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Case study

Pre-Columbian tuberculosis in Tierra del Fuego? Discussion of the paleopathological and molecular evidence

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ABSTRACT

This work contributes to ongoing discussions about the nature of tuberculosis in the Western Hemisphere prior to the time of European contact. Our example, from the extreme south of South America was, at the time of our study, without firm temporal association or molecular characterization. In Tierra del Fuego, Constantinescu (1999) briefly described vertebral bone lesions compatible with TB in an undated skeleton from Myren 1 site (Chile). The remains of Myren are estimated to represent a man between 18 and 23 years old at the time of death. The objectives of this research are to extend this description, to present molecular results, to establish a radiocarbon date, and to report stable isotopic values for the remains. We provide further description of the remains, including tuberculosis-like skeletal pathology. Radiocarbon dating of 640 ± 20 years BP attributes this individual to the precontact fourteenth-fifteenth centuries. Isotopic ratios for nitrogen and carbon from bone collagen suggest a mixed diet. Molecular results were positive for the rpoB quantitative PCR (qPCR) assays but negative for two independent IS6110 and IS1081 qPCR assays. Further testing using genomic methods to target any mycobacteria for specific identification are needed.

1. Introduction and background

Tuberculosis (TB) is an infectious disease whose origin and co-evolutionary relationships with *Homo sapiens* are subjects of ongoing debate (Comas et al., 2013; Hershberg et al., 2008; Hershkovitz et al., 2015; Gagneux, 2012; Pálfi et al., 2015; Roberts and Buikstra, 2003; Stone et al., 2009a,b; Wirth et al., 2008). Any such discussion of human–pathogen co-evolution must consider TB's global dispersion and the variety of vectors that can transmit it. Addressing this complex scenario requires the collaboration of paleopathologists, epidemiologists, microbiologists, immunologists, bioarchaeologists, zooarchaeologists, and historians. Cold regions, with their potential for preserving bone and/or soft tissue, as well as preservation of ancient DNA, are especially auspicious sites for this form of paleopathological study. Our focus here is on the "Island of Tierra del Fuego" where there is preliminary bioarchaeological evidence of TB.

The long-term goal of our research program is to explore the molecular nature of TB in Tierra del Fuego, before and after the arrival of Europeans. Importantly, during the late 19th and during the 20th century, with the development of the first urban centers in southern Patagonia, TB was the leading cause of death in the Rio Grande valley, as recorded for Ushuaia in Argentina and Punta Arenas in Chile (Casali, 2013).

In 1999, Constantinescu briefly described vertebral bone lesions compatible with TB in a skeleton from the Myren 1 site (Fig. 1), excavated by the archaeologist Massone. Although there was no radiocarbon dating of the human remains at that time, Massone attributed this site to a pre-European contact period (Massone et al., 1999). In 1995, as part of a collaborative project between teams

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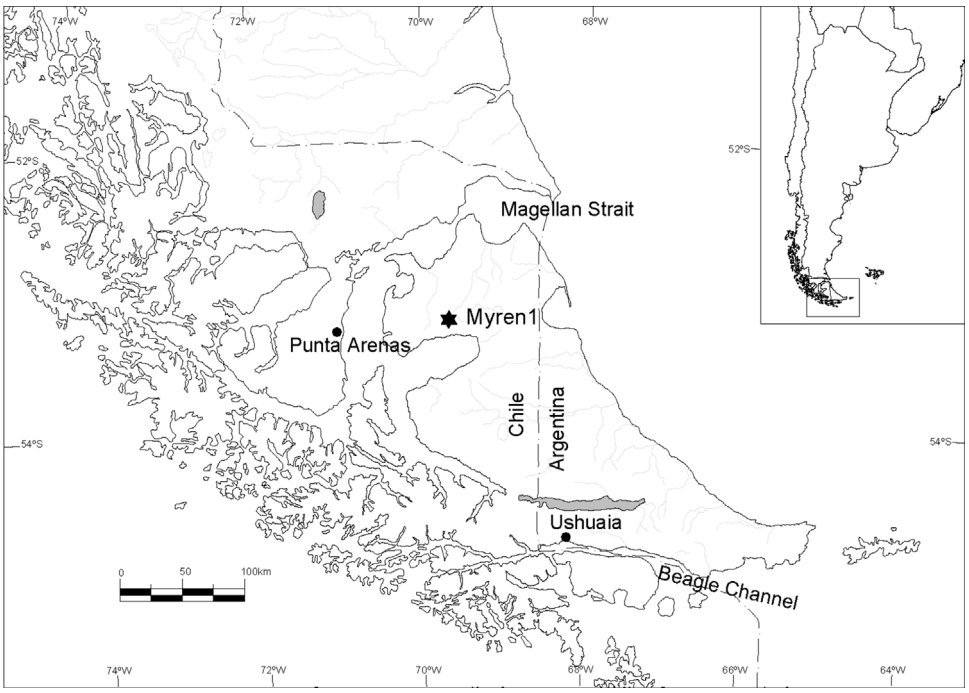

Fig. 1. Location of Myren 1 in "Isla Grande" of Tierra del Fuego.

from Spain, Argentina, and Chile, the D haplogroup was identified for this individual, which corresponds with haplotypes common for native peoples in this region (Lalueza-Fox, 1995; Lalueza-Fox et al., 1997).

Clinical models describe a low frequency of bone involvement as a result of disseminated TB, usually associated with destructive lesions in the spinal column and appendicular skeleton, and occasionally in the skull (Resnick and Niwayama, 1995). New bone formation on the pleural surfaces of ribs is considered suggestive but not pathognomonic for tTB (Roberts and Buikstra, 2003; Santos and Suby, 2012).

Analyses of ancient DNA have been used to investigate the causative agent of the observed TB bone lesions in South America since the 1990s (Arriaza et al., 1995; Salo et al., 1994). These early studies recovered and amplified ancient DNA from the *Mycobacterium tuberculosis* complex (MTBC), which was inferred to have been most closely related to the TB species that are found today in humans, possibly derived from an Asian strain. However, recent studies from the Osmore river valley of southern Peru have identified TB in ancient South American human remains most closely linked to the form that affects pinnipeds (seals and seal lions), suggesting that these sea mammals served as the vector that transferred the pathogen from the Old World to South America (Bos et al., 2014). Studies of historic period South American human remains thus become crucial for evaluating the nature of TB–human coevolution in the Western Hemisphere. The remains from Tierra del Fuego, due to their location, skeletal evidence of TB, and antiquity, thus become especially important in this regard. Our study was conducted to clarify the chronological placement of the remains, to provide a detailed description of the remains, and to generate genetic information about the pathogen.

2. Archaeological context

By approximately 8000 BP, a land bridge connected Tierra del Fuego and the mainland (McCulloch and Morello, 2009), which was potentially first used by hunter–gatherers who arrived at least 10,000 years ago (Massone, 2003). Beginning in the 16th and extending into the 18th century AD, contact between indigenous groups and Europeans was almost exclusively coastal. According to documentary sources, European exploration of Tierra del Fuego's interior began at the end of the nineteenth century (Borrero, 1992; Fugassa and Guichón, 2004).

The Myren 1 site is located approximately 14 km north of Inútil Bay (53°14′S–69°28′W), at the northern aspect of a wide glacial valley that connects this cove to the Bay of San Sebastián, located in the Chilean territory north of Tierra del Fuego. Myren 1 is a large (approximately 200 × 150 m) archaeological site, positioned on the eastern shore of Banty lagoon. In 1998, Massone and Jackson noted four areas with concentrations of cultural material. A few years earlier, farm workers had found a skull located on the eastern side of the lagoon, 38 m from the edge of the water (now called Sector 1). Partially buried human remains also were discovered in a deposit of silt near the lagoon (Sector 1), within a flat area near the bay where the herbaceous cover had been partially eroded (Fig. 2). Massone recovered long bones, phalanges, teeth, and fragments of ribs and vertebrae scattered on the silt surface within a radius of up to 20 m surrounding the buried remains. Both the surface scatter and the buried remains appear to be from the same individual. The skeleton was removed due to its progressive deterioration and dispersal due to a very active erosion front located near the edge of the lagoon. The skeletal remains discovered *in situ* was in a flexed position, with the upper part oriented towards the southeast. No cultural remains associated with the interment were discovered (Massone and Jackson, 1998). The skull, recovered in 1986, thought to be associated with the same individual, had been culturally modified in the tabular erect style (Guichón, 1994; Berón and Luna, 2009).

Sector 2, established to further explore site stratigraphy, was located 80 m east of Sector 1. It is a 31 × 19 m flat, irregularly shaped, raised surface with a noticeable concentration of stone artifacts and remains of a guanaco exposed on the eroded silt surface and in the cuts adjacent to the raised area. In January 1999, a 1 × 1 m survey and a 2 × 2 m excavation next to the survey region were carried out. In this sector the excavations exposed four natural silt strata containing small freshwater snails. Only Stratum III, situated between 45 and 75 cm below the surface, contained cultural mate-

Table 1
Primer and probe sequences used in qPCR screening of skeletal samples.

Primer/Probe	Final concentration	Sequence (5′–3′)	Amplicon size (bp)
IS1081_F	900 nM	*GCACTCCATCTACGACCAG*	84
IS1081_Probe	300 nM	*6FAM-ATTGGGCAACAACTGATTCGGCGTCG-TAMRA*	
IS1081_R	900 nM	*GGGAGTTTGTCGGTCAGAG*	
IS6110_F	50 nM	*GGGTAGCAGACCTCACCTATGTG*	63
IS6110_Probe	300 nM	*6FAM-ACCTGGGCAGGGTT-MGBNFQ*	
IS6110_R	300 nM	*CGGTGACAAAGGCCACGTA*	
rpoB_F	50 nM	*GGTCGCCGCGATCAAG*	66
rpoB_Probe	300 nM	*6FAM-CTCAGCTGGCTGGTG-MGBNFQ*	
rpoB_R	300 nM	*GGGTTGTTCTGGTCCATGAATT*	
rpoB2_F	300 nM	*CAACGTCGAGGTGCTATCG*	70
rpob2_Probe	300 nM	*FAM-TCGCCGCACCGTCACT-NFQMGB*	
rpoB2_R	300 nM	*CTCCAGGTCCTCGTCCTCA*	

Fig. 2. Myren 1 Sector 1 (Massone and Jackson 1998).

rial: including a cut guanaco epiphysis, a fragmentary stone point tip, and some red pigment. A sample of guanaco bones removed from Level III was radiocarbon dated to 870 + 70 BP[1] (Massone et al., 1999). This date, along with the associated material culture links the site to the Selk'nam Culture (Massone and Jackson, 1998; Massone et al., 1999; Massone, 2003).

3. Material and methods

The skeleton Myren 1 (# 30477 curated at the Center for the Study of Southern Man, Instituto de la Patagonia, Universidad de Magallanes, Punta Arenas, Chile) was excavated in 1998. A complete inventory of the remains was conducted, as well as an overall assessment of preservation. Sex estimation was based upon pelvis and skull morphology (Buikstra and Ubelaker, 1994). Age at death was estimated by observing the pubic symphysis (Brooks and Suchey, 1990) and auricular surface (Lovejoy et al., 1985; Falys et al., 2006). The AMS radiocarbon dating was performed at the Center for Applied Isotope Studies at the University of Georgia, Athens, Georgia. Sample preparation for isotopic analyses followed standard protocols and procedures (Ambrose, 1990). Isotopic analyses were carried out at the Stable Isotope Ratio Facility for Environmental Research (SIRFER) at the University of Utah, Salt Lake City, Utah.

Recording pathological lesion of the skeleton followed the criteria suggested by (Aufderheide and Rodriguez Martin, 1998; Ortner, 2003; Santos and Roberts, 2006; Baker et al., 1999; Pálfi et al., 2012; Haas et al., 2000) for TB. Abnormal bone formation, bone destruction, and lesion distribution were assessed.

3.1. Destructive analyses

The aDNA bone sample from Myren was processed at Arizona; State University's (ASU) laboratory designated for ancient DNA work and following protocols to prevent contamination as is standard in the field (*e.g.* Cooper and Poinar, 2000; Pääbo et al., 2004). The ancient DNA laboratory contains a class 10,000-HEPA filtered clean room. All personnel wear appropriate personal protective equipment, including a coverall with hood, facemask, shoe covers, double gloves and a face shield. The clean room is UV irradiated after use and every night as well as cleaned with bleach to reduce the potential for contamination by exogenous DNA. The ancient and modern DNA laboratories at ASU conform to a unidirectional workflow to eliminate contamination of post-PCR products into the clean room environment; this includes all personnel, equipment and reagents.

A portion of the vertebral body adjacent to a lesion was excised and rinsed to remove surface contaminants with 1.5% sodium hypochlorite and sterile water. The bone was pulverized in a SPEX ceramic mill. 50–200 mg of powder were left overnight in extraction buffer, 0.5 M EDTA and Proteinase K. The sample was purified alongside extraction blanks with a modified silica protocol (Rohland and Hofreiter, 2007).

The MTBC includes several closely related "species" or ecotypes: *M. tuberculosis, Mycobacterium canettii*, *Mycobacterium bovis*, *Mycobacterium microti*, *Mycobacterium africanum*, and *Mycobacterium pinnipedii* (Bos et al., 2014). Four quantitative PCR (qPCR) assays to test for the presence and quantity of a single-copy and two multi-copy regions within the MTBC DNA were designed using the primers and probes listed in Table 1 (Harkins et al., 2015). The assays target two nearby regions in the beta subunit of the RNA polymerase gene, referred to here as rpoB-1 and rpoB-2, and the multi-copy mobile insertion elements, IS6110 and IS1081, which are considered to be exclusive to the members of the MTBC (Dziadek et al., 2001; Thierry et al., 1990; Eisenach et al., 1990; van Soolingen et al., 1993), although evidence suggests that some

[1] Beta 128091 measured C14 age 880 +/− 70 BP; C13/C12 ratio −19.0*‰; conventional C14 calibrated results (2 sigma, 95% probability): cal AD 965 to 1220 (Cal BP 985–730).

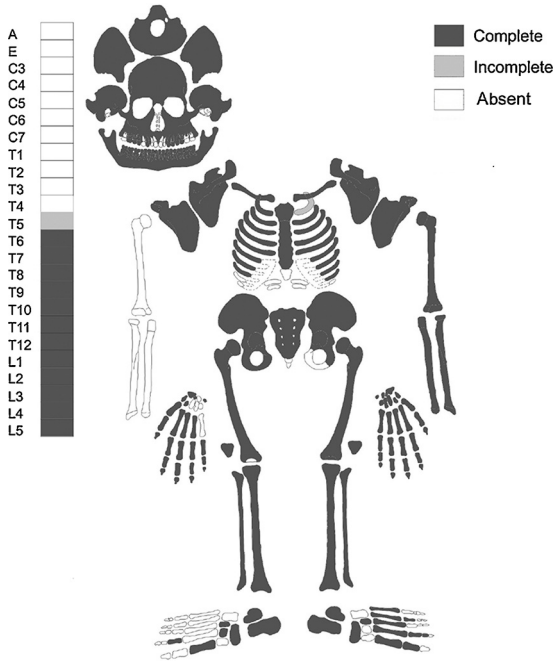

Fig. 3. Surviving elements of Myren 1.

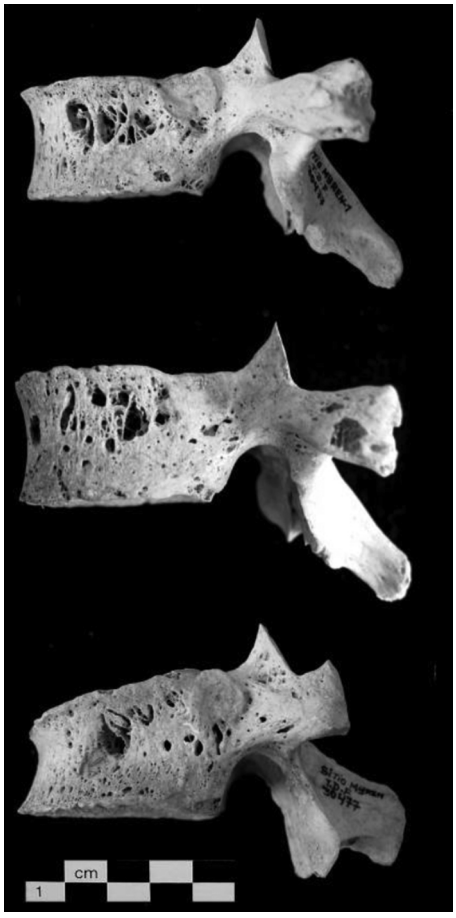

Fig. 4. Thoracic 11th (above), 12th (middle) and lumbar 1st (below) vertebrae of Myren 1 site, showing circumferential resorptive areas on left anterior-lateral surfaces of the bodies.

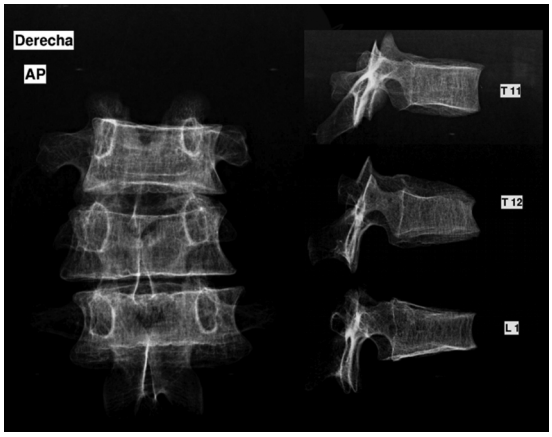

Fig. 5. X-ray images of T11, T12 and L1 of Myren 1. No lytic lesions were observed in the images. These vertebrae were apparently affected by osteopenia and anterior compression of L1 is clearly visible (below right).

mobile elements are present in other mycobacteria (Kent et al., 1995). Nevertheless, these loci have been used to detect tuberculosis in ancient DNA studies (e.g. Salo et al., 1994; Taylor et al., 2007; Müller et al., 2014). Specifically, the IS6110 and rpoB-1 primers/probe sets used here were designed recently and described in Klaus et al. (2010) and Harkins et al. (2015). All qPCR assays employed TaqMan® hydrolysis probe chemistry, which hybridizes a probe to a target sequence within the amplicon, releasing fluorescence proportional to the amount of starting DNA template. This process increases the assay's specificity compared to other quantitation technologies, which detect any amplifiable double-stranded DNA, including primer-dimers and non-specific product. The fluorescence signal is recorded as it crosses a threshold during the linear phase at a given cycle number, called the cycle threshold (C_t). The assay's efficiency is determined by testing a dilution series of DNA with known starting copy numbers, called a standard curve. Theoretically, a PCR efficiency of 1, or 100%, would result in a doubling of product for each cycle, reflected in a slope of the standard curve at −3.32. In other words, sample dilutions that differ by 10-fold should cross the C_t∼3.32 cycles apart.

Ancient samples often contain substances co-extracted with the endogenous DNA (King et al., 2009). Co-extractors negatively affect PCR efficiency (Wilson, 1997), which can be determined from the slope of the standard curve. Inhibition in ancient samples is evaluated by including 10-fold dilutions, here 1:10 and 1:100, and examining deviations from the expected C_t values, or ΔCq. 90–110% efficiency, or a slope of −3.58 to −3.10 of the standards is required to examine the ancient sample results. In the clean room, the reaction was prepared and qPCR plate loaded with 2 × Taqman Universal PCR Mastermix, primers, probe and 2 ul sample and sample dilutions in triplicate, each in a 20 ul reaction; the plate remained covered during the addition of the standard 10-fold serial dilutions, which were added in the post-PCR DNA lab. Standard curves for absolute quantitation were created by diluting DNA from *M. tuberculosis* H37rv with known copy numbers (1 to 1e05 copies/μl).

The Myren sample was also sent to the Paleogenetics Laboratory at the Institute for Archaeological Sciences (IAS) at the University of Tübingen, Germany, where targeted enrichment and sequencing (MiSeq 2000) were performed and reported in Bos et al. (2014), Table S1. Quantitative PCR results are discussed below.

4. Results

4.1. Preservation

The Myren 1 skeleton was fairly complete (Fig. 3), although the upper section of the vertebral column, the right arm and some

bones of the hands and feet were not recovered. Macroscopically, bone preservation is very good to excellent, with only minor post-depositional alteration of the 4th thoracic vertebrae and the left first rib.

4.2. Sex and age of death

This individual's estimated sex was male, through observations of pelvic and cranial morphology (Buikstra and Ubelaker, 1994). Age-at-death was estimated to have been between 18 and 23 years, due to incomplete fusion of long bone epiphyses (head and greater trochanter of the femora and the proximal tibiae) and the iliac crests. Also evaluated were the pubic symphyses (phases 1–2 of Brooks and Suchey, 1990) and auricular surfaces coincident with Fig. 6A & B (Lovejoy et al., 1985).

4.3. Pathological changes

In a previous paper, Constantinescu (1999) mentioned that bone characteristics of lower thoracic and the first lumbar vertebrae of this skeleton could be suggestive of having been affected by TB, although detailed descriptions or images were not presented. Our observation showed irregular and asymmetrical hypervascularization on the antero-lateral aspect of the bodies of T6–T10 vertebrae, with small resorptive foci (less than 3 mm) and cortical remodeling (Fig. 4). Similar hypervascularized areas are present on the left side of the bodies from T11 to L1. The end plates of the bodies and the neural arches are not affected. No collapsed vertebrae typical of TB were identified, although the body of L1 shows a 15° anterior compression of the body, which could have resulted in slight angular kyphosis. The vertebra, as can be seen in (Fig. 5), showed reduction of the trabecular matrix, probably due to osteopenia, mentioned by Constantinescu (1999).

No non-specific lesions associated with TB were found in other bones, including on the articular surfaces of long bones (Aufderheide and Rodriguez Martin, 1998; Ortner, 2003), dactiyitis (Teo and Peh, 2004), periosteal reactions of rib ends (Kelley and Micozzi, 1984; Roberts et al., 1998; Mays et al., 2002; Santos and Roberts, 2006) and symmetrical new bone formation in long bones (Assis et al., 2011). Endocranial lesions, mentioned as possibly related to tuberculous meningitis (*e.g.* Roberts and Buikstra, 2003; Giacon, 2008; Pálfi et al., 2012) could not be studied, although facial bones that in some cases could be affected, were normal in this skeleton.

Hypervascularization of vertebral bodies has been suggested to be associated with TB in skeletons from archaeological sites and in documented collections (Baker, 1999; Haas et al., 2000; Pálfi et al., 2012). As stated by Pálfi et al. (2012) vertebral hypervascularization, endocranial lesions and rib and long bone periosteal reactions may be useful for diagnosis of TB, but especially when these lesions are all associated together. However, vertebral hypervascularization is also associated with the normal developing spine in non-adult and young adult individuals (Baker, 1999; Roberts and Buikstra, 2003; Pálfi et al., 2012).

Other pathological conditions that could affect vertebral bodies, mainly brucellosis, trauma and neoplasia, have to be considered in a differential diagnosis of TB (*e.g.* Aufderheide and Rodriguez Martín, 1998; Ortner, 2003; Pálfi et al., 2012). Vertebral hypervascularization identified in this skeleton seems not to be related to trauma and bone tumors. On the other hand, it is similar to vertebral lesions attributed to brucellosis described by Mutolo et al. (2012), although no lysis of the antero-superior rim of the vertebral bodies described in this disease was observed (Curate, 2006; Mays, 2007).

Thus, in the young individual studied here with no other bone lesions suggestive of TB, the observed vertebral hypervascularization could be the result of the normal developing spine, although infectious diseases as TB or brucellosis cannot be completely discarded as causative. Other methods, such as molecular analyses (see below), are needed to discuss its etiology.

4.4. Dating

An AMS radiocarbon date of the remains (2005, Center for Applied Isotope Studies, University of Georgia UGAMS#03353) of 640 ± 20 years BP (Caliber Rev 7.0.2 Calibration data set: Marine/So Hem. Two Sigma Ranges Cal AD 1445: cal AD 1504) indicates that these remains predate direct European contact with the indigenous people of Tierra del Fuego.

4.5. Diet

The results obtained from SIRFER, in collaboration with other work in progress, suggest a possible mixed diet (d15N col 16.8 and d13C col −15.7). These values must be considered within the context of the resources available in this region, such as guanaco (*Lama guanicoe*) and sea lions (*Otaria flavescens*), which have values of d13C al −21 and −8 respectively (Borrero et al., 2001; Barberena et al., 2009). The values for the Myren individual are within an expected range for a hunter-gatherer diet in this area.

4.6. Biomolecular identification

Four qPCRs designed to target MTBC were used to test for the presence of TB DNA. Multiple replicates of the sample showed the presence of rpoB in both the rpoB-1 and rpoB-2 assays; however, neither IS1081 nor IS6110 were detected during the qPCR analyses. The rpoB-1 and rpoB-2 qPCR analyses displayed an overall slope of −3.29 and −3.34, respectively, with the coefficient of correlation ($R2$) for the standard curves being >0.99. All non-template controls were negative. An undiluted (1:1) extract was tested for rpoB-1 on three separate dates wherein every replicate (total 9 of 9) recorded a signal, as well as three replicates each at 1:10 and 1:100 dilutions. In the rpoB-2 assay, the signal was recorded for two (of two) 1:1 replicates. Only one of three 1:10 replicates recorded a signal, thus resulting in a negative result for this dilution, but it is interesting to note that the replicate recorded a higher quantity and C_t value than all undiluted extracts. No replicates recorded a signal at 1:100.

These qPCR data, presented in Table 2 and shown in Fig. 6, suggest inhibition or primer/probe mismatch in the Myren sample extract, which not only affects efficient reactions but also renders the estimates of starting molecules unreliable. For ancient samples, inhibition is not necessarily surprising. Inhibition can be detected by changes in the threshold cycle or slope as compared to an uninhibited reaction or unexpected ΔCq values in a dilution series. The potential inhibition in the Myren sample is demonstrated by a consistent difference across qPCR plates in the slant of the amplification curves, as compared to those of the standards or other positive ancient samples (Fig. 6). Additionally, within the rpoB-1 assay, sample dilutions of the extract showed presence of the gene regions at the same or higher C_t values than the undiluted extract, further supporting the presence of inhibition. Although only one of three 1:10 replicates recorded a signal for rpoB-2, it occurred higher than the undiluted replicates. Nevertheless, the qPCR assays detect low quantities of the conserved region of rpoB. Unfortunately, the final amplicon size for the rpoB-1 and rpoB-2 is 66 and 70 bp, hindering our ability to successfully Sanger sequence the small products. Without sequence data for the qPCR product, we must then consider the concerns of relying on an evolutionarily conserved element such as the rpoB gene (Stadhouders et al., 2010; Yao et al., 2006). Despite its homology in many organisms, rpoB gene sequence-based identification has been shown to be an effective tool to characterize emerging nontuberculous mycobacte-

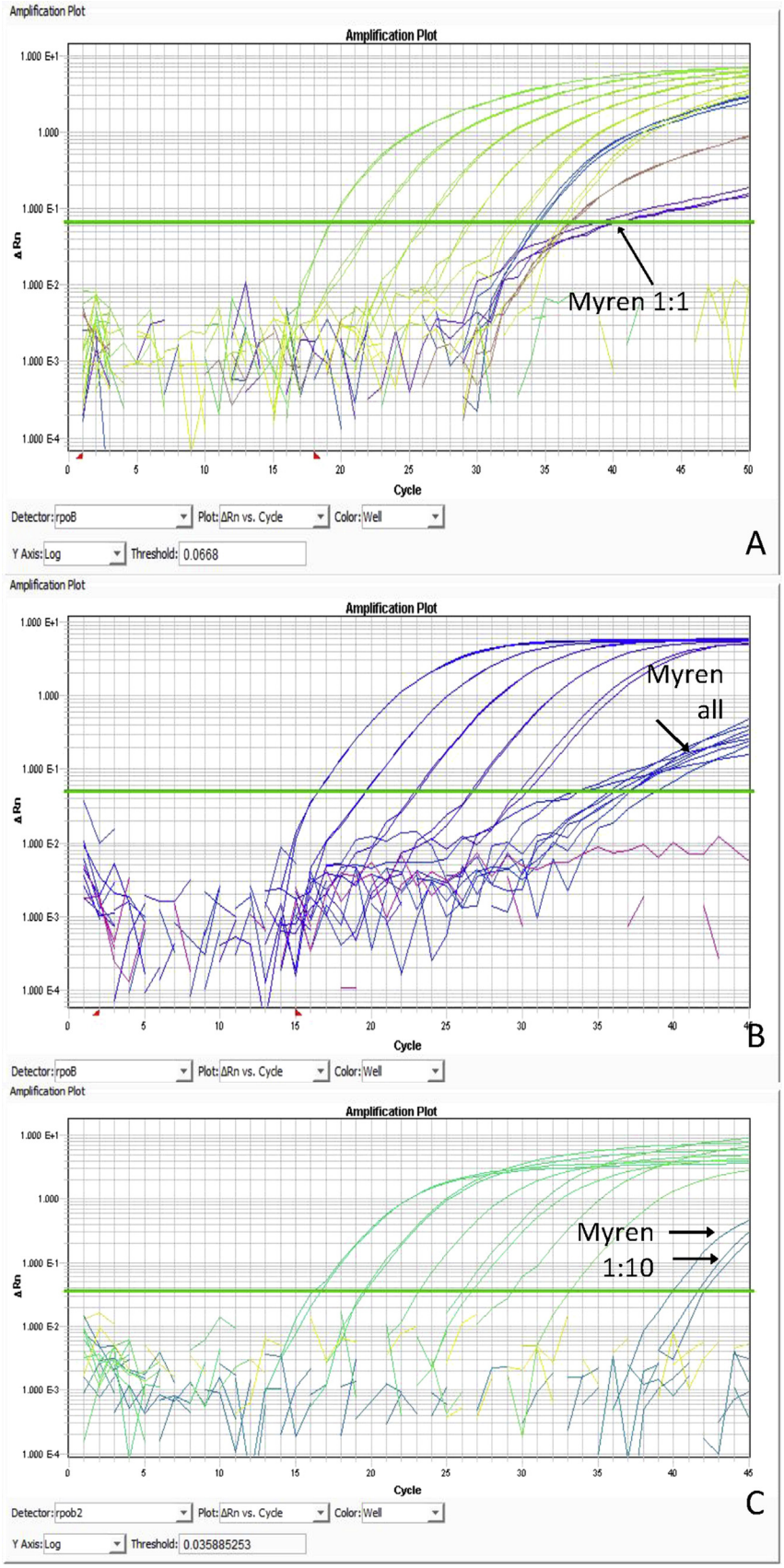

Fig. 6. qPCR amplification plots. (A) rpoB-1 assay, depicting standards (green), inhibited Myren samples and dilutions (purple), and two uninhibited ancient samples (brown and blue). NTCs–yellow. (B) rpoB-1 assay depicting late amplification of all Myren samples and dilutions (blue) with standards (purple). (C) rpoB-2 assay, Myren–blue. Green–standards. (For interpretation of the references to color in the legend, the reader is referred to the web version of this article.)

Table 2
qPCR results for rpoB-1 and rpoB-2 of Myren extracts and dilution series.

Assay	Locus	Lab sample name	Dilution factor	Quantity copies/ul	Mean copies/ul	Mean copies X dilution	Quantity copies std dev	Positive replicates	C_t value	C_t std dev	Ct mean	Cq
aTBassay8	rpoB1	AD216.1	1	0.058000047	0.110536	0.11053572	0.07570621	3 of 3	40.63561	0.960075	39.88925	>3.227
aTBassay8	rpoB1	AD216.1	1	0.19731332					38.806175			
aTBassay8	rpoB1	AD216.1	1	0.076293774					40.225975			
aTBassay11	rpoB1	AD216.1	10	1.0434722	1.620302	16.203026	0.71972317	3 of 3	34.79895	0.62125	34.25308	
aTBassay11	rpoB1	AD216.1	10	1.3905947					34.383213			
aTBassay11	rpoB1	AD216.1	10	2.4268408					33.57707			
aTBassay18	rpoB1	AD216.2	1	2.0081918	1.550058	1.5500581	0.647899	2 of 2	31.914865	0.624106	32.35617	
aTBassay18	rpoB1	AD216.2	1	1.0919243					32.797485			
aTBassay18	rpoB1	AD216.2	10	0.27564484	0.175781	1.7578085	0.09255814	3 of 3	34.7916	0.787973	35.58311	>1.378
aTBassay18	rpoB1	AD216.2	10	0.09287314					36.367496			
aTBassay18	rpoB1	AD216.2	10	0.15882461					35.59023			
aTBassay18	rpoB1	AD216.2	100	0.11141992	0.070585	7.058489	0.04080977	3 of 3	36.10375	0.970045	36.96132	
aTBassay18	rpoB1	AD216.2	100	0.029800422					38.014133			
aTBassay18	rpoB1	AD216.2	100	0.07053434					36.766056			
aTBassay18	rpob2	AD216.2	1	0.001929223	0.002331	0.002331	0.0005682	2 of 2	42.93358	0.3555576	42.68216	>−1.868
aTBassay18	rpob2	AD216.2	1	0.002732785					42.430744			
aTBassay18	rpob2	AD216.2	10	0		0		1 of 3	Undetermined			
aTBassay18	rpob2	AD216.2	10	0		0			Undetermined			
aTBassay18	rpob2	AD216.2	10	0.008372532		0.08372532			40.8139			
aTBassay18	rpob2	AD216.2	100	0		0		0 of 3	Undetermined			
aTBassay18	rpob2	AD216.2	100						Undetermined			
aTBassay18	rpob2	AD216.2	100						Undetermined			

ria (NTM) and mycobacterial infections because of the high amount of sequence diversity harbored within parts of the gene (Adekambi et al., 2006). However, to target the correct species accurately, primer/probe design must take into account the ever-increasing number of genome projects that produce new sequence data for previously unknown environmental bacteria, including species of NTM.

An additional challenge to molecular paleopathology is that other pathogenic species outside of the MTBC can cause clinical symptoms resembling tuberculosis, namely *Mycobacterium kansasii* and *Mycobacterium avium.* In fact, in tests of our assays, these two species were detected by our rpoB-1 primer/probe, at low levels; with an input of ~1E05 known starting copies/ul of *M. kansasii* or *M. avium* DNA, approximately 40 and 8 copies were recorded, respectively, underestimating the known value by 99%. These results indicate the rpoB-1 primer/probe set can record signals from closely-related NTM. The rpoB-2 assay, however, was specific to the MTBC. Given what we observe with mismatches in an alignment between the reference genome and other mycobacterial species within the complex present at the 3′ end of the primers and probe, any sequence variability or damage at these sites in the ancient template could explain the late, non-exponential qPCR signal. If rpoB sequence information for the Myren sample became available, the high intra- and interspecies variability within the rpoB gene could help refine the classification as a member of the MTB complex, or as one of the pathogenic species causing tuberculosis like clinical outcomes (Adekambi et al., 2003). Independently, DNA from the Myren bone sample was extracted at the Paleogenetics lab at the IAS in Tübingen, Germany, and enriched *via* in-solution capture targeting five gene regions within the *M. tuberculosis* genome. The capture, product was then sequenced; the capture results are reported in Table S1 of Bos et al. (2014). The capture sequence reads map to <50% of each targeted region, with a fluctuating range of coverage across each gene, from 0 to over 100×. These coverage values did not meet the threshold criterion used for full genome capture, indicating that the Myren sample does not have adequate DNA preservation. Unfortunately, if an ancient tuberculosis-causing pathogen is present, it is difficult to discern endogenous pathogen DNA from other endogenous but contaminant (myco) bacteria DNA that may have hybridized to the probes during enrichment. Improvements in methods may allow further analysis and independent confirmation of the presence of MTBC DNA.

5. Discussion and conclusions

The extreme south of the American continent has only recently entered conversations about the presence of TB among indigenous peoples prior to direct contact with Europeans (Gómez et al., 2003). The results presented here are discussed with regard to the possible presence of tuberculosis in humans during the 14th and 15th centuries in this region. The geographically closest evidence of TB in a pre-contact skeleton is recorded in remains recovered from the Salitroso–Basin Inn site in Santa Cruz province, Argentina, 650 km north and dated to between 1224 and 1380 cal. AD (Goñi et al., 2003; Garcia Guraieb, 2006). Locating other possible evidence of pre-contact TB in Patagonia with better preservation is the future agenda. This is important in relationship to other pre-Columbian evidence, *e.g.* the TB genomes sequenced from the Osmore River Valley, Peru (Bos et al., 2014). At present, it is assumed that this represents the form identified in other South and North American contexts (Roberts and Buikstra, 2003; Stone et al., 2009a,b). Its relationship to TB documented in the region during the late 19th and early 20th centuries in death certificates from the Salesian Mission in Rio Grande, Tierra del Fuego will be the subject of further inquiry (Garcia Laborde et al., 2010). Other hypothesized sources of TB infection for these groups of hunter-gatherers of southernmost South America also need to be explored (Bastida et al., 2011).

Two decades ago TB in several otariid pinniped species in Australia and Argentina was first diagnosed (Bernardelli et al., 1996; Bastida et al., 1999). In 2003, molecular studies determined that pinniped TB was caused by a new species described as *M. pinnipedii*, which is now included in the MTBC. This new species could have been responsible for TB in humans and in wild and domestic animals from prehistoric and historic Tierra del Fuego, although its membership in the MTBC should have resulted in strong signals for all three of the qPCR tests performed here. The distribution and mobility of hunter–gatherers and their prey, including ethnographic, archaeofaunal, and ecological data will be considered to assess the source and antiquity of this type of TB, which could be caused by other types of mycobacteria, including *M. kansasii* and *M. avium.* Its possible relationship to the disease spectrum of other hunter–gatherers in specific geographical regions will also be examined. Finally, further study using targeted enrichment and next-generation sequencing could shed light on the relationship between the causative pathogen and modern mycobacteria.

As emphasized in the introduction, discussions of the nature and spread of infectious diseases such as TB in the Americas requires a consideration of pan-continental patterning and rigorous tests of alternative hypothesis concerning the nature of ancient TB and its relationship to more recent epidemics. This study represents a first step in addressing these issues from the far south of the Western Hemisphere.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpp.2015.09.003.

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