosy is apparent (Scollard et al., 2006).

It was long thought that leprosy was only a disease of humans, but in the mid-20th century, it was also found to be prevalent in the nine-banded armadillo in parts of the southern United States (Walsh et al., 1988). The disease was not present in the New World prior to the 16th century (Feldman and Sturdivant, 1975), and contact with infected humans is the most likely original source of the armadillo infection, although the transmission mechanism is still unclear (Truman, 2005). It is suggested that 65% of armadillos experimentally inoculated with *M. leprae* will develop disseminated infection (Scollard et al., 2006), and it is known that the infection is present in armadillos in Arkansas, Louisiana, Mississippi, and Texas in the United States today. Leprosy has also been observed in armadillos from Mexico and Brazil (Amezcuca et al., 1984; Daps et al., 2007). It is important to realize that extensive surveys of leprosy in modern animal populations have not been undertaken, and the disease has been documented in nonhuman African primates (Meyers et al., 1985, 1992; Walsh et al., 1988; Rojas-Espinosa and Lovik, 2001), with possible monkey-monkey transmission (Gormus et al., 1988). Given that the highest prevalence (number of existing cases) of leprosy today occurs in areas of the world where nonhuman primates are also abundant, surveys of these populations as potential reservoirs will be important avenues of future research.

In 1991, leprosy was targeted for elimination by the World Health Assembly, but despite the availability of effective chemotherapy and extensive international efforts, this goal remains elusive. The WHO's targeted prevalence rates were calculated to interrupt transmission of the bacteria, but this alone may not suffice. Lockwood (2002), for example, explains that while the target prevalence has been reached for leprosy in many areas,

incidence has remained constant or even has increased. In fact, even in areas where the prevalence has been below the target for decades, transmission and new cases still occur (Lockwood and Suneetha, 2005). There is some hope, however. At the beginning of 2006, the global registered prevalence of leprosy, based on reports from 115 countries and territories, was 219,826 cases, and the new cases detected during 2005 stood at 296,826 (a decline of over 100,000 compared to 2004). This represents a 20% decline in new cases, a situation seen for the last four years (www.who.int/lep/en/; accessed April 2, 2007). Most previously, highly endemic countries have now eliminated leprosy, but the main countries that still have leprosy as a problem are areas of Angola, Brazil, Central African Republic, Democratic Republic of Congo, India, Madagascar, Mozambique, Nepal, and the United Republic of Tanzania. The frequency of leprosy has declined because of the concerted effort of public health workers to find, diagnose, educate, and treat people with leprosy and also to educate the general public about the facts of this still very stigmatized infection.

In the 1950s, dapsone was introduced for treatment, but pathogen resistance eventually developed; following this, rifampicin and clofazimine were used, but again resistance occurred. In 1981, the WHO recommended a combination of all three drugs over a period of 2 years (Scollard et al., 2006). Since 1998, the duration of recommended treatment has been reduced due to lack of funding by governments. Vaccines have also been used for preventing leprosy, especially the BCG vaccine, but its efficacy varies around the world. As the vaccine has variable effects on the prevention of TB (Fine, 1995), it is not surprising that its preventative effect on leprosy varies; this might be due to biological differences in BCG strains, exposure to environmental mycobacteria, or ineffective boosting against reinfection with, or reactivation of, TB (Scollard et al., 2006). It is suggested that with a better understanding of the molecular nature of *M. leprae* and the human response to infection, new vaccines will be developed. Multiple drug therapy (antibiotics) at an early stage in the disease is the key to treatment and cure, and it is essential that this occurs before the development of disability as a result of damage to hands and feet because of peripheral nerve involvement. However, even if cure rates for leprosy are increasing, there are still thousands of people who continue to suffer the debilitating physical, social, and psychological consequences of leprosy (Meima et al., 2004; Scollard et al., 2006), thus underscoring that the consequences of leprosy will be with us for many years.

SKELTAL EVIDENCE FOR MYCOBACTERIAL DISEASE IN THE OLD AND NEW WORLDS

Recognizing TB in archaeological remains (for extensive reviews, see Aufderheide and Rodriguez-Martin (1998), Ortner (2003), Roberts and Buikstra (2003).

Identifying skeletal TB requires knowledge of the manner in which the tubercle bacilli circulate through the system. *M. tuberculosis* within the circulatory system tends to localize within hemopoietic tissues, which is more widely distributed in juveniles than in adults. Thus, TB in children may even cause destructive, expansive lesions within the tubular bones of the hands and feet (*spina ventosa*), the skull, and other flat bones of the body. Among adults, characteristic skeletal lesions of TB include the classic "Pott's Disease" manifesting as de-

structive focal lesions of the spine, typically within the lower back, such that the column collapses and will secondarily fuse or ankylose. Other frequent sites of focal lesions include the sacro-iliac articulations and the joint surfaces of the limb long bones. As reviewed by Ortner (2003), any joint surface may be affected in adults, and even the skull may show focal lesions, though these are relatively uncommon.

Although nonspecific, bone addition to the internal aspects of the ribs may be associated with TB (Kelley and Micozzi, 1984; Roberts et al., 1994, 1998; Roberts, 1999). In clinical contexts, rib destruction is usually described and radiographically displayed in TB (Tatelman and Drouillard, 1953). As Roberts and Buikstra (2003) emphasize, studies of skeletons with clinically diagnosed TB (Kelley and Micozzi, 1984; Roberts et al., 1994; Santos, 2000) suggest that this mycobacterial disease is the most likely culprit, but not all cases of TB present rib lesions and not all lesions are associated with clinically documented TB. As this attribute only became of interest to paleopathologists within the last quarter-century, relatively few reports of rib lesions in archaeological materials exist.

Within mummified soft tissues, diagnoses of TB are typically based upon diagnostic lesions of the lungs, including the ghon complex (Allison et al., 1981; Salo et al., 1994). Acid-fast bacilli have also been considered diagnostic of TB in mummified remains (Allison et al., 1973).

Skeletal studies of TB in the Old World

Most evidence for TB in human remains from the Old World comes from Europe, which we believe reflects the prevalence of paleopathologists there compared to the rest of the Old World. This may also reflect differential survival of human remains, limited excavations of ancient cemeteries, or destructive funerary rites (Roberts and Buikstra, 2003), as well as the fact that only 3–5% of people with TB develop bone changes (Resnick and Niwayama, 1995). However, those Old World areas with no evidence may truly be areas with no TB. The earliest historical references to TB come from Egypt in a medical papyrus dated to 1550 BC, from India in a Sanskrit hymn (Rig Veda) dated to 1550 BC (Evans, 1998), and from a medical text from China dated to 2700 BC (Keers, 1981). When considering evidence from human remains, however, we can divide the data into three broad areas in the Old World, “Northern Europe,” the “Mediterranean,” and “Asia,” which reflect similar climate and environmental features.

The Mediterranean. Although some have claimed that TB was present in *H. erectus* (Kappelman et al., 2008), the earliest convincing evidence of skeletal TB in humans has been discovered in Italy. One example is a female skeleton, aged around 30 years at death, dated to 5800 ± 90 BP and excavated from the Neolithic cave of Arma dell’Aquila in Liguria (Canci et al., 1996). A second Italian case, also dating to the first centuries of the fourth millennium BC and presenting convincing skeletal evidence of TB, involves the remains of an adolescent male from the site of Arene Candide, in Liguria (Formicola et al., 1987). Jordan has also revealed two early examples of TB from Bab edh-Dhra at 3150–2200 BC (Ortner, 1979; Ortner and Frohlich, 2008), although Israel does not present evidence until AD 600 at the monastery of John the Baptist in the Judean Desert

(Zias, 1991). There is no evidence in skeletal remains from sub-Saharan Africa (Santos, personal communication), but in Egypt and the Sudan there has been considerable research on all aspects of heritage, including analyses of TB in human remains; these studies have been published since the early-20th century (Elliot-Smith and Ruffer, 1910). Probably, the most famous example is the mummy Nesperehân, excavated in Thebes, wherein a psoas abscess and spinal changes were recorded and established TB’s presence in Egypt by between 1069 and 945 BC (Morse et al., 1964). However, Morse et al. (1964) also record the earliest evidence for TB from Nagada dated to as early as 4500 BC. Before this study, Derry’s (1938) summary of the skeletal TB data recognized the earliest occurrence at 3300 BC. Spain is next in date, with possible TB in skeletal remains dated to the Neolithic (Santoja, 1975), and in Greece TB appears by 900 BC (Angel, 1984).

France, like Lithuania, and Austria (“Northern Europe”) reveal TB around the 4th century AD (Moyart and Pavaut, 1998). Data are focused in specific regions and reflect research intensity. Evidence has appeared in early, late, and post-Medieval south-east France (Berato et al., 1991; Pálfi et al., 1992, 1995; Brun et al., 1997; Molnár et al., 1998; Ardagna et al., 1999). Northern France has probably seen the most extensive work (Moyart and Pavaut, 1998; Blondiaux et al., 1999) with nearly 2,500 skeletons being examined from 17 sites dated between the 4th and 12th centuries AD. Twenty-nine cases of TB were identified and most came from urban sites. Other “Mediterranean” countries such as Serbia, Turkey, and Portugal do not present evidence for the infection until much later in the Medieval period (from around the 12th century AD). In fact, it is not until that period that there appear to be significant numbers of populations with TB (Roberts and Buikstra, 2003), as described earlier.

Northern Europe. In “Northern Europe,” Poland has the first evidence for TB from the Neolithic site of Złota dated to 5000 BC (Gładkowska-Rzeczycka, 1999), but frequencies, as for many other countries in Europe, increased in the later Medieval period. Data from Russia suggest that TB was present by 1000 BC at the Bronze Age site of Manych, Southern Russia (Rokhlin, 1965). Denmark became visibly at risk for TB, beginning with the Iron Age (500–1 BC) at a site at Varpelev, Sjælland (Bennike, 1999), and in Britain, the first evidence is from an Iron Age site at Tarrant Hinton, Dorset, dated to 400–230 BC (Mays and Taylor, 2003; Taylor et al., 2005). Austria and Lithuania have skeletal evidence by the 4th century AD. Lithuania has seen extensive research documenting the frequency of TB in skeletal remains with the early Marvelé site producing late-Roman data (Jankauskas, 1999). During more recent periods, frequencies increase along with population density and intensification of agriculture. Norway (Holck, personal communication) and Switzerland present evidence of skeletal TB by the 7th century AD (Morel et al., 1961), along with Hungary, and Sweden and the Netherlands in the 11th and 13th centuries AD, respectively. In Hungary, there have been extensive published reports that document diachronic changes in the frequency of TB (Pálfi and Marcsik, 1999). Clearly, from these data, TB was fairly common in the 7th–8th centuries and also in the 14th–17th centuries; an obvious gap in the evidence in the 10th century was, it is suggested, due to

the seminomadic way of life the population had at that time. In Sweden, an extensive study of over 3,000 skeletons from Lund dated to between AD 990 and 1,536 showed TB of the spine in one individual (AD 1050–1100) although over 40 had possible TB in one or more joints (Arcini, 1999). The Czech Republic also provides its first evidence in the later Medieval period (Horácková et al., 1999).

Asia

In Asia, skeletal TB is reported much later than both the “Mediterranean” and “Northern European” areas. China presents early evidence dating from the 2nd century BC (Suzuki and Inoue, 2007), but the first written description of TB treatment is dated to 2700 BC (Morse, 1967) and the first accepted description of the disease to 2200 BC (Kiple, 1993). Japan reports the earliest skeletal evidence dated to 454 BC to AD 124 (Suzuki and Inoue, 2007). Thailand is reputed to have possible evidence a little earlier and dated to 300 BC to AD 300 (Tayles, personal communication). Papua New Guinea and Hawaii describe pre-European TB data for more recent periods (Pietreuwsky et al., 1991; Pietreuwsky and Douglas, 1994; Trembly, 1997), with possible TB being recorded on Tonga and the Solomon Islands (Pietreuwsky, personal communication).

Summary of skeletal evidence for TB in the Old World

Although the data for TB across the Old World appears quite plentiful, there are many areas where there is no evidence. This may be because (1) it really does not exist, (2) skeletal remains are not traditionally studied in a particular country, (3) disposal of bodies at a particular time may not preserve them well for the evidence to be observed (e.g. cremation in Bronze Age Britain), (4) skeletal remains do not survive burial due to the climate or environment in a specific geographic area (e.g. the freeze/thaw cycles in the climate of Finland, or the acidic soils of Wales or Scotland), or (5) for some periods of time, in some countries, there just have not been any skeletal remains excavated. Of course, what should also be noted is that the skeletal evidence described here is that recorded from remains that have been excavated and analyzed. Thus, the picture of TB that we see will reflect these facts, and what we understand of its origin and evolution may change considerably with each new find. However, on the basis of the evidence to date, we see that TB has an early focus in the Mediterranean and Northern European areas and specifically in Italy in the Neolithic period. There are later appearances in Asia and other parts of Northern Europe and the Mediterranean. However, it is not until urbanization and an increase in population size and density in the later Medieval period that we see a rise in the frequency of the disease in most places. Elevated skeletal frequencies, therefore, appear to be associated with the hazards of urban living and closely packed communities.

Skeletal studies of TB in the New World

As discussed in Ancient Biomolecules and the Study of Mycobacterial Diseases Section, identification of ancient TB in the Americas remained controversial until aDNA evidence began to accumulate. Although a few cases

were described during the 19th century (e.g. Whitney, 1886), Hrdlicka (1909) discounted skeletal evidence of TB among ancient Indian remains. By the mid-20th century, however, additional examples had been reported for North America (Lichtor and Lichtor, 1952; Ritchie, 1952; Judd, 1954; Crane and Griffin, 1959) and South America (Garcia-Frías, 1940; Requena, 1945). Yet, during the 1960s, Morse (1961, 1967, 1969), a medical doctor with considerable clinical experience argued vigorously against such attributions. Buikstra (1976, 1981) has, however, pointed out that Morse developed standards based upon his clinical experience in a chemotherapy era and also applied more restrictive standards to New World cases than those from the Old World.

Skeletal evidence continued to accumulate throughout the Americas during the 20th century, along with the cases found in mummified remains (Allison et al., 1981; Roberts and Buikstra, 2003). A variety of approaches were used to identify TB, including those that were primarily clinical and others wherein population-based discussions of lesion patterning was combined with age-at-death and cultural factors that might promote risk of mycobacterial disease (Buikstra and Cook, 1978, 1981; Milner and Smith, 1990; Buikstra and Williams, 1991).

South America. Among the earliest convincing cases of TB from the Americas are those recovered from the site of Casarones, located within the Tarapacá valley of northern Chile, within the arid Atacama desert (Allison et al., 1981). Even if the radiocarbon dates have been affected by the old carbon problem, the three remains are unlikely to postdate AD 700. Allison et al. (1973) also report destructive lesions and acid-fast bacilli in a child from the Nazca culture of coastal southern Peru. These remains are also said to date to ~AD 700.

Within South America, the largest numbers of TB cases tend to be associated with arid coastal locations of southern Peru and northern Chile (Garcia-Frías, 1940; Allison et al., 1981; Buikstra and Williams, 1991; Burgess, 1992; Almonacin, 1992; Owen, 1993). A few identifications originate in Venezuela (Requena, 1945) and Colombia (Boada, 1988; Rodriguez, 1988; Arregoces, 1989; Correal and Florez, 1992; Martinez de Arateco Hoyo, 1999). Those from northern South America tend to be few when compared to the more southern examples.

North America. All evidence of TB in North America postdates AD 900, with marked increases beginning at AD 1000. As reported by Buikstra (1999) and Roberts and Buikstra (2003), two clusters of cases can be defined, one in the eastern North America (Whitney, 1886; Lichtor and Lichtor, 1952; Ritchie, 1952; O'Bannon, 1957; Crane and Griffin, 1959; Morse, 1961; Anderson, 1964; Wright and Anderson, 1969; Perino, 1971; Nash, 1972; Seet, 1976; Buikstra, 1977; Katzenberg, 1977; Perzigian and Widmer, 1979; Cook, 1980; Rathbun et al., 1980; Buikstra and Cook, 1981; Widmer and Perzigian, 1981; Milner, 1982; Walker, 1983; Pfeiffer, 1984; Murray, 1985; Eisenberg, 1986; Williams and Snortland-Coles, 1986; Kelley and Eisenberg, 1987; Milner et al., 1988; Powell, 1988; Milner and Smith, 1990; Powell, 1990; Pfeiffer, 1991; Garten, 1997) and the other in the Southwest (Hooton, 1930; Morse, 1969; El-Najjar, 1979; Fink, 1985; Micozzi and Kelley, 1985; Ortner and Putschar, 1985; Sumner, 1985; Akins, 1986; Stodder, 1990; Lahr and Bowman, 1992; Regan et al., 1993; Stodder, 1996). The eastern North America examples are more widely distributed than those of the Southwest. A few cases have

also been reported near Mexico City (Cuesta, 1982) as well as some from West Mexico (Gill, 1971). The sites from near Mexico City date to the late precontact and early contact periods, with one of the west Mexican sites being 12th century while the other dates to ~AD 1300. To some degree, the larger numbers of remains from North America may be an artifact of the large number of paleopathologists working in this area.

Mesoamerica. Interestingly, there are virtually no skeletons presenting classic lesions of TB from Mesoamerica. Large collections of remains have been excavated, and while the notorious poor preservation within this region may be invoked, one might expect at least a few cases of ankylosed, dense Pott's disease. Thus, this virtual absence appears enigmatic, given the presence of large population clusters contemporary with North and South American expressions of the disease. Buikstra (1999) and Roberts and Buikstra (2003) speculate about trade routes that bypassed Mesoamerica, the burial of "deformed" individuals in remote areas or perhaps destructive burials customs for those of distinctive physical conditions. In Wilbur et al. (2008), as summarized here in *Coevolution of Humans and M. tuberculosis* Section, a novel explanation invoking diets low in iron is presented.

Key unresolved issues in considering American TB include (1) how we explain the absence of ancient TB in Mesoamerica, given the South and North American examples; (2) why the earliest cases are in South America, when humans migrated North to South; (3) the genetic relationship between the North and South American forms of TB; (4) the genetic relationship between the American and Old World ancient forms, and (5) the course taken by the American form of TB following the Era of Exploration. The answers to these and other related questions will require the fusion of molecular and bioarchaeological approaches.

Recognizing leprosy in archaeological remains (for extensive reviews, see Aufderheide and Rodriguez-Martin (1998), Ortner (2003), and Roberts and Manchester (2005)).

Identification of leprosy in skeletal remains recognizes the fact that the bacilli are first directly inhaled into the facial area, with the sensory, motor, and autonomic nerves also being indirectly involved. Bone changes occur in about 5% of people with untreated leprosy (Resnick and Niwayama, 1995), but the changes that develop depend on the type of immune response. Low resistance to *Mycobacterium leprae* will predispose to lepromatous leprous bone changes, those most often seen in archaeological contexts and usually involving the facial bones, the bones of the hands and feet, and the tibia and fibula; bone changes are usually bilateral and symmetrical. However, high resistance to the bacteria (tuberculoid leprosy) may not predispose to any bone changes, but if such changes are present, they may consist solely of hand and/or foot bone changes. These are usually unilateral, or if bilateral, they are asymmetrically expressed. The bone changes recognized in the skull include absorption and remodeling of the margins of the nasal aperture, recession of the alveolar process of the maxilla, loss of the anterior maxillary teeth, inflammation of the palatal surfaces, and loss of the anterior nasal spine. The indirect effects of the bacilli on the nerves can lead to osteomyelitis, septic arthritis, concentric atrophy, and/or absorption of the hand and foot bones, dorsal tarsal exos-

toses due to foot drop, palmar grooves in the proximal hand and foot phalanges due to claw hand and foot deformity, and "pencil" of metatarsals and metacarpals. Many of the bone changes in leprosy can be caused by other pathological conditions, and thus, diagnosis can be challenging.

Skeletal studies of leprosy in the Old and New Worlds

The earliest evidence of leprosy in historical documents is from India in the Sushruta Samhita, dated to 600 BC (Dharmendra, 1947), and a little later from China in a 3rd century BC bamboo book (Skinses, 1980). As we summarize below, all the pre-16th century archaeological and historical records for leprosy originate from Old World contexts. There is no skeletal evidence for leprosy in the pre-Columbian New World. This indicates that leprosy's origins should be sought in the Old World, with spread to the Western Hemisphere following the Era of Exploration. It has been apparent that, at the time of European voyages to the east coast of the Americas in the late 15th century, leprosy was a declining disease in Europe, and it is unlikely that lepromatous leprosy would have reached the Americas via European contact (Roberts and Manchester, 2005). As Browne (1970) in Ortner (2003) indicates, because leprosy is one of the least contagious of the transmissible infectious diseases, if it had been transported across the Atlantic, it would be unlikely to have had as severe an impact on the native populations of the Americas as other infectious diseases. As in our discussion of TB, we can divide the data into three broad areas in the Old World, "Northern Europe," the "Mediterranean" and "Asia," which reflect similar climate and environmental features.

The Mediterranean. Based on historical data, it has long been suggested that leprosy was brought to the Mediterranean from the Indo-Gangetic basin by the armies of Alexander the Great (356–323 BC) returning from the Alexandrian campaign. However, there is no evidence for leprosy in any human remains analyzed from India, although evidence from skeletal and mummified remains has been observed in Israel, and Egypt and Nubia. Dzierzykra-Rogalski (1980) described leprosy in four individuals dated to 250 BC from the Dahkleh Oasis of Egypt, and Molto (2002) discussed more recent results of excavations at the Roman Kellis 2 cemetery, specifically two males with leprosy dated to the early-mid 4th century AD. In Nubia, Elliot-Smith (1908) described leprosy in the hands and feet of a 4th–7th century AD Coptic mummy, excavated in 1907 from cemetery 5, which was located a short distance from the temple of Biga, near Aswan, Nubia. Later, Møller-Christensen and Hughes (1966) discussed leprotic skeletal changes in another skull from the same cemetery (~AD 500).

In evaluating the evidence from Israel, we underscore that although leprosy was long thought to have been described in biblical texts, it is now argued that this derivation came about because of a poor translation of the Hebrew word *Tsar'ath*. This term is now accepted as being associated with any disfiguring skin disease, and therefore the association of leprosy with a punishment for impurity and sin, transmission by sexual intercourse, and stigma and ostracism cannot be supported as originating in biblical texts. In fact, there is little evidence for leprosy in human remains from Israel, as reviewed

by Zias (2002). However, Zias does describe skeletal remains with leprosy changes from a mass grave representing an estimated 300 individuals at the Monastery of St John the Baptist in the Judaeen Desert and dated to the 9th century AD. Dating to the same period (AD 300–600), another skeleton was identified from Bet Guvrin with leprosy (Manchester, 1993), and Donoghue et al. (2005) report that aDNA analysis indicates that the person had harbored leprosy during life.

In France, Blondiaux et al. (1994, 2002) discuss bone changes of leprosy in two individuals dated to AD 500 in northern (Neuville-sur-Escaut) and southern (Vaison-la-Romaine) France. In more recent years in Italy, Mariotti et al. (2005) have reported a 3rd–4th century AD skeleton with probable leprosy bone changes from Casalecchio di Reno in Bologna, and Belcastro et al. (2005) describe a young adult male with leprosy from the 7th century AD necropolis of Vicenne-Campochiaro, Molise. There currently is no evidence for leprosy in Greece, Jordan, or Portugal, but Angel (1969) described what appears to be a skeleton with bone changes of leprosy in Turkey dated to 2700–2300 BC. If accepted, this would be the oldest case of leprosy yet identified.

Northern Europe. In Northern Europe, the evidence for leprosy in human remains is plentiful in some countries, again reflecting bioarchaeological activity levels and preservation quality. In the British Isles, the earliest evidence for leprosy dates to the early-Medieval period. The skeletal data for Britain are summarized in Roberts (2002) and illustrate that most individuals presenting skeletal evidence for leprosy were buried in nonleprosarium cemeteries. The data also show evidence for leprosy increasing from the early Medieval (5th–mid to 11th century AD) through the late-Medieval period. The evidence is geographically located from York in the north and as far south as the Isles of Scilly off the south-west coast of England, for example, at Eccles in Kent (Manchester, 1981) and Cannington in Somerset (Brothwell et al., 2000). However, it is in the late-Medieval period (mid-11th to mid-16th century AD), prior to the malady's decline beginning in the 14th century that leprosy is even more prevalent [see also Rawcliffe (2006) on leprosy in medieval England from an historical perspective]. Individuals with leprosy bone changes are geographically located from the Orkney Islands in Scotland to Chichester in Sussex, southern England (Magilton et al., 2008), for example, at St. John, Timberhill (Anderson, 1998) and in Ireland at Armoy, County Antrim (Murphy and Manchester, 2002). At this time, many leprosaria were founded in Britain (e.g. Magilton et al., 2008), with more than 200 being established between the 11th and 16th centuries AD (Roberts, 1986).

There are two examples of skeletal leprosy in a post-medieval context (AD 1550–1850), recovered from Bristol in southwest England and from London (Roberts and Cox, 2003; Walker, 2008). However, leprosy appears to have survived in Scotland until the late 19th century, which is a lot later than in England. Given that leprosy appeared later in Scotland than the southern United Kingdom, with later survival, perhaps due to trade connections with Scandinavia, the foundation of new leprosy hospitals only occurred there after the 14th century. Leprosy was present in Scandinavia until the middle of last century although not in high numbers; 10,000 cases being recorded in Norway, Sweden, Finland, and Iceland between 856 and 1956 (Richards, 1977). In Denmark, of

course, there is much evidence for leprosy in skeletal remains, but it is focused in the late-Medieval period.

Møller-Christensen (1953, 1967, 1969) published widely from his research on skeletal remains with leprosy bone changes recovered from the 13th to 16th century leprosarium of St. Jørgen, Naestved. In the more recent years, other authors have brought to our attention further evidence for leprosy. Boldsen and Møllerup (2006) record leprosy in the nonleprosarium cemeteries of Odense dated from AD 1000 to AD 1400, after which there was a dramatic decline, as seen in other Northern European countries. Boldsen (2005) also describes leprosy in the mid-12th to mid-14th century cemetery of Tirup. For Finland, there is no evidence of skeletal leprosy, but the first historical sources recount a leprosy hospital in AD 1355 (Vuorinen, 2002); environmental factors probably account for the lack of skeletal evidence for leprosy due to nonsurvival of remains for identification, as is the case of TB (Vuorinen, personal communication). In Sweden, Arcini (1999) describes a 10th century skeleton with leprosy from Lund, but there is no evidence of leprosy in any skeletal remains yet from Iceland, Norway, or Switzerland.

In Germany, Boldsen (2008) describes leprosy in the early-medieval site of Lauchheim in southern Germany and dated to the second half of the 5th century AD to around 680, and Schmitz-Cliever (1972) discusses excavation and analysis of late-medieval skeletons with leprosy from a leprosarium in Aachen. Haas et al. (2002) also report on two female skulls with leprosy changes from an ossuary at Rain/Lech in Southern Germany dated between 1481 and 1632 AD. In Eastern Europe, Strouhal et al. (2002) document only one individual with leprosy bone changes from an ossuary at Křtiny, near Brno (13th–18th centuries AD), in the Czech Republic, although leprosy hospitals are documented from the late-Medieval period. More recently, however, Likovsky et al. (2006) record two individuals with leprosy from the 12th century Romanesque church cemetery of Chelčického nám at Zatec (north-west—Bohemia). In Hungary, Pálfi et al. (2002) describe the extant skeletal evidence for leprosy in Hungary as commencing in the 10th century AD with an elderly female affected at the cemetery of Sárrétudvari-Hízófold (Pálfi, 1991), with further possible cases from the 10th–11th centuries (Puspokladany) and the 14th and 15th centuries (Opusztaszter). More recently, Marcsik (2007) provides 7th–9th century evidence for leprosy, thus pushing back the date for the origin of leprosy in Hungary. Poland has less evidence; for example, Gladykowska-Rzeczycka (1976) describes a late-medieval case of leprosy in a male from Suraz, Lapy County, Poland. In Russia and the Former Russian states, Buzhilova (2002) has documented through historical evidence the presence of leprosy at the end of the 19th and beginning of the 20th centuries. Nevertheless, the only skeletal evidence for this “area” is that of a middle-aged female skeleton dated to the early 1st millennium AD found in a burial mound (kurgan) at Devkesken 6 on the Ustyurt Plateau (between the Aral Sea and an inlet of the Caspian Sea) in Northern Uzbekistan (Blau and Yagodin, 2005). Finally, Kozak and Schultz (2006) describe 10th century skeletal evidence for leprosy in Kiev in the Ukraine.

Asia. Despite early historical sources suggesting the presence of leprosy in India and China, no evidence from human remains have been found in either country. Fur-

thermore, there have been no reports from Australia, New Zealand, or virtually any of island Southeast Asia or Polynesia. However, in Thailand, Tayles and Buckley (2004) describe two probable leprosy adult males from the site of Noen U-Loke in north-east Thailand dated to 300–200 BC and the first two centuries AD, and Tremblay (1995) reports on at least six individuals with leprosy from pre-Spanish contexts on the islands of Guam and Saipan in western Micronesia (radiocarbon dated to between the 7th and 15th centuries AD). Finally, Hirata (2000) describes leprosy in Medieval Japan.

Summary of skeletal evidence for leprosy

The skeletal evidence for leprosy in human remains in the Old World is quite extensive but, as for TB, there are many parts where leprosy is absent. Again, this probably reflects a number of factors already discussed with regard to TB. On the basis of the skeletal evidence, leprosy has an early focus in the Mediterranean and Northern European areas, and specifically Egypt and Nubia, and the British Isles. If the data from Thailand are accepted then an early focus for leprosy is also noted there. There are later appearances of leprosy in Micronesia and other parts of Northern Europe and the Mediterranean. However, it is not until (again) the later Medieval period that we see a rise in the frequency of the disease in Northern Europe. Like TB, it appears to be associated with urban living and poverty, although its presence in early-Medieval Britain where settlements were rural in nature correlates with the fact that it is a disease of rural communities today. Leprosy declined from the 14th century onward in Europe, in particular, possibly due to the rise of TB and the nature of cross immunity between the two infections (Chaussinand, 1953; Manchester, 1991) but this continues to be debated (Leitman et al., 1997; Wilbur et al., 2002). Of course, it has been maintained in some parts of the Old World today such as Asia.

THE PHYLOGENY OF MYCOBACTERIA

In the late-19th century, the causative agents of two of the most devastating diseases known to humans were identified: Hansen (1874) described the bacillus causing leprosy, while Koch (1882) identified the TB bacillus. In 1896, Lehmann and Neumann proposed the genus *Mycobacterium* to differentiate *M. tuberculosis* and *M. leprae* from closely related organisms (Goodfellow and Magee, 1998). Extensive research in subsequent decades elucidated the biochemical and immunogenic properties of these organisms. With the advent of the AIDS crisis in the 1980s, opportunistic infections from typically harmless environmental mycobacteria highlighted the pathogenic abilities of this largely overlooked class of mycobacteria. During the same period, advances in molecular genetics techniques made it possible to define species and study their evolutionary relationships based on genetic sequences. For example, 16S rRNA sequences have aided in the identification of new species (Boddington et al., 1990; Tortoli, 2003), the sequencing of several mycobacterial genomes (e. g. Cole et al., 1998, 2001; Garnier et al., 2003; Stinear et al., 2008) has facilitated studies of genome structure, evolution, and biology in these organisms, and molecular epidemiology has allowed a better understanding of the spatial patterning of variation within *M. tuberculosis* (Gutacker et al.,

2002; Filliol et al., 2003, 2006; Baker et al., 2004; Gutierrez et al., 2005; Gagneux et al., 2006). However, many questions remain regarding the origin, evolution, and future coevolutionary trajectory of mycobacteria and humans.

Evolutionary studies of the genus *Mycobacterium*

At present, the genus *Mycobacterium*, which is the single genus within the family of *Mycobacteriaceae*, comprises over 100 species including several that are pathogenic in humans and animals. Mycobacteria are distinguished from most other bacteria in the order *Actinomycetales* by the ability to synthesize mycolic acids. Members of the closely related *Corynebacteriaceae*, *Gordona*, *Nocardiaceae*, and *Tsukamurella* families also synthesize mycolic acids, but these have a simpler structure compared to mycobacteria. Mycobacteria are gram-positive and have been traditionally classified based on phenotypic characteristics, including mycolic acid types, slow or rapid growth, and presence or absence of pigmentation (Runyon, 1959). However, these methods can result in misidentification and underestimation of mycobacterial species diversity (Springer et al., 1996).

The phylogeny of the genus *Mycobacterium* based on molecular evidence is not yet clear, with many species examined at only one locus and weak statistical support for the phylogenetic relationships. In general, molecular phylogenies of bacteria have been based on 16S rRNA gene sequences (Woese and Fox, 1977; Fox et al., 1980; Woese, 1987; Woese et al., 1990). However, many bacterial strains that are identical at 16S rRNA (including members of the *M. tuberculosis* complex) have been classified as separate species based on phenotypic analyses (Fox et al., 1992; Drancourt et al., 2000; Hamid et al., 2002) and many internal branches are unresolved or have low-statistical support (Pitulle et al., 1992; Clayton et al., 1995; Roth et al., 1998; Adekambi and Drancourt, 2004). The molecular phylogenies generated to date, however, also can show different topologies with different methods and/or different gene sequences. Discrepancies also exist when genotypic- and phenotypic-based mycobacterial phylogenies are compared (Springer et al., 1996; Brown-Elliott and Wallace, 2002), although phylogenies based on 16S rRNA gene sequences do support a division between fast and slow-growing species (Rogall et al., 1990; Stahl and Urbance, 1990). Another weakness is that few studies have included most or all of the mycobacterial taxa identified to date, focusing instead on particular taxa of clinical relevance or on a particular group within the mycobacteria. As a result, the closest relatives or outgroup taxa for both the TB complex and *M. leprae* are unclear. Finally, these studies have typically not included analyses of evolutionary rate differences among lineages.

The *ad hoc* committee for the re-evaluation of the species definition in bacteriology has recommended that phylogenetic data be obtained from a minimum of five housekeeping genes (Stackebrandt et al., 2002), and some studies are even using hundreds of gene sequences generated from genome sequencing (Daubin et al., 2002; Coenye et al., 2005; Gevers et al., 2005; Gophna et al., 2005; Hershberg et al., 2008). Housekeeping genes are those that encode proteins used in basic cell functions. However, to date, few studies of mycobacteria have examined more than one housekeeping gene and even fewer have investigated whether these genes are subject

to stabilizing selection and thus, evolve at a relatively constant rate across species. Genes that have been examined in mycobacteria include the DNA gyrase genes (*gyrA* and *gyrB*), chaperonin GroEL (now known as *GroEL* but also called 65 kDa antigen, *Cpn60-2* or *hsp65*), superoxide dismutase (*sodA*), RNA polymerase B (*rpoB*), recombinase A (*recA*), the 32 KD protein (now called *fbpA*), and chaperone protein (*dnaJ*) (e. g. Takewaki et al., 1993; Zolg and Philippi-Schulz, 1994; Soini and Viljanen, 1997; Gingeras et al., 1998; Ringuet et al., 1999; Blackwood et al., 2000; Dauendorffer et al., 2003; McNabb et al., 2004; Richert et al., 2005). The focus of most of these studies has typically been species identification for clinical purposes rather than resolving the relationships between taxa. However, Kasai et al. (2000) examined 15 species of slow-growing mycobacteria using 1.2 kb of the *gyrB* gene. They constructed an unrooted neighbor-joining tree using a distance matrix generated with the Kimura two-parameter model (Kimura, 1980), but found only a few branches with bootstrap values >50% (these included the MTBC and the *M. avium* complex branches). More recently, Adekambi and Drancourt (2004) used complete sequences from the 16S rRNA, *recA*, and *rpoB* genes and partial sequences from *hsp65* (*GroEL*) and *sodA* to examine the relationship between 19 species of fast growing mycobacteria. In the combined analysis (~7,000 bp), they were able to resolve most interior branches with >88% bootstrap support. The three branches leading to *M. immunogenum*, *M. mageritense*, and *M. wolinskyi* and their respective sister groups were supported by low-bootstrap values (55%, 35%, and 44%, respectively). A conditional combined analysis after using the incongruence length difference test (Farris et al., 1994) was conducted with only the *recA* and *rpoB* sequences; here, two of these branches were better resolved although another branch (*M. porcinum* and its sister taxa) was less resolved (46%). Both of these analyses used all coding positions and neither study used relative rate tests to examine whether the molecular clock hypothesis can be rejected.

Lateral or horizontal gene transfer may also affect the phylogeny of bacteria (e.g. Dykhuizen and Green, 1991; Smith et al., 2000; Ochman et al., 2000; Daubin et al., 2003; Gogarten and Townsend, 2005). Whole genome comparisons between bacteria suggest that only a small percentage (~3%) of the *M. tuberculosis* genome is foreign (Ochman et al., 2000), although Nakamura et al. (2004) estimated that ~12% of open reading frames had undergone lateral gene transfer. Gamielien et al. (2002) even indicated that 19 genes in *M. tuberculosis* had been acquired from eukaryotes, although in a re-examination of the data, Kinsella and McNerney (2003) suggested that stronger statistical support is necessary and that alternative explanations were more likely. In a study of genes involved in fatty acid synthesis in *M. tuberculosis*, Kinsella et al. (2003) found that five of the eight gene families were more closely related to α -proteobacteria than expected. Most analyses of *M. tuberculosis* and *M. bovis* have suggested that these organisms are primarily clonal (Smith et al., 2003; Supply et al., 2003; Smith et al., 2006; Hershberg et al., 2008). However, Krzywinska et al. (2004) found evidence for lateral gene transfer and homologous recombination between *M. avium* strain sequences from the glycopeptidolipid biosynthesis gene cluster. Recently, Liu et al. (2006) examined synonymous single nucleotide polymorphisms (SNPs) in strains of *M. tuberculosis* and presented evidence for a recombination

hotspot between MT0103 and MT0105, a PPE protein gene that may be involved in virulence or evading the host immune system. However, in the TB genome as a whole, the estimated recombination rate was very low.

In sum, phylogenetic studies of species within the *Mycobacteriaceae* that include multiple housekeeping genes are necessary to identify the closest relatives of pathogenic species and to provide a robust framework for investigating questions about the extent of lateral gene transfer, the congruence with phylogenies generated using morphological characters, and the ecological differentiation between species (including changes that lead to pathogenicity in humans).

Evolutionary studies of the *M. tuberculosis* complex

Until recently, genetic analyses indicated that a population bottleneck of the progenitor strain of the *MTBC* 15,000 to 20,000 years ago coincided with a speciation event that ultimately produced the five extant members of the *MTBC* (Kapur et al., 1994). This analysis was based on short DNA sequences from four genes in 31 *M. tuberculosis* isolates, and little genetic variation was found. It was also assumed that the progenitor strain was more closely related to *M. bovis*, which has a broad host range, than it was to *M. tuberculosis sensu stricto*, which is a pathogen almost exclusive to humans. Current investigations, however, support a different evolutionary sequence. These analyses indicate that the progenitor strain of the *MTBC* was a human pathogen from which other species, including *M. bovis*, diverged, and they suggest a much older age for the origin of *M. tuberculosis* (Brosch et al., 2002; Mostowy et al., 2002; Garnier et al., 2003; Baker et al., 2004; Gutierrez et al., 2005).

Gordon et al. (1999) were the first to suggest that *M. tuberculosis* did not evolve from *M. bovis* using genetic evidence. They identified seven deletions in *M. bovis* relative to *M. tuberculosis*. Using these, as well as two previously identified deletions, they examined their presence in isolates of the TB complex. Gordon et al. (1999) found that *M. tuberculosis* and *M. africanum* were most closely related, while *M. microti* was more distantly related and that *M. bovis* was further distant from *M. tuberculosis* but closely related to *M. microti*. Thus, they concluded that a simple evolution of *M. tuberculosis* from *M. bovis* is unlikely, but that they both evolved from a common ancestor.

Initial DNA analyses found little sequence variation within *M. tuberculosis* that is useful for phylogenetic analyses. Sreevatsan et al. (1997) examined sequences from 26 structural genes in strains of *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*. They assigned strains to three groups based on one polymorphism in the catalase-peroxidase gene, or *katG* (a T to G substitution causing a change from leucine to arginine at codon 463) and one polymorphism at the DNA gyrase subunit A gene, or *gyrA*, (a C to G substitution at codon 95 causing an amino acid change of threonine to serine). Although both changes are nonsynonymous and located in genes linked to antibiotic resistance, these particular sites do not appear to be involved in resistance (Takiff et al., 1994; Rouse et al., 1996). Group 1 included strains of all four species, while groups 2 and 3 included strains only from *M. tuberculosis*. Because group 1 strains have the highest amount of variation in copy number of the repeat element, IS6110, and in synonymous substitu-

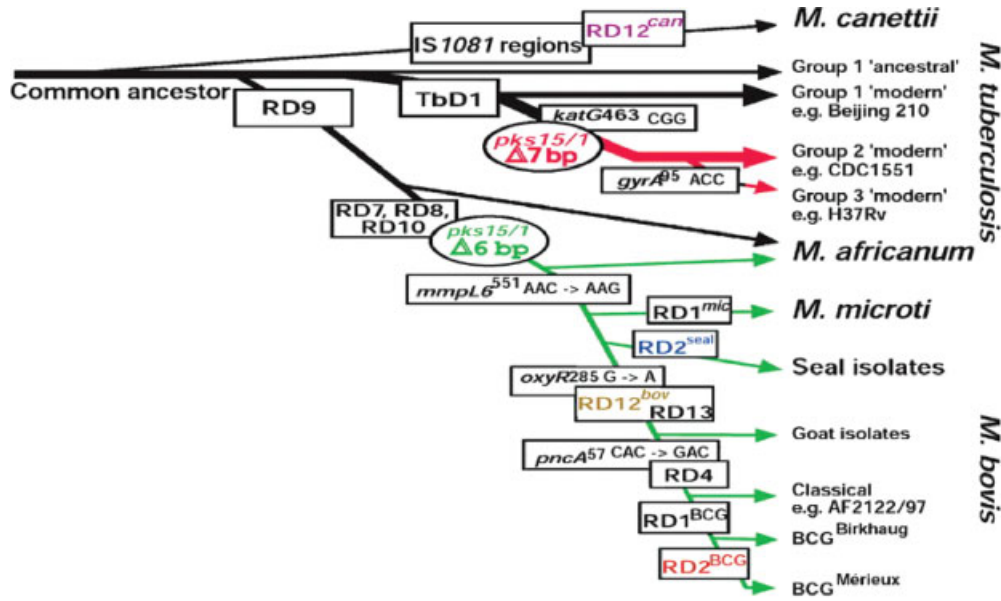


Fig. 1. Depiction of the relationships between members of the TB complex [from Brosch et al. (2002) and Marmiesse et al. (2004)]. Boxes indicate deletions or sequence changes. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tions, Sreevatsan et al. (1997) suggested that they are most ancestral. Musser et al. (2000) also found limited DNA variation in 24 genes in 16 *M. tuberculosis* strains.

Although Sreevatsan et al. (1997) and Musser et al. (2000) did not question the hypothesis that *M. tuberculosis* evolved from *M. bovis* and assumed a recent origin, the analyses of Brosch et al. (2002) and Hughes et al. (2002) did question this, and Hughes et al. (2002) were the first to suggest that the age of *M. tuberculosis* was older than 15,000–20,000 years. Hughes et al. (2002) compared synonymous substitutions between the two complete *M. tuberculosis* genomes, H37Rv and CDC1551 to estimate a divergence time of ~35,000 years. Because this estimate is based on two lineages within the species that are not, in fact, the most divergent from each other, the divergence time of the last common ancestor of *M. tuberculosis* from other mycobacteria is likely much older.

Brosch et al. (2002) investigated 20 insertion–deletions in 100 diverse isolates of the *MTBC*. From their results, they proposed that *M. canettii* and *M. tuberculosis* were more ancient, whereas the *M. microti*, *M. africanum*, and *M. bovis* lineage is more derived (see Fig. 1). Within *M. tuberculosis*, a 2,153-base-pair segment of DNA that includes the *mmpS6* gene and part of the *mmpL6* gene was deleted in almost 90% of the strains tested. The strains without this deletion, named *M. tuberculosis* specific deletion 1 (TbD1), have fewer copies of IS6110 and are primarily of African and Indian origin. Brosch et al. (2002) noted that strains in all three groups defined by Sreevatsan et al. (1997) have TbD1, while all strains without this deletion fall into group 1. These results were supported by analyses of additional samples (Marmiesse et al., 2004) as well as additional deletions (Mostowy et al., 2002). An analysis of 68 deletions using microarrays has also noted several large phylogeographic clusters of lineages within *M. tuberculosis* and suggests that these are stably associated with host populations (Hirsh et al., 2004).

Gutaker et al. (2002) identified 230 synonymous SNPs (sSNPs) using two complete *M. tuberculosis* genome sequences (H37Rv and CDC1551), one partial *M. tuberculosis* genome sequence, and the complete *M. bovis* genome sequence. These sSNPs were then examined in 432 TB complex strains. On the basis of the analysis of 148 confirmed sSNPs in a subset of isolates selected to represent the diversity of *M. tuberculosis*, Gutaker et al. (2006) found eight major clusters of related genotypes (see Fig. 2). More recently, Gutaker et al. (2006) used 36 of these sSNPs to examine over 5,000 strains from patients and found that these could be grouped into nine genetic clusters (cluster II was divided into two groups). Filliol et al. (2006) used four complete genomes (*M. tuberculosis* H37rV, CDC1551, and 210 and *M. bovis*) to identify 212 SNPs, which they then analyzed in a global collection of 323 *M. tuberculosis* and *M. bovis* strains (see Fig. 2). Cluster analysis identified one group of *M. bovis* lineages and six major groups of *M. tuberculosis* lineages (see Fig. 2). The most ancestral group, cluster 1, was common in patients from India. Thus, Filliol et al. (2006) suggest that *M. tuberculosis* first arose in India. One limitation of these studies is that the identification of SNPs from only a few genome sequences has the potential to lead to ascertainment bias, where false results are produced by nonrandom sampling, particularly if the genomes are from closely related isolates (Alland et al., 2003; Pearson et al., 2004; Clark et al., 2005).

Baker et al. (2004) sequenced seven unlinked loci (*rpoB*, *katG*, *oxyR*, *ahpC*, *pncA*, *prnL*, and *gyrA*) in 317 strains of *M. tuberculosis*, four *M. bovis* isolates, two *M. africanum* strains, and one *M. microti* isolate. The strains were also characterized by resistance phenotype, IS6110 repeat element profile and typing of the direct repeat (DR) locus. Of the 8,318 bp sequenced, 115 variable sites were found of which 36 were silent mutations. These sSNPs were used for phylogenetic analyses, which identified four major *M. tuberculosis* lineages as well as

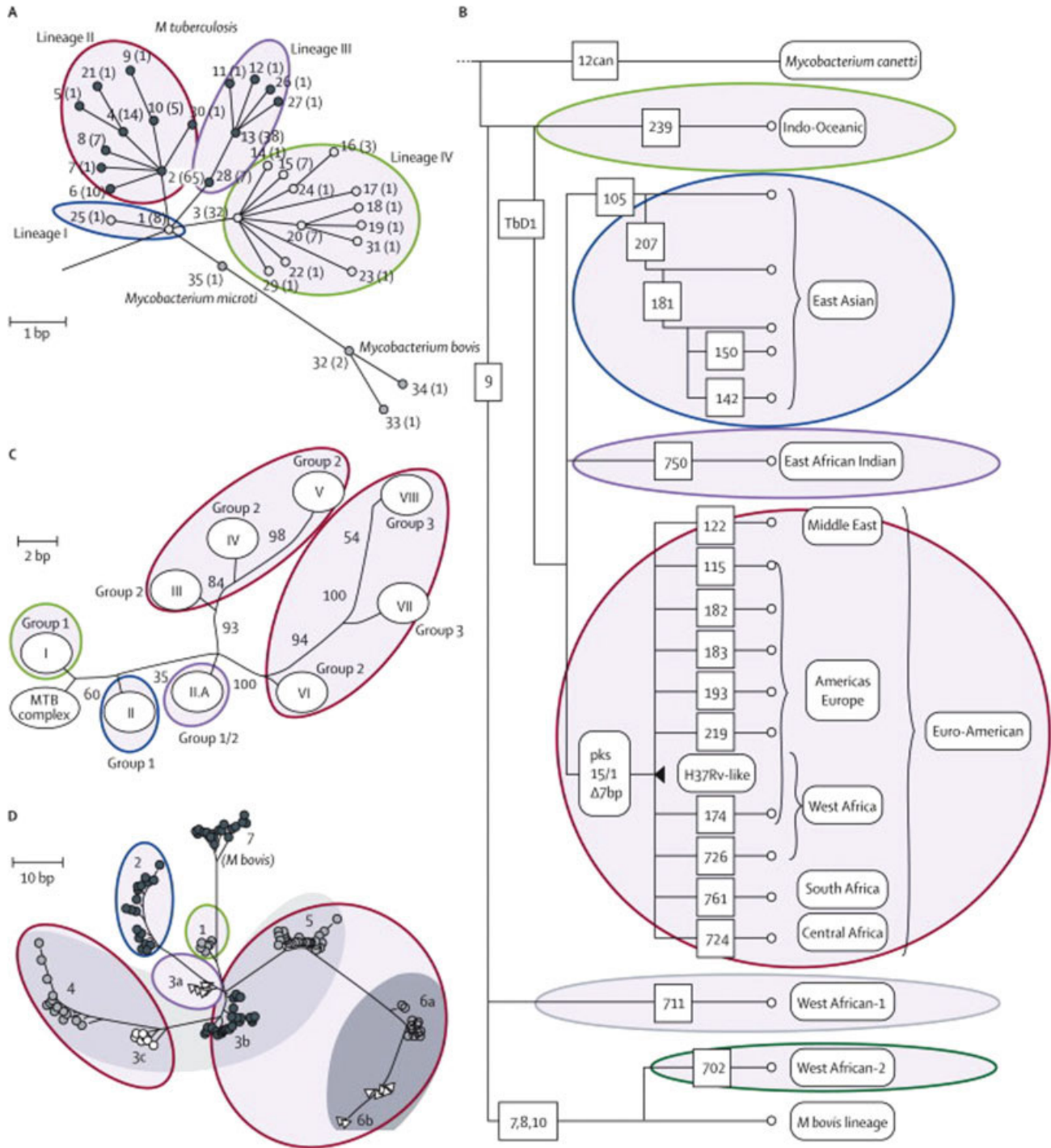


Fig. 2. The colors indicate corresponding lineages in the four phylogenies (Gagneux and Small, 2007). **A:** Figure is adapted from Baker et al. (2002). Numbers next to nodes refer to specific synonymous SNP combinations. Numbers in parentheses refer to number of isolates. **B:** Figure is adapted from Gagneux et al. (2006). Numbers in rectangles refer to specific deletions. **C:** Figure is adapted from Gutaker et al. (2006). Group refers to those described by Sreevatsan et al. (1997), roman numerals refer to phylogenetic lineages and numbers on the branches indicate bootstrap values. **D:** Figure is adapted from Filliol et al. (2006). Numbers refer to phylogenetic lineages. The light gray and dark gray show strains belonging to Sreevatsan et al. groups 2 and 3, respectively.

a *M. bovis*/*M. microti* lineage (see Fig. 2). The *M. africanum* strains were indistinguishable from *M. tuberculosis* lineage I. Both the complete genome sequences (H37Rv and CDC1551) were found to have the same profile (#2) within lineage II, suggesting that they are closely

related. They note that lineage IV has the fewest number of IS6110 insertion elements, is the most widely distributed, and, like *M. bovis*, *M. microti*, and *M. africanum*, does not have TbD1, suggesting that it is the most ancient lineage of *M. tuberculosis*.

More recently, Hershberg et al. (2008) sequenced over 7 Mb of DNA from 108 strains in the MTBC to assess genetic diversity and the evolutionary history of the complex. This study focused on 89 housekeeping and antigen genes. Hershberg et al. (2008) found greater diversity in the MTBC than expected. In particular, they noted that human-adapted strains have as much diversity as the animal-adapted strains (including *M. bovis*, *M. pinnipedii*, *M. caprae*, and *M. microti*) and that the animal adapted strains form a subgroup within the human adapted strains. This supports previous studies indicating that the animal strains are more derived. Analyses of these data also indicate that the ratio of nonsynonymous substitutions to synonymous substitutions (dN/dS ratio) is high, but this is the result of reduced selective constraint that is likely due to serial bottlenecks, subdivided population structure, and reduced horizontal transfer (i.e. high clonality), which reduce the effective population size and amplify the effects of genetic drift over natural selection. Hershberg et al. (2008) also investigated the population history of the MTBC. They found that the data support an African origin of the MTBC (where two of the three most ancestral strain groups are found today) and suggest that the third group of ancestral strains common today in India and the Philippines spread from Africa during the initial migrations out of Africa by anatomically modern humans ~50,000 years ago. Subsequent to the spread of the ancestral lineages, "modern" lineages of the MTBC that also have the TBD1 deletion (Brosch et al., 2002), are hypothesized to have spread more recently as human population sizes increased. These strains then expanded more quickly and broadly, including to the Americas, subsequent to the Age of Exploration.

In addition to Brosch et al. (2002), a few studies have used deletion or insertion/deletion polymorphisms for evolutionary analyses within the MTB complex. Gagneux et al. (2006) investigated geographic structure in a worldwide sample of MTBC strains using large insertion/deletion polymorphisms and found six main lineages, confirming several of the previous lineage groups (Fig. 2). Although they are unable to date the origin of the MTBC, they indicate that it most likely arose in East Africa and has an older origin (i.e. prior to 10,000 years ago). They also suggest that these main lineages are associated with and adapted to specific human populations. Recently, Wirth et al. (2008) used data from 24 microsatellite loci known as mycobacterial interspersed repetitive units in 355 strains of MTBC to examine MTBC diversity and divergence times. Using phylogenetic analyses and STRUCTURE (Wirth et al., 2008), they were able to identify two major clades within the MTBC, one corresponding to human pathogens and clade two corresponding to both human and animal pathogens. Because these microsatellites primarily mutate according to the stepwise mutation model, they were able to estimate mutation rates from serial or epidemiologically linked strains. In addition, they assumed generation times of 1 day and used these measures to estimate a mean time to the most recent common ancestor (TMRCA) of ~40,000 years, with the TMRCA for clades 1 and 2 of 21,000 and 33,000 years, respectively.

Most of the phylogenetic analyses of the MTBC have not included significant sampling of non-TB or bovis lineages. In particular, the strains with the smooth colony phenotype (i.e. the colonies have smooth edges rather than rough) classified as *M. canettii* are not well studied.

Gutierrez et al. (2005) investigated 37 smooth tubercle bacilli using repetitive DNA markers. These strains were clustered in eight clonal groups. Of these, two groups (A and C/D) corresponded to *M. canettii* strains, while group B was closely related. The remaining groups were somewhat distinctive. They then sequenced segments of seven genes in isolates in all these groups as well as members of the TB complex. They found that the TB complex (not including *M. canettii*) forms a single bifurcating branch rooted within the much larger tree of smooth bacilli and they suggest that this branch of the TB complex is a recent, successful clonal population that derived from an ancient and diverse bacterial species (which they name *M. Prototuberculosis*). This phylogeny with *M. canettii* as the most basal branch of the MTBC was also supported by an analysis using insertion/deletion markers and SNPs (Huard et al., 2006). Gutierrez et al. (2005) also estimated the time to the most recent common ancestor of the tubercle bacilli as 2.6–2.8 million years ago based on the average pairwise distance of synonymous substitutions. Because most smooth tubercle bacilli isolated to date are from East Africa, they suggest that these bacilli emerged in Africa and may have affected early humans and spread with them around the world. However, Smith (2006) noted that recombination affects their estimation of diversity and casts doubts on their assertions that the MTBC derives from the smooth isolates and that it originates in Africa at an early date.

Great progress has been made during the last 10 years in understanding the diversity present in the MTBC and the relationships between different strains. This research raises interesting questions about the time depth that humans (and other primates) have been affected by TB, the relationship between domestication and the closely related *M. bovis* and *M. caprae* strains, and the diversity and host species of closely related "smooth colony" strains of the MTBC.

Evolutionary studies of *M. leprae*

In 2001, the Wellcome Trust Sanger Institute released the complete genome sequence of the Tamil Nadu, India (TN) strain of *M. leprae* (Cole et al., 2001). From an evolutionary perspective, the genome of *M. leprae* is highly unusual. It is smaller than that of other mycobacteria, and it has a large number of pseudogenes (~27% of the genome) and noncoding sequences (~23.5% of the genome). This paring down of the genome may reflect its niche, because such reductive genome processes are also seen in other obligate intracellular parasites and endosymbionts (Frank et al., 2002).

The genetic variation present within *M. leprae* is not well studied. Monot et al. (2005) sequenced 142 kb of DNA in a Brazilian strain and compared this to the genome sequence revealing five SNPs in noncoding and pseudogene regions. These SNPs were examined in seven strains of *M. leprae*, and two were found to be present only in the TN strain. The remaining three SNPs were found in two or more of the seven strains, and these were then examined in 175 strains from all over the world. Their results showed that there were only four SNP configurations that corresponded with the geographic origin of the strain. In addition, Monot et al. (2005) investigated VNTRs that were previously identified for molecular epidemiology. The SNP configurations did not, however, correspond with the variation found in the VNTRs. On the basis of the SNP configurations, the

authors hypothesized two evolutionary scenarios for the origin and global dissemination of leprosy. The two scenarios differ in that either East Africa or Central Asia provided the origin of the disease, with subsequent spread to the other region. After this, the disease then traveled to the New World and to Western Africa following its arrival in Europe and during the era of European colonization.

Although Monot et al.'s (2005) study is the first to use molecular analyses to investigate the population history of the species, the ascertainment bias in SNP discovery and the limited number of SNPs used does not allow statistically supported conclusions about the origins of leprosy. This work was criticized for a presumed estimate of origin during the Pleistocene with the dispersal of modern humans out of Africa (Pinhasi et al., 2006), although in truth, Monot et al. (2005) do not state a time of origin, as this type of inference is not possible with their data. Instead, they show the timing of human migrations as known from genetic, archaeological, and historical data to illustrate that leprosy distribution routes track known routes of human population movement. Pinhasi et al. (2006) conclude that a Holocene origin and distribution of *M. leprae* is more likely than one during the Pleistocene for three main reasons: (1) the lack of paleopathological evidence for leprosy before the development of urban life; (2) the timing of the appearance of evidence for leprosy is consistent with active spread of the disease during urbanization; and (3) the assumption that *M. leprae* requires high levels of human contact to be maintained. In reality, it is probably not possible to decide between a Holocene and an earlier origin given the current data; however, the lack of congruence between the SNPs and VNTRs may be more consistent with an older origin.

The florescence of studies on the biogeography and phylogeny of *M. leprae*, the MTBC, and the genus as a whole promises to shed light on the population history and evolution of mycobacteria and other pathogenic bacteria. Over the past 20 years, the application of molecular methods has aided greatly in this endeavor, though much remains to be done. Such necessary work includes expanding our knowledge about environmental mycobacteria, understanding the evolutionary forces affecting the distribution of mycobacterial species and ecotypes, and increasing our insight into the rate of mycobacterial evolution. In addition, these analyses provide the necessary context in which to place results from aDNA analyses.

ANCIENT BIOMOLECULES AND THE STUDY OF MYCOBACTERIAL DISEASES

The application of methods for studying ancient biomolecules has great promise for addressing questions about the evolutionary history of mycobacterial diseases. In particular, using DNA to diagnose infectious diseases in ancient remains is intriguing, because it holds potential to look at the evolution of an infectious agent, investigate the history of a global disease, and identify the causative agent in cases where skeletal or soft tissue signals are ambiguous. Sequence information from ancient pathogens may also provide information to help determine the causes of viral and bacterial strain variation in virulence.

Archived medical specimens and samples from individuals buried in permafrost have yielded pathogenic DNA from viruses such as the human papilloma virus, the

HIV, and the 1918 flu virus (Shibata et al., 1988; Taubenberger et al., 2005; Worobey et al., 2008). In older remains, recovery of DNA from pathogenic agents is especially challenging, because the total amount of viral or bacterial DNA present is likely to be relatively small even in a heavily infected person. Despite this difficulty, several studies have examined archaeological samples for the presence of bacteria such as *Yersinia pestis* (Drancourt et al., 1998; Raoult et al., 2000), *Escherichia coli* and *Corynebacterium* spp., *Salmonella enterica* serovar Typhi (Papagrigorakis et al., 2006), and *Treponema pallidum* (Kolman et al., 1999). Many of these results, however, are controversial based on phylogenetic comparisons with additional species/strains, the questioned utility of tooth- or bone-based aDNA methods to diagnose fatal bacteremias, and the possibility of contamination by soil bacteria (Gilbert et al., 2004; Bouwman and Brown, 2005; Barnes and Thomas, 2006; Shapiro et al., 2006). Nevertheless, an increasing number of reports chronicle successful amplification and analysis of DNA from ancient pathogens.

Ancient TB

Of particular interest to the paleopathological and bioarchaeological communities has been the study of ancient mycobacterial DNA. Leprosy and TB have plagued human populations for millennia, and their ability to leave characteristic lesions on skeletons render them ideal for studies of the appearance and distribution of the diseases in various world areas. TB researchers have made ample use of the presence of repetitive elements in the genome, diagnostic of a particular species or complex. Projects on *M. tuberculosis* have focused primarily upon the isolation of a fragment of the TB-complex specific repeat element, IS6110 (Thierry et al., 1990). This element is recognized as the gold-standard (e.g. van Embden et al., 1993; van Soolingen et al., 1998) for epidemiological typing of the MTBC and is present in 0–27 copies in the genome. These two characteristics, specificity for the *M. tuberculosis* complex and the presence of multiple copies in most strains, have made IS6110 the most popular target for ancient TB genome studies. A second repeat element, IS1081 (Collins and Stephens, 1991), has also been used for the identification of MTBC, as the IS6110 element is not present in all strains. IS1081 tends to occur in five or six copies per MTBC strain and has been helpful for identification of non-IS6110 containing MTBC strains.

In 1994, the recovery of a small (123 bp) fragment of the IS6110 insertion element from a calcified subpleural nodule in a 1,000-year-old mummy from Peru confirmed the presence of the *M. tuberculosis* complex in pre-Columbian South America (Salo et al., 1994). The fragment was later amplified from a diseased vertebra in an adolescent from Chile who died ~900 years ago (Arriaza et al., 1995). The recovery of IS6110 from 15th century Canadian, 11th–13th century late Woodland/Mississippian, and 11th century Middle Mississippian bone samples by Braun et al. (1998) subsequently established the presence of *M. tuberculosis* in pre-Columbian North America. More recently, *M. tuberculosis* was identified in two of 12 Andean samples examined for IS6110 (Konomi et al., 2002). In addition, they amplified a 441-bp fragment of the *hsp65* (*GroEL*) gene in seven of the 12 samples. Restriction fragment length polymorphisms (RFLPs) at this locus indicated the presence of mycobac-

teria found in soil and water. Finally, Bathhurst and Barta (2004) amplified IS6110 in a 16th century Iroquoian dog, and Raff et al. (2006) reported successful amplification of IS6110 *M. tuberculosis* DNA from ribs of seven Mississippian period human skeletons that evidenced signs of vertebral TB.

Most molecular studies of ancient TB, however, have focused on the Old World. To demonstrate the utility of the IS6110 fragment for aDNA analyses, Baron et al. (1996) tested whether IS6110 could be successfully amplified from 100-year-old bone samples collected at autopsy from patients diagnosed with TB. All three samples yielded positive results from affected bone tissue and two samples of unaffected bone from two of these individuals also gave positive results. In contrast, Barnes and Thomas (2006) were unable to successfully amplify MTBC DNA from bone samples from 18th to 20th century individuals whose cause of death was TB. Despite multiple tests of seven different primer sets targeting several genes and IS6110, no repeatable results were obtained from any of the 37 individuals tested.

To date, successful recovery of IS6110 has also been reported in samples from Austria (19th century), Borneo (pre-European contact), China (202 BC–AD 200), Egypt (2120–500 BC), England (Iron Age, Medieval), Germany (AD 1400–1800), Hungary (7th–8th and 17th–19th centuries), Israel (AD 600), Lithuania (15th–17th centuries), Scotland (17th–18th centuries), and Turkey (Byzantine) (Spigelman and Lemma, 1993; Taylor et al., 1996, 1999, 2005; Faerman et al., 1997; Donoghue et al., 1998; Haas et al., 2000a; Germaey et al., 2001; Mays et al., 2001, 2002; Fletcher et al., 2003a,b; Fusegawa et al., 2003; Mays and Taylor, 2003; Zink et al., 2003, 2005; Bachmann et al., 2008; Hershkovitz et al., 2008).

Two other types of mycobacterial loci have also been examined in a few published studies: single-copy gene sequences and a polymorphic DR region. The single-copy sequences include fragments of several different genes, and the analyses have attempted to confirm the presence of the MTBC to distinguish infections stemming from *M. tuberculosis* and *M. bovis* or to identify the phylogenetic group within *M. tuberculosis* to which a strain belongs. For example, Haas et al. (2000a) amplified a fragment of the GroEL gene from 7th to 8th and 17th century burials from Hungary to discern whether mycobacterial sequences were present. A positive PCR result indicated the presence of mycobacteria in 13 of 14 samples, and further assay with IS6110 confirmed the presence of *M. tuberculosis* complex DNA in eight of them. Direct sequencing of the fragment of the GroEL gene from three positive samples indicated that nontuberculous (environmental) mycobacteria (possibly *M. agri*) had been amplified in two of them and that MTBC had been amplified in one.

Many studies have focused on distinguishing between *M. tuberculosis* and *M. bovis*. Taylor et al. (1999) examined IS6110 and fragments of the *oxyR*, *rpoB*, and *mtp40* genes in two medieval bone samples from London. They found the wild-type sequence at *rpoB* (the region examined does not contain phylogenetically informative synonymous SNPs, which do not change the amino acid, but does contain nonsynonymous changes linked to antibiotic resistance) in both individuals. The *mtp40* and *oxyR* results were consistent with *M. tuberculosis* rather than *M. bovis*. Moreover, they were not able to amplify a *M. bovis* specific sequence (Rodriguez et al., 1995). Fragments of *oxyR*, *rpoB*, and *mtp40* were also used by Mays

et al. (2001) to analyze nine medieval English burials, and they found results in agreement with the presence of *M. tuberculosis* rather than *M. bovis*. Zink et al. (2003) have investigated the *mtp40* and *oxyR* loci in samples from Egypt as well as a *M. bovis* specific IS6110 flanking sequence. Of 25 individuals positive for IS6110, none produced clear results for the other markers. An analysis of an Iron Age individual with classic morphological indications of TB as well as positive results for IS6110 was not successful in attempts to amplify other loci, including fragments of the *oxyR*, *gyrA*, and *katG* genes as well as the RD7 deletion (Mays and Taylor, 2003); however, a subsequent analysis successfully investigated smaller fragments of the *oxyR* and *pncA* genes, a smaller fragment flanking the RD7 deletion, and a smaller segment of the IS1081 repeat (Taylor et al., 2005). IS1081 amplification was positive, confirming the IS6110 results. Analysis of *oxyR* and *pncA* sequences showed that the strain present was *M. tuberculosis* rather than *M. bovis*. In addition, they examined whether the RD7 deletion (also known as TB deletion 1 or TbD1) was present to distinguish which major phylogenetic group the strain belonged to. This indicated that the strain present in this Iron Age individual was related to modern strains in lineages 1, 2, and 3 (Baker et al., 2004). This analysis of the phylogenetic affiliation of an ancient strain of TB is one of only a few such studies to date. The first example of such a study was that by Fletcher et al. (2003a) who examined fragments of the *gyrA* and *katG* genes to show that the strains present in eleven individuals buried in crypts from 1731–1838 in a church in Hungary belong to groups 2 and 3 as defined by Sreevatsan et al. (1997).

In four Iron Age skeletons from South Siberia, DNA consistent with *M. bovis* was amplified (Taylor et al., 2007). If verifiable, this is the first evidence of skeletal TB from ancient *M. bovis*. The insertion sequences IS6110 and IS1081 were first used to screen the samples for the presence of *M. tuberculosis* complex DNA. The *oxyR* and *pncA* loci were then used to distinguish between *M. tuberculosis* and *M. bovis*, as these differ at base 285 and 159, respectively. The authors also report successful amplification of several regions of difference (RD) that help distinguish members of the complex.

Another type of repeat sequence, the polymorphic DR region, also known as the DR locus, has been investigated in a limited number of studies. The DR locus contains polymorphic short DRs interspersed with nonrepetitive spacers, and in addition to variation in the number of DRs, strains also vary in the presence and absence of particular spacers (Kamerbeek et al., 1997). Analysis of the DR locus uses a method known as spacer-oligonucleotide typing (spoligotyping). In spoligotyping, PCR primers target the DRs to amplify multiple fragments from this locus and then multiple oligonucleotides that hybridize to the different spacer sequences are used to discern patterns of variation. Taylor et al. (1999) used spoligotyping in addition to the analysis of the gene regions mentioned above. In this analysis, the medieval samples showed similar hybridization patterns although the results from the two vertebrae from the same individual differed slightly. From these results, Taylor et al. (1999) suggest that the ancient strains were more closely related to *M. tuberculosis* than *M. bovis*, in agreement with the other analyses. In 2001, Rothschild et al. reported recovery of *M. tuberculosis* complex DNA from a 17,000-year-old bison bone from the Natural Trap

Cave in Wyoming. They included a spoligotype analysis in their study and found that it matches most closely with modern *M. africanum*. Mays et al. (2001) obtained positive spoligotyping results for three cases, which also suggested the presence of *M. tuberculosis*. Of these, only one was examined twice from independent extracts and two somewhat different patterns were found. Spoligotyping of three remains from a Hungarian church crypt (Fletcher et al., 2003b) showed patterns 50 and 53. Strains with pattern 53 belong to Sreevatsan et al. groups 2 or 3 which is in agreement with data from the *katG* gene (Fletcher et al., 2003a). Strains with pattern 50 belong to group 2; however, the individual with this pattern had a *katG* sequence consistent with group 3. This may indicate allele dropout (i.e. fragments that do not amplify due to low quantity) for one or more spacers in the DR region. Zink et al. (2003) also used spoligotyping on 4,000-year-old Egyptian samples and obtained reproducible patterns in 12 individuals. The patterns from three of these were identical or most closely related to type 53, a basal type common in modern strains. Five samples appeared to be similar to a pattern found in *M. africanum*, whereas the remaining types were not found in the database but do appear to be more closely related to the *M. tuberculosis* types. The most recent ancient *M. tuberculosis* study from a 9,000-year-old submerged site in the Eastern Mediterranean (Hershkovitz et al., 2008) used the insertion sequences *IS6110* and *IS1081*, the *TbD1* and *RD2* deletion regions, and spoligotyping to assign *M. tuberculosis* as the causative agent of pathological skeletal remains at the site. Unfortunately, the claims of TB presence at this early site are not convincing from a paleopathological perspective or from a molecular genetics perspective. The skeletal lesions present are those indistinguishable from other infectious diseases, insufficient procedures to prevent contamination are reported, and the spoligotypes are not reproducible. Furthermore, the “consensus spoligotype” reported matches a modern spoligotype that is ubiquitous throughout today’s world.

Although the goal of subspecies and strain identification in ancient remains for phylogenetic analysis is an excellent one, spoligotyping is an inappropriate tool for this purpose. Spoligotyping is problematic both from an aDNA methodological perspective and from a phylogenetic perspective. As noted earlier, different patterns may result from independent replications of aDNA; this is likely the result of allele dropout where the various segments within the locus may or may not amplify due to low-DNA quality and quantity in independent PCRs. In aDNA research, absence of evidence is not evidence of absence, which makes interpretation of the hybridization patterns problematic, particularly when results are not consistent. In addition, spoligotyping has been found to be less informative for phylogenetic analyses than DNA sequencing or *IS6110* RFLP pattern analysis, because the evolution of the region is complex, involving deletion events, strand slippage during replication leading to duplication, point mutations, and *IS6110*-mediated mutation and may result in lineages that are identical by state but not by descent (van Embden et al., 2000; Warren et al., 2002; Gori et al., 2005; Filliol et al., 2006). Several studies have now shown that convergence is common even in modern epidemiological contexts (van der Zanden et al., 2002; Brudey et al., 2006; Filliol et al., 2006; Guernier et al., 2008; Mathuria et al., 2008). This an important issue to be considered when comparing an-

cient spoligotypes to patterns seen in modern epidemics: identity by state may be more common when strains are separated by longer time periods. Thus, spoligotypes generated from these ancient remains are essentially meaningless.

An additional problem with much of the ancient pathogen genetics literature is the lack of adherence to proper authentication procedures. A series of minimal criteria for scientific investigation of aDNA that focus primarily upon two essential issues, control of contamination and independent reproducibility of results, has been established in the ancient field as paramount (Handt et al., 1994, 1996; Richards et al., 1995; Stoneking, 1995; Cooper and Poinar, 2000). A recent survey of ancient pathogen DNA publications found that fewer than 90% of studies addressed even the most basic issues of contamination, and fewer than 85% of studies had their results independently replicated (Roberts and Ingham, 2007). Although some authors have conjectured that mycobacterial DNA may be more resistant to damage than DNA from other organisms (e.g. Zink et al., 2005), as yet no evidence to support this has been given, and the fact that some replication attempts with ancient mycobacterial DNA have been discordant or irreproducible would not be in support of this idea. Given the irreplaceable nature of the samples as well as the destruction and expense caused by aDNA analysis, it is crucial that future studies fully adopt the rigorous scientific procedures embodied by these authentication criteria, and further, that important and answerable problems and questions be addressed.

Ancient pathogen DNA studies have also been limited by the available data for modern strains. Although several phylogenetic studies offer new sequence-based markers for the phylogenetic analysis of *M. tuberculosis*, these markers are often not easily applied to ancient samples, because the information about where in the phylogeny each SNP falls, and the raw sequence data necessary for comparison to any data generated from ancient samples are not publicly available in some cases (i.e. not deposited in GenBank). In addition, at present, we know very little about genetic variation in the environmental mycobacteria, which are found in soil and water, and thus, are likely contaminants of ancient samples.

A final problem with ancient pathogen DNA research is the issue of optimal sampling from preserved remains. It is unclear whether MTBC bacteria should be found at detectable levels in all tissues of an ancient infected person. Experimental evidence available from Lurie’s (1964) rabbit studies indicate that the number of *M. tuberculosis* bacilli depends on the location in the body, with the highest numbers typically in the lungs and very low amounts in bone marrow. Optimal sampling would obviously occur in areas in which mycobacteria were directly replicating. In humans, this is typically in the vertebral areas or those adjacent to other mycobacterial abscesses. Proliferative bone on the interior aspects of ribs have also been associated with TB, but these are typically due to inflammatory processes secondary to pulmonary infection and may be found in severe pulmonary diseases other than TB, TB of the rib is rare (Tatelman and Drouillard, 1953), and in clinical contexts, it is usually bone destruction of a single rib that is reported and, of course, is visualized on radiographs. This makes sense if TB is transmitted haematogenously from its initial focus in the lung or gastrointestinal tract (as is seen in the de-

structive lesions of Pott's disease of the spine). These expectations are supported by the work of Mays and colleagues (2002) who reviewed the clinical literature on the types of rib lesions found in TB and then tested archaeological ribs with visceral surface lesions from skeletons in which affected vertebrae had previously rendered ancient *M. tuberculosis* complex DNA. Ribs from all seven individuals showed proliferative woven bone, indicating active disease at time of death, and three also showed destructive lesions that might have resulted from direct mycobacterial replication at the site. Only one rib with clastic lesions was positive for *M. tuberculosis* complex DNA, and this was likely from direct mycobacterial replication at the site. More recently, in a large skeletal population from the pre-Columbian Americas, Raff et al. (2006) reported successful amplification of *M. tuberculosis* DNA from ribs of seven skeletons, but these were individuals with other skeletal evidence of TB. We agree with the final statements of both Mays et al. (2002) and Raff et al. (2006), who caution against diagnosing TB solely from rib analysis.

Ancient leprosy

Leprosy has been of great interest to paleopathologists because of its well-documented historical presence and because of the characteristic lesions that it may leave on a skeleton. In the case of the arguments for a Holocene origin noted earlier, it must be emphasized that the absence of paleopathological evidence for leprosy before urbanization is not evidence of absence of leprosy. In fact, leprosy is today a rural disease, and there are archaeological sites that were rural in nature that have leprosy skeletons, for example, early-Medieval England (Roberts, 2002). Furthermore, many factors likely affect the appearance of leprosy in the skeletal record: relative abundance of skeletons in urban cemeteries, relative prevalences of tuberculoid, and lepromatous leprosy in ancient times (and thus contemporary "diagnoses" of the disease as well as modern paleopathological diagnosis from skeletal manifestation), and potential animal reservoirs for the disease. Extensive surveys of leprosy in modern animal populations have not been undertaken, and the documented existence of naturally acquired leprosy in nonhuman African primates (Meyers et al., 1985; Walsh et al., 1988; Rojas-Espinosa and Lovik, 2001), with possible monkey-monkey transmission (Gormus et al., 1988), does not conflict with an hypothesized African origin for the disease. Further data analysis and examination of assumptions is necessary before the origin of the disease can accurately be determined.

In spite of the debates centered on the origins of the disease, what is clear is that there is abundant paleopathological evidence for leprosy following approximately the third century BC (Roberts and Manchester, 2005). In 1994, the first extraction and amplification of ancient *M. leprae* DNA was reported from a metatarsal with characteristic lesions from Jerusalem (Rafi et al., 1994). This work purports to have amplified a 530 base segment of the 36 kDa antigen as well as a 439 base pair segment of the 65 kDa protein. Later attempts to detect *M. leprae* in ancient bone samples likewise report amplification of large PCR fragments of elements such as the RLEP repetitive element or the 18 and 36 kDa antigens (Haas et al., 2000b; Taylor et al., 2000; Spigelman and Donoghue, 2001; Donoghue et al., 2002; Spigelman and Donoghue, 2002) or smaller fragments of these genes (Haas

et al., 2000b; Spigelman and Donoghue, 2002); unfortunately, in these studies no cloning was performed to confirm that the sequences match *M. leprae*. Furthermore, in several cases, the bands shown on the figures give a stronger signal than those of the modern positive controls, a typical sign of contamination. Coinfection with both *M. tuberculosis* and *M. leprae* was also reported in several European samples dated from the Roman period to the 13th century, including in samples that showed no overt indications of infectious disease (Donoghue et al., 2005).

In general, most researchers studying ancient leprosy (Haas et al., 2000b; Taylor et al., 2000; Spigelman and Donoghue, 2001, 2002) find that *M. leprae* DNA may be recoverable from the rhinomaxillary areas of the skull, whereas insufficient bacilli are present in affected areas of the hands and feet for PCR to detect. This finding is biologically plausible, as large numbers of bacilli are typically present in nose and mouth in the lepromatous form of the disease (Chimenos-Küstner et al., 2006), whereas tuberculoid leprosy is characterized by few bacilli and damage to the hands and feet secondary to nerve damage.

Unfortunately, like many of the ancient *M. tuberculosis* studies, the ancient leprosy DNA literature has largely failed to appropriately address anticontamination and authentication procedures. Of great concern is the reported length of amplified fragments from many of these studies: a study by Pruvost et al. (2007), for example, determined from a large sample of ancient bones that freshly excavated and nontreated bones are significantly more likely to yield DNA (46% of samples) than those that have been excavated, treated with standard museum prestorage protocols, and stored (15% of samples). Furthermore, fragments of relatively large size (201 bp) were amplifiable from only 15% of these fresh bones, and this number is drastically reduced (4%) in older materials. These experimental data underscore the need to critically examine studies that report amplification of large fragments and especially with success rates in more than 50% of samples.

COEVOLUTION OF HUMANS AND *M. TUBERCULOSIS*

M. tuberculosis and *M. leprae* have likely been human pathogens for millennia. To date, however, most of the research on coevolution between humans and mycobacteria has focused on *M. tuberculosis* (Gagneux et al., 2006; Hershberg et al., 2008). A comparison of genetic sequence differences between two closely related strains of modern *M. tuberculosis* conservatively estimated a minimum age for the complex of 35,000 years (Hughes et al., 2002). Historic and paleopathologic data also confirm that the association between *H. sapiens* and *M. tuberculosis* is a very old one, and indeed, a very common one since at least classical Greek and Roman times (Hughes et al., 2002; Roberts and Buikstra, 2003). TB is known to have spread throughout the Old World, and by the mid-16th century, it is thought to have been responsible for 20% of deaths in England (Lutwick, 1995; Roberts and Buikstra, 2003). The disease remained at epidemic proportions in Europe and the New World until the end of the 19th century and early 20th century. With the discovery of antibiotics in the mid-20th century, hopes were raised that it would be eradicated once and for all. Certainly, morbidity and mortality rates from

infectious diseases declined dramatically in developed nations during the first few decades of widespread antibiotic availability, but by the early-1990s, it became painfully obvious that TB was again on the rise.

Indigenous groups who are immunologically naïve for mycobacterial disease regularly suffer high rates of TB relative to their nonindigenous neighbors (Sousa et al., 1997; Donald, 1998; Hurtado et al., 2003; Grace and Chenhall, 2006; Coimbra and Basta, 2007; Chuang et al., 2008; Larcombe et al., 2008), and under the “conventional wisdom” of host-pathogen coevolution (see below), this has been used as evidence of an evolutionarily recent relationship, one which began only after contact with European explorers and settlers. However, we now know, at least for the prehistoric New World, that the first encounter between humans and *M. tuberculosis* was not so recent. Abundant skeletal (Requena, 1945; Ritchie, 1952; Ackerknecht, 1955) and mummified (Allison et al., 1973, 1981) evidence for pre-Columbian TB was later confirmed by genetic evidence (Salo et al., 1994; Arriaza et al., 1995; Braun et al., 1998). The conventional model of host-pathogen coevolution thus cannot explain the relationship between humans and TB.

The “conventional wisdom” of host-pathogen coevolution (May and Anderson, 1983) states that as the amount of time a pathogen and host species coexist increases, the virulence of the pathogen should decrease. (Here, virulence is defined as parasite-induced host mortality.) Because the host is the pathogen’s environment, a pathogen that kills its host is harming itself by eliminating the environment in which it grows and reproduces. In this view, a virulent pathogen represents an evolutionarily recent association with the host. This model has been criticized for many reasons: it does not address variation within hosts or environments, there is no phenotype defined upon which selection could be acting, and perhaps most importantly, the model relies heavily upon the assumption that no one mutant genotype can overgrow the others—in other words, it relies upon group selection (Lenski and May, 1994).

In response to the “conventional wisdom,” several other host-pathogen coevolution models were formulated. The “trade-off” model, first developed by Anderson and May (Anderson and May, 1982; May and Anderson, 1983), posits that the evolution of pathogen virulence involves tradeoffs between counterbalancing selection pressures. Here, transmission and virulence are considered together: optimal parasite virulence is functionally coupled with transmissibility and has been shown to depend greatly upon host population density (Anderson and May, 1982; Lenski and May, 1994). Although a strain that can reproduce and infect individuals rapidly is at an evolutionary advantage relative to more slowly reproducing strains, an evolutionary dead-end is reached if hosts are immobilized before the pathogen can be transmitted to a new susceptible host.

Pathogen virulence has also been modeled in relationship to mode of transmission (Ewald, 1987, 1994). Ewald posited that a direct (person to person) mode of transmission should impose high-fitness costs on organisms that immobilize (and therefore reduce transmission opportunities) their hosts; such limitations on virulence can be alleviated, however, if the pathogen is vector-borne, because a mobile host is not required for transmission. The “sit-and-wait” hypothesis predicts that such fitness costs can also be alleviated if a pathogen can survive outside of a host while waiting for a new host. This

logically led Walther and Ewald (2004) to hypothesize that the higher the survival time in the external environment, the more virulent the pathogen; and indeed, they found a strong correlation between survival time in the external environment and mortality per infection in human respiratory pathogens. This study included *M. tuberculosis* in the high-durability, high-survival category (from 1 to 309 days in darkness or indirect sunlight, and in temperatures ranging from 15 to 30°C). We note here that such conditions may be more prevalent in some sorts of living conditions (slums and prisons) known to be risk factors for TB.

Host-pathogen coevolution has also been viewed as an evolutionary arms race, which led to a hypothesis that Van Valen (1973) called the Red-Queen Hypothesis after Lewis Carroll’s *Through the Looking Glass* (Carroll, 1872). Because “competitors” and “enemies” are constantly adapting to one another, there is change (evolution), but no progress. Hence “... it takes all the running you can do, to keep in the same place” (Carroll, 1872). Here, if one imagines a resistant host genotype and a virulent parasite genotype, over generations the allele frequencies of both will oscillate, as each successively adapts to the other. A difficulty with incorporating the trade-off and Red-Queen models into a study of human-mycobacterial coevolution is that the outcome of human infection with mycobacteria relies upon an extremely complex interplay of host genetics, host environmental conditions, and host social conditions. *Homo sapiens*, in the present, is largely a geographically diverse set of subpopulations that interact to varying degrees. This would have been the case in the ancient past as well, with the main difference being that only the most geographically proximal human subpopulations would have been likely to interact.

In this regard, one of the most promising conceptual frameworks for understanding human and mycobacterial coevolution is the geographic mosaic theory (Thompson, 1997; Gomulkiewicz et al., 2000). The theory incorporates population differences in selection, gene flow, and random genetic drift across space to understand how coevolution contributes to biodiversity on the landscape. A key feature here is the existence of “coevolutionary hotspots,” the communities of host-pathogen interaction in which reciprocal selection is occurring (Thompson, 1999; Gomulkiewicz et al., 2000). Three ecological predictions follow from the theory: that populations will differ in traits shaped by host-pathogen interactions; that there will be variation among communities in levels of adaptation between hosts and pathogens; and that there will be few species-level coevolved traits. One problem with this model is that it is difficult to empirically test (Forde et al., 2004). In an attempt to do so, Forde et al. (2004) used *Escherichia coli* (host) and the T7 bacteriophage (pathogen) to examine coevolutionary dynamics across a spatially structured landscape. Differences in resource availability influenced the development of host resistance to the pathogens, which, in turn, influenced the development of pathogen mutants that could infect resistant hosts. The study also demonstrated that gene flow among parasite populations acted as a source of beneficial mutations, and in high-productivity host communities, there was greater variation in pathogen adaptivity in open communities than in closed communities. These results suggest that it may be possible to predict geographic variation in both hosts and mycobacteria through time based on what is known of human popula-

tion sizes, and trade and migration routes from the archaeological record and from modern epidemiological studies.

Factors involved in recognition of mycobacterial disease in ancient populations

Wilbur et al. (2008) examined the complex relationship between diet and osseous manifestation of TB. The ability to recognize infectious disease in the skeleton is of paramount importance to paleopathologists, but the relationships among exposure to an infectious agent, development of disease, and skeletal involvement depend on multiple factors including ecology, nutrition, and immune function. TB has been much studied in ancient remains, but it is especially influenced by these factors and, following onset of active disease, individual and population differences in severity and course are apparent. Such complexities have particular implications for the osteological record, as within- and among-individual skeletal lesion distribution represents the confluence of host and pathogen characteristics, many of which are assessable independently through the archaeological record.

The study by Wilbur et al. (2008) examined the contributions of two nutrients, protein, and iron to immune function and to the course and outcome of infection with *M. tuberculosis*, particularly in regard to the dissemination of bacilli to the skeleton and subsequent formation of lesions. Based upon that understanding, models and hypotheses are informed by this interplay. Discrepancies between the expectations and the observed skeletal record in four prehistoric New World areas served as a basis for further hypotheses concerning the origins and evolution of human TB. The biggest discrepancy between expectations and observations occurred during the early hunter-gatherer periods, when groups are presumed to have had a nutritionally varied and generally adequate diet, a situation in which osseous TB can be expected if the population was exposed to *M. tuberculosis*. When protein and iron are replete in the general population, cell-mediated immunity to mycobacteria is possible, but availability of iron to mycobacteria in infected individuals also allows for possible growth and dissemination of bacilli to other organ systems, including the skeleton. In this case, development of TB following infection, as well as dissemination to extrapulmonary locations in the body will largely depend upon both host and pathogen factors such as host genetics, reduced immunity due to trauma or other infectious agents, and level of exposure to the pathogen (dose as well as number of exposure episodes) (Wilbur et al., 2008).

Thus, the model predicts that foraging groups with adequate iron and protein stores would be susceptible to disseminated TB. The hunter-gatherer populations of west-central Illinois, for example, are presumed to have had a nutritionally varied and generally adequate diet, but there is no osseous evidence for TB anywhere in the New World until ~300 A.D. (Allison et al., 1981; Roberts and Buikstra, 2003). This issue is not unique to a study of the disease in the Americas, because skeletal TB also does not appear in the Old World until relatively late. The earliest paleopathological evidence comes from 6th millennium BC Neolithic Italy (Canci et al., 1996).

How then, can the absence of TB among skeletal remains until 5800 BC in the Old World and AD 300 in the New World be explained? How could the disease

have been maintained in small populations of hunter-gatherers? Was the *M. tuberculosis* complex common ancestor instead maintained in animal populations until humans began living in large, permanent settlements? The latter seems unparsimonious, as one would then have to posit at least two zoonotic transmissions into humans—one in the Old World, and one in the New World. Indeed, one such transmission was already hypothesized for the Old World (Rich, 1944), with *M. bovis* suggested as the ancestral organism (Cockburn, 1963) that gave rise to human TB following cattle domestication. However, as discussed earlier, strong genetic evidence from multiple laboratories and multiple types of genetic polymorphisms indicates that the common ancestor of the complex gave rise to the human pathogens *M. canettii* and *M. tuberculosis* and that the other species arose later (Brosch et al., 2002; Gutacker et al., 2002; Baker et al., 2004; Gutierrez et al., 2005).

One hypothesis is that early *M. tuberculosis* strains were characterized by low virulence and that strains that arose following development of large settlements developed increased virulence. Studies of virulence have demonstrated strain variation in virulence as defined by the ability to access the host lymphatic and circulatory system (McDonough and Kress, 1995), acquire drug-resistance mutations (Drobniewski et al., 2002; Shemyakin et al., 2004), compete for limited resources (Shemyakin et al., 2004), and spread globally (Anh et al., 2000; Caminero et al., 2001; Glynn et al., 2002; Kubica et al., 2004; Victor et al., 2004), and there is molecular evidence that virulence in *M. tuberculosis* has increased over time. An analysis of 20 variable regions in 100 modern strains of the *M. tuberculosis* complex (Brosch et al., 2002; Victor et al., 2004) indicates that modern virulent strains causing major epidemics such as Beijing, Haarlem, and Africa (van Soolingen et al., 1995; Kremer et al., 1999; Brosch et al., 2002) possess a large deleted region termed TbD1, which is hypothesized to have arisen sometime in Europe prior to the 18th century—this upper bound for the date was arrived at by the purported recovery of TbD1-deleted strains of *M. tuberculosis* from 18th and 19th century Hungarian mummies (Fletcher et al., 2002 cited in Brosch et al. (2002)). The most promising avenues for future work will involve examination of host and pathogen genetic diversity in light of the host-pathogen coevolutionary models discussed in the previous section.

CONCLUSIONS

We have here reviewed the several classes of evidence that inform concerning the coevolution of our species and pathogenic mycobacteria, with special emphasis upon the MTBC and *M. leprae*. These mycobacteria, obligate pathogens for millennia, have left diagnostic signatures upon human remains, and their symptoms are discussed in historical sources. In addition, ancient remnants of their genomes have been recovered, amplified, and compared with modern counterparts to assess their relationships to contemporary, global variation. Thus, history, bioarchaeology, molecular biology, and genomics are integrated to address host-pathogen coevolution for pathogenic mycobacteria and our species.

It is fair to say that within the past quarter century, accelerating markedly within the last decade, knowledge gained through the study of contemporary molecular variation in the MTBC has overturned accepted wisdom

about the phylogenetic relationship between *M. tuberculosis* and *M. bovis*. Building upon Rich's (1944) hypothesized relationship, scholars had readily accepted the notion that the agropastoralists of the Mediterranean put themselves at risk for acquiring the more ancient zoonosis, which then adapted efficiently to its human hosts. Cockburn (1963), for example, argued for an avian form as precursor to both the bovine and human pathogens, which became virulent with intensified agriculture and pastoralism. As we have seen here, the archaeological record for the Old World supports this model very well, given the early presence of diagnostic TB lesions in Mediterranean Neolithic groups.

Viewed against this model, however, the archaeological record for the Western Hemisphere remained enigmatic until recently. Diagnostic lesions from precontact North and South American bone and desiccated soft tissues were readily linked to TB, and aDNA studies during the 1990s and more recently have proved to be more convincing. This evidence was largely ignored by scholars developing global coevolutionary scenarios, however, as no ready explanation for the presence of pre-Columbian New World TB could be offered. Wandering Vikings, Polynesians, and pinnipeds failed to provide truly convincing models.

As we have reviewed here, 21st century genetic evidence called into question the assumption that *M. tuberculosis* evolved from *M. bovis* (Brosch et al., 2002; Gutierrez et al., 2005). In parallel, our notion of a relatively limited history of coevolution—less than 10,000 years—has been seriously questioned (Kapur et al., 1994; Brosch et al., 2002; Garnier et al., 2003; Baker et al., 2004; Gutierrez et al., 2005). One scenario, combining model phylogenies with knowledge of diversity, suggests that human ancestors and a *M. tuberculosis* progenitor species began their coevolutionary pathway in Africa, perhaps as early as 2.5–3.0 mya (Gutierrez et al., 2005). Humans migrated thence to South, Southeast, and East Asia, ultimately carrying the persistent pathogen to the Western Hemisphere, perhaps along with some of the earliest groups to traverse land, ice, or water.

Although this global model no longer renders ancient New World TB enigmatic, important questions remain. When did the modern, virulent form evolve? Fletcher et al. (2003a) and Taylor et al. (2005) have argued for the TbD1 deletion in 17th–18th century Hungarian remains and for Iron Age (late 8th century to AD 100) UK, respectively. To date, no studies have addressed this issue in examples from the Western Hemisphere, though one could hypothesize the unlikely scenario that the deletion was present or was independently derived. Although we know now that the current distribution of skeletal lesions does *not* map the full course of humanity's coevolutionary history with the MTBC, perhaps the presence of skeletal lesions indicates the presence of the virulent, modern form? If so, then virulence appears to have evolved separately in the New and Old Worlds.

A further cautionary note must be raised about the interpretation of skeletal lesions, even the most diagnostic forms, in relationship to population prevalence. Although the field of paleopathology has well appreciated that we cannot infer "incidence" in paleopathological contexts (Waldron, 2007), it also appears that many factors, such as diet, heritage, and host immunocompetence can affect the degree to which *M. tuberculosis* will disseminate from the lungs to other parts of the body, including the skeleton. Therefore, to use proportions

affected within an archaeological sample to infer prevalence appears unwarranted without consideration of these other, intervening variables. We here allude to a model that considers diet, protein-calorie malnutrition, and iron-deficiency in relationship to the dissemination of TB within the human body. This model has been tested in the archaeological record of the Western Hemisphere and confirmed for all but the earliest groups (Wilbur et al., 2008). The Wilbur et al. study underscores the multifactorial nature of TB dissemination and the need for caution when attempting to infer prevalence in paleopopulations.

Here, we have also reviewed evidence for *M. leprae* in ancient Old World materials. As noted, the disease appears to have originated, based upon historical documents, in South or East Asia, though skeletal evidence for such an early development is absent. Although contemporary wisdom suggests that Alexander the Great brought leprosy back to the Mediterranean from whence it spread to northern Europe (356–323 BC), the archaeological record only convincingly supports the skeletal presence of the disease slightly more than two millennia ago. The absence of skeletal evidence from South Asia is also troublesome. Prevalent in the United Kingdom during the Medieval Period but rare in postmedieval times, the disease persisted in Scotland, Scandinavia, and Iceland until recent times.

Certainly, questions also surround the coevolution of *M. leprae* and human hosts. First, we have no clear picture of the circumstances that led to its development as a human pathogen. The genetic structure is attenuated, suggesting a focused adaptation as an obligate pathogen. The phylogenetic relationship to other mycobacteria is also not clearly understood. Cross-immunity has been cited in the decreased prevalence of the disease in post-Medieval Europe, but this theory remains to be carefully scrutinized in the context of current molecular biological knowledge.

As we also emphasize here, phylogenies within the *Mycobacteriaceae*, especially for the nonpathogenic forms, are incompletely understood. Lateral gene transfer remains a problematic issue and requires that studies include multiple housekeeping genes. Relationships between morphological phylogenies and those based upon molecular genetics remain incompletely understood, as well. Thus, to appreciate the coevolutionary history of this diverse bacterial taxon, further work should emphasize the complete genus and model the evolutionary history in environmental and ecological terms.

A particularly thorny issue is the rate of evolution for the various members of this genus. Evolutionary clocks are notoriously difficult to calibrate (e. g. Ochman and Wilson, 1987; Battistuzzi et al., 2004). Even so, that Gutierrez et al. (2005) hypothesized an antiquity for the *M. tuberculosis* progenitor of ~2.5–3 mya is tantalizing evidence for the antiquity of humankind's coevolutionary history with this form of bacterium.

Although studies of aDNA are key to testing models for the history of TB in humankind and defining the course of mycobacterial evolution, a number of concerns must be raised about research methods and standards for reporting. As we have emphasized earlier, a number of workers have advocated rigorous protocols (Handt et al., 1994, 1996; Richards et al., 1995; Cooper and Poinar, 2000), which are followed in only a minority of cases (Roberts and Ingham, 2007). Rigorous adherence to widely accepted techniques for avoiding contamination

and spurious results are essential and should be reported fully either within the body of texts or as web-based supplements.

Standards for reporting of pathological lesions should also be rigorous, including using accepted descriptive terminology (Buikstra and Ubelaker, 1994; Ortner, 2003). The distinction between “pathognomonic for” and “consistent with” should be carefully drawn, and in the latter case, differential diagnosis is necessary. If such details cannot be supported by journals, web-based supplements should provide images and extended descriptions of lesions and the biological profile of the individual(s) studied. Reporting archaeological or historical contexts as well as the location of collections and all attached identifying information will ensure that researchers can independently validate observations. Finally, we underscore the fact that archaeological and historical collections of remains are nonrenewable resources. Research problems should be tightly focused and significant before destructive procedures, such as those required for aDNA analysis, are contemplated. This is particularly crucial in that the most predictable location for aDNA requires sampling skeletal lesions. Systematic documentation and recording prior to destructive procedures is, of course, essential.

In sum, the power of molecular studies combined with historical and archaeological evidence has been amply illustrated by the recently developed models that push the antiquity of the *M. tuberculosis* progenitor into deep time and provide insight into the biogeography of both *M. tuberculosis* and *M. leprae*. As we report here, many issues—both theoretical and methodological—remain to be resolved if we are to fully model the coevolutionary relationship between our species and these persistent pathogens. We also hope that our developing knowledge of this long-term coadaptive history will prove useful in designing effective medical and public health interventions in today’s and tomorrow’s worlds.

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