Beyond Tuberculosis: Diversity and implications of non-tuberculous mycobacteria at the wildlife-livestock interface

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Summary

Non-tuberculous mycobacteria (NTM) circulate between the environment, animals and humans entailing a double concern: their ability to interfere with tuberculosis diagnosis and their potential to cause infections in their hosts. However, published records on NTM infections in animals are still scarce. The aims of the present study were to describe the diversity of NTM circulating among wild and domestic species from Spain, and to analyse their implications as potential pathogenic microorganisms or as sources of interferences in the diagnosis of bovine tuberculosis. Overall, 293 NTM isolates of 277 animals were obtained from tissue samples collected between 2012 and 2019, and analysed through a multigene approach for mycobacteria identification. Thirty-one species were identified, being *M. avium* subsp. *avium* (*Maa*) and *M. avium* subsp. *hominissuis* (*Mah*), but also *M. bouchedurhonense*, *M. nonchromogenicum* and *M. lentiflavum*, the most abundant ones. *Maa* and *M. lentiflavum* were isolated in several animals showing tuberculosis-like lesions. *Maa*, *Mah* and *M. nonchromogenicum* were recovered from many cattle that had reacted to the tuberculin skin test (TST). Other NTM were also associated to these phenomena. These four mycobacterial species were geographically associated between wild boar and other hosts. The findings of the present study suggest that a high diversity of NTM circulates among wildlife and

livestock. Wild boar and *M. avium* seem to play a relevant role in this epidemiological scenario.

Keywords: Non-tuberculous mycobacteria, tuberculosis, wildlife, cattle, diagnosis, epidemiology

1. Introduction

According to the List of Prokaryotic names with Standing in Nomenclature (www.bacterio.net) while writing this manuscript, the genus *Mycobacterium* contained 259 species and 24 validly published subspecies that range from innocuous saprophytes to relevant pathogens (Parte et al., 2020). Some well-known members of the *Mycobacterium tuberculosis* complex (MTC), such as *M. tuberculosis* or *M. bovis*, have historically stood out because of their medical and veterinary relevance, but nowadays so do several species of non-tuberculous mycobacteria (NTM) (Biet and Boschiroli, 2014; Saxena et al., 2021).

NTM are widely distributed in the environment, being isolated from a broad variety of sources including water, feed, soil and dust (Falkinham III, 2016). Their cell wall composition and their adaptability allow them to survive in different habitats for long periods of time (Hruska and Kaevska, 2012) even under adverse conditions. In veterinary medicine, NTM entail a twofold problem: the interference in the diagnosis of bovine tuberculosis (TB) and the potential to cause infections that may lead to economic losses and deprivation of animal welfare (Bercovier and Vincent, 2001; de la Rua-Domenech et al., 2006; Michel, 2008; Biet and Boschiroli, 2014). However, excluding *M. avium* subsp. *paratuberculosis* (*Map*), published records on NTM infections in animals are scarce and mainly report secondary findings of MTC research, since active surveillance for the detection of NTM is

not contemplated nor is far less mandatory in livestock health control campaigns and wildlife surveillance programs in general. Even though many of those publications have contributed with substantial information on the implications of a wide range of NTM detected in terrestrial or aquatic, domestic, farmed and/or wild animals (Bercovier and Vincent, 2001; Balseiro et al., 2011; Rónai et al., 2016; Gcebe and Hlokwe, 2017; Ghielmetti et al., 2018; Gcebe et al., 2018; Ghielmetti et al., 2021), there is still a long way to go before being able to understand the relevance of many of these microorganisms. In the Iberian Peninsula, few studies have reported the isolation of NTM in wildlife: Among *M. avium* subspecies, *M. avium* subsp. *avium* (*Maa*) and *M. avium* subsp. *hominissuis* (*Mah*) have been detected in badger (*Meles meles*) and wild boar (*Sus scrofa*) (Domingos et al., 2009; Balseiro et al., 2011; Muñoz-Mendoza et al., 2013), while *Map* has been found in more species: fallow deer (*Dama dama*), otter (*Lutra lutra*), wild boar and wild rabbit (*Oryctolagus cuniculus*) (Álvarez et al., 2005; Balseiro et al., 2008; Maio et al., 2011; Matos et al., 2013; Matos et al., 2016). Besides, *M. avium* complex (MAC)-infected roe deer (*Capreolus capreolus*) and foxes (*Vulpes vulpes*) have been also reported (Muñoz-Mendoza et al., 2013). Apart from *M. avium*, other NTM have been isolated from wildlife. For instance, *M. intracellulare* was found in red deer (*Cervus elaphus*), wild boar and wood mouse (*Apodemus sylvaticus*); *M. interjectum* in red deer and wild boar, and *M. scrofulaceum* in fallow deer, red deer and wild boar (Gortázar et al., 2011; García-Jiménez et al., 2015; Varela-Castro et al., 2020). Some species have been detected in one host only, such as *M. xenopi* in fallow deer (Gortázar et al., 2011), *M. chelonae*, *M. nebraskense* or *M. triplex*, reported in wild boar (García-Jiménez et al., 2015); and *M. fortuitum*, *M. gordonae* or *M. celatum*, found in wood mice (Varela-Castro et al., 2020). Regarding livestock from the Iberian Peninsula, *M. avium* subspecies such as *Map* have been reported in cattle, goat and sheep, while *Mah* being detected in pigs (Sevilla et al., 2005; de Juan et al., 2006; Domingos et al., 2009; Álvarez et al., 2011), but records of

NTM other than *M. avium* have been anecdotally described only: For instance, *M. kansasii* was isolated from a tuberculin-positive goat, while *M. intracellulare* has been described in cattle (Acosta et al., 1998; Gortázar et al., 2011).

In the last decade, NTM have been isolated from several wild and domestic species during wildlife TB surveillance programmes, bovine TB eradication campaigns or TB-related research. Taking advantage of the material obtained from several regions of Spain, the aims of the present study were 1) to describe the diversity of NTM circulating in wild and domestic species and 2) to assess the possible implications of these mycobacteria as potential pathogens or as a source of interferences in the diagnosis of bovine TB.

2. Materials and Methods

2.1 Mycobacterial isolates: Study sites and sample collection

The 293 cultures used in this study were recovered from tissue samples belonging to wild and domestic animals from seven different sites in Spain (AR, BA, BC, BU, CR, GR and RI, see Figure 1) that were received in the laboratory between 2012 and 2019. Information on the total number of wild animals analyzed per host species and region was only available for the Basque Country (BC). According to a previous study covering the period between 2010 and 2019 in the Basque Country, MTC infection prevalence was 1.12% and 2.40% for wild boar and red deer, respectively (Varela-Castro et al., 2021). The same study reported annual bovine TB herd prevalences decreasing from 0.37% to 0%. Wildlife NTM from this region were isolated from 123 animals in the course of a wildlife health surveillance program for which samples from 818 wild boars, 208 roe deer, 165 red deer, 84 badgers, 41 foxes, 37 carnivores other than badger and fox, one lagomorph (*Lepus* spp.) and one common buzzard (*Buteo buteo*) were cultured. Wildlife samples were collected from road-killed animals or through wildlife population management and hunting activities

following different collection protocols according to the entities involved and to the reasons for which they were collected (surveillance/control programs or confirmation of cases compatible with TB). Although not always possible, routine sampling consisted in collection of mandibular, parotid, retropharyngeal, tracheobronchial, mediastinal, hepatic and mesenteric lymph nodes (LN), as well as any tissue with TB-compatible lesions. Samples collected from livestock belonged to animals (19 cattle and one goat) slaughtered at abattoirs from the Basque Country for being suspected of TB through *in vivo* official diagnostic methods (TST; specifically, the single intradermal tuberculin test) and/or inspection at slaughter. All cattle except for one cow from Granada (GR) and two from Burgos (BU; from Burgos Province but less than 10 Km apart from the Basque Country) came from herds of the Basque Country. In agreement with the Spanish National Bovine TB Eradication Program, in the presence of TB-compatible lesions, at least the lesioned site including adjacent tissue was analyzed in the laboratory. If lesions were not visible, at least one of the LNs from each of the following sites were analyzed: head (retropharingeal or mandibular), thoracic cavity (mediastinic or bronquial), thoracic body (cervical or prescapular), abdominal cavity (mesenteric or hepathic) and mammary gland (supramammary). These animals were subsequently ruled out as MTC-infected or confirmed as coinfected with *M. bovis* and NTM. No animal was killed specifically for this study.

2.2 Pathological examination

A *post mortem* examination of carcasses (if available) or samples received was performed for the detection of gross pathological changes. Histological analysis was also performed in samples from livestock and in some samples with lesions from wildlife. Samples were fixed in 10% buffered formalin, dehydrated and embedded in paraffin wax. Sections (3-5 μm thick) were stained with Carazzi's hematoxylin and eosin for histopahological analysis, and Ziehl-Neelsen for acid-fast bacilli detection.

2.3 Culture of samples

Samples were processed for culture at the Biosafety level 3 Laboratory of NEIKER (Bizkaia, Basque Country). Samples were individually processed if macroscopic lesions were observed, provided that they were not pooled originally. Otherwise, they were processed individually or in convenient pools, depending on the host species, the year of collection and the region of origin. The composition of pools was not always systematic, but mainly comprised LNs of the head or LNs from the thoracic cavity. Samples (LN or pool) were homogenized in sterile distilled water (2 g in 10 ml or equivalently) (Serrano et al., 2018) and subsequently decontaminated using the BD BBL™ MycoPrep™ kit, following manufacturer's instructions. BBL™ mycobacteria growth indicator tubes (MGIT™) (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with BACTEC™ MGIT™ growth supplement (oleic acid-albumin-dextrose-catalase, or OADC) and PANTA™ antibiotic mixture were inoculated with the resulting decontaminated samples and incubated in an automated BACTEC MGIT 960 system (Becton, Dickinson and Company, Sparks, MD, USA) at 37ºC for 42 days (Varela-Castro et al., 2021). *Map*-specific media were not used.

2.4 Tetraplex real-time PCR screening of positive cultures

Pelleted material obtained from 1 mL of MGIT cultures with positive BACTEC time to detection (TTD) readouts was washed and resuspended in 0.25 ml of distilled water, inactivated at 90ºC for 20 min and disrupted with zirconia/silica beads (0.1 mm) for 10 min in a TissueLyser II (Qiagen, Hilden, NRW, Germany). Hereafter, it was centrifuged and the supernanant used in a previously described (Sevilla et al., 2015) and improved (Sevilla et al., 2017) tetraplex real-time PCR for the simultaneous detection of the *Mycobacterium* genus, the subspecies of *M. avium* and the species of the MTC. The concentration and quality of extracted DNA was not measured before amplification. Finally, extracted DNA from 293

NTM isolates coming from 277 animals (255 wild and 22 domestic) was preserved at -20 °C for further species or *M. avium* subspecies identification: 202 belonged to 192 wild boar, 23 to 21 badgers, 21 to 19 cattle, 17 to 17 red deer, 17 to 16 roe deer, 3 to 3 foxes, 2 to 2 stone martens (*Martes foina*), 2 to 2 American minks (*Neovison vison*), 2 to 2 domestic rabbits, 2 to 1 common buzzard, 1 to 1 goat and 1 to 1 Iberian wild goat (*Capra pyrenaica*).

2.5 Species Identification of NTM isolates

2.5.1 Identification of Mycobacterium sp.-positive and M. avium-negative cultures

PCR amplification and sequence analysis of a portion of the 16S ribosomal RNA (16S rRNA) and the *rpoB* genes was performed for the identification of *Mycobacterium* sp. isolates using three previously described protocols: two for 16S rRNA gen (Wilton and Cousins, 1992; Harmsen et al., 2003) and one for *rpoB* gen (Adékambi et al., 2003). PCR mixtures of 50 µl contained 5 µl of 10X *Taq* buffer, 2.5 µl of 50 mM MgCl2, 0.4 µl of 25 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 0.2 µl of 5 U/µl *Taq* DNA polymerase (Invitrogen S.A., Barcelona, Spain), 1 µl of 10 µM of each forward and reverse primers, 38 µl of RNase/DNase-free water and 2 µl of DNA. PCR amplification conditions and primer sequences are shown in Table 1. A proportion of samples with weak or no amplification were reanalyzed under the same PCR conditions but using the CERTAMP Kit for Complex Amplifications (Biotools, Madrid, Spain) following manufacturer's instructions.

PCR products were treated with the ExoSAP-IT™ kit (Thermo Fisher scientific Inc., Waltham, MA, USA) or purified from agarose gels with the Genelute Gel Extraction kit (Sigma-Aldrich Co. Ltd., St. Louis, MO, USA), as recommended by the manufacturers. Purified amplicon and appropriate primer were mixed at requested concentrations and sent to EuroFins GATC Biotech GmbH (Konstanz, Germany) for sequencing. Sequencing primers

were the same used for amplification in both cases. Inspection, edition and alignment of sequences was performed using the Unipro UGENE 40.1 free software (Unipro, Novosibirsk, Russia) and then compared with other published sequences using online BLAST analysis (NCBI, NLM, Bethesda, MD, USA). Because the 5' end sequence of 16S rRNA gene is considered sufficient for species identification of most mycobacteria (Harmsen et al., 2003), the 5' end region flanked by 16S27F/MycGen_F and BKL1-R primer was retained for BLAST and phylogenetic analysis (≈ 477 bp) (Monteserin et al., 2016). To assign species, ≥ 99.7% similarity to reference sequences was required for 16S rRNA and ≥ 97% for *rpoB* (Monteserin et al., 2016). Afterwards, a consensus for species identification was performed as follows: When species assignation derived from 16S rRNA and *rpoB* gene sequences was concordant and achieved the percentage of similarity for both of them, the species identification consensus was recorded. When genes were not in complete agreement in assigning species and both achieved the percentage of similarity, both results, excluding *Mycobacterium* sp. hits, were recorded for species identification. When the percentage of similarity was not accomplished for one of the genes, species assignation was conducted with the other gene only. If the percentage of similarity was not accomplished for any of the genes, isolates remained unidentified at species level and they were recorded as *Mycobacterium* sp., but the hit with highest percentage of similarity of the BLAST search was recorded to state to which NTM species was the sequence closest to. If the final consensus was an unclassified mycobacterium (e.g., *Mycobacterium* sp. GN-9680) this result was recorded, but the next BLAST hit with species designation displaying the highest percentage of similarity was also recorded to indicate to which species could be most related to.

Some isolates could not be identified through the aforementioned methods due to failing of PCR amplification or to poor-quality sequences (mycobacteria with no sequencing result). To discard non-specific results in the screening PCR and confirm that these isolates

belonged to the genus *Mycobacterium*, the Genotype Mycobacterium CM kit (Hain Lifesciences GmbH, Nehren, Germany) was used following the indications of the manufacturer. The presence-absence of a band in the Genus Control position of the membrane strips of the kit was used for this purpose.

2.5.2 Identification of M. avium subsp.-positive cultures

For *M. avium* subspecies identification, DNA was analyzed by two real-time PCR methods described earlier to amplify IS*1245* and IS*901* insertion sequences (Slana et al., 2010). Identification was performed on the basis of presence–absence signatures obtained for these genomic targets (Bartos et al., 2006): *Maa*, including the variant called *M. avium* subsp. *silvaticum* is IS*1245*+, IS*901*+, while *Mah* is IS*1245*+, IS*901*−. Although their occurrence is rare, some IS*Mav6*-positive *M. avium* subsp. *hominissuis* strains have been described as IS*901*-positive because sequence identity between the original IS*901* and IS*Mav6* is very high (95%) (Scherrer et al., 2019). Using the IS*901* real-time PCR method employed in this study, IS*Mav6* detection is not expected due to relevant mismatches of forward primer and probe with this sequence.

2.6 Phylogenetic analyses

For phylogenetic analyses, incomplete sequences missing some informative base positions at 5' and/or 3' ends were removed to avoid loosing information of complete sequences. Two phylogenetic trees, one per gen, were constructed with MEGA-X 10.2.0 software, using the neighbour-joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and all positions containing gaps and missing data were eliminated (complete deletion option). Bootstrap tests were calculated on 1000 replicates and trees were rooted using *Nocardia*

farcinica as the outgroup. The style of the trees was edited using the online tool iTOL 6.4 (Letunic and Bork, 2021).

3. Results

Out of the 293 isolates, 83 belonged to *M. avium* subspecies and 210 to other NTM. For NTM other than *M. avium* we obtained valid sequences of both genes in 50.50% of the isolates (106). We obtained mycobacteria sequences of 16S rRNA gene alone in 25/210 isolates and of *rpoB* gene alone in 28/210 isolates, either because genes were not amplified or were weakly amplified despite several PCR attempts, because sequences did not display sufficient quality or because other microorganisms contaminating the cultures were co-amplified (mainly with the 16S rRNA PCR protocol described by Harmsen et al. 2003). Altogether, isolate identification was possible to conduct in 76% of the NTM analysed through sequencing (159/210) and in 83% of all the isolates (242/293) (see Supplementary Table 1). Still, the Genotype Mycobacterium CM kit confirmed the presence of mycobacterial DNA in all the cases with no sequencing result.

Out of 242 isolates, 166 belonged to 31 known species, 37 displayed homology with more than one species (in 28 cases 16S rRNA and *rpoB* species assignation was not in full agreement and in the other 9 cases *rpoB* was lacking and 16S rRNA sequence was identical for more than one NTM species), 27 belonged to 7 unclassified mycobacteria strains (e.g., *Mycobacterium* sp. GN-9680 or *Mycobacterium* sp. 34028-3) and 12 did not meet the established similarity criteria with any deposited sequence (#39, #56, 219#, #225, #226, #227, #328, #367, #376, #386, #449 and #454 (unidentified mycobacteria) (See Supplementary Table 1 for further detail).

Regarding the single region with information on the number of wild animals analysed per host species (BC region), an overall NTM prevalence of 9.10% was detected. At host

species level, this prevalence was 21.42% for badger, 10.64% for wild boar, 5.29% for roe deer, 4.88% for fox, 3.66% for red deer and 2.70% for carnivores other than badger and fox. The NTM found in the different hosts considering all study sites is shown in Table 2. NTM belonging or related to *M. avium* complex (MAC) were the most abundant, particularly *Maa* (n=48) and *Mah* (n=35), but also *Mycobacterium* sp. GN-9680 (n=15) and *M. bouchedurhonense* (n=12). The predominance of MAC was also true for the hosts with more isolates (badger, cattle, red deer, roe deer and wild boar), being *Maa* or *Mah* again the most abundant except for red deer, for which strain *Mycobacterium sp.* 8115-1 was the most frequent. Out of the MAC, the species most frequently isolated were *M. nonchromogenicum* (n=12) and *M. lentiflavum* (n=10). Two cases of coinfection with different NTM were detected, both of them in the Basque Country: one badger was coinfected with *M. kumamotonense* and *M. septicum* and one roe deer with *Maa* and *Mah*. Besides, coinfection with *M. bovis* and NTM was identified in 14 wild boar from CR infected with *M. alvei*, *M. bouchedurhonense* (n=3), *M. paraffinicum*, *M. porcinum*, *M. vulneris/ colombiense/ intracellulare/ bouchedurhonense* (n=2), *Mycobacterium* sp. GN-9680 (n=2), *Mycobacterium* sp. IEC1808 (related to *M. interjectum*), *Mycobacterium* sp. 2 (n=2) and a NTM with no sequencing result, and two cattle from the Basque Country infected with *Maa* and *Mah*.

Maa, *Mah*, *M. nonchromogenicum* and *M. lentiflavum* were isolated from different hosts within the same municipality, being wild boar always one of the implicated hosts (see Table 3).

Records on lesions compatible with TB and on mycobacteria isolated from the animals showing those lesions are summarized in Table 4. Lesions were detected in a total of 47 individuals (mainly wild boar). Macroscopic findings consisted in encapsulated or diffuse lesions such as single or multiple caseous, necrotic or calcified nodules ranging in size from 1 to 30 mm. Histological findings were mainly observed as necrotic granulomas. Among

animals for which pathological analysis was performed at both macroscopic and microscopic levels, gross and histological lesions were found in 18 individuals, while other six only had microscopic (n=3) or macroscopic (n=3) lesions. The remaining animals with lesions (n=23) were only macroscopically examined. In 37 out of the 47 individuals showing lesions (78.72%), NTM were isolated from the sample where lesions were detected, being *Maa* (in 11 animals) and *M. lentiflavum* (in 8 animals) the most frequent species. Among the remaining 10 individuals, only *M. bovis* was isolated from the lesioned tissue (4 wild boar), no mycobacterium was isolated from those tissues in 5 animals and the lesioned tissue was not available for culture in one animal.

Different NTM were isolated from 20 domestic ruminants that were analysed under TB surveillance procedures. The causes for having been submitted to TB microbiological diagnosis (TST reactivity, TB-compatible lesions detected at slaughter or follow-up or depopulation operation of herds where TB-positive animals were detected) are summarized in Table 5, together with the mycobacteria isolated. All cattle included in the study belonged to officially tuberculosis-free herds where TB prevalence was zero except for four animals from four herds where TB had already been detected and for which a depopulation operation (two herds) or a follow-up (two herds) was ordered by the competent authority. The other 15 cattle were identified as TST-reactors during annual test and cull procedures of the National Eradication Program. Only two of them were TB-positive (Table 5). *Maa* and *M. nonchromogenicum* were the species most frequently isolated from TST-reactor cattle, followed by *Mah*. *M. scrofulaceum* and *M. triplex* were also isolated from reactor cattle, and *M. senuense* from one reactor goat. One non-reactor cow that was slaughtered during the depopulation operation of a *M. bovis*-infected herd showed lesions compatible with TB but *M. bouchedurhonense* was isolated from the lesioned tissue while *M. bovis* infection was

discarded. Similarly, tuberculous lesions were detected at slaughter in one of the reactor cattle and *Maa* was isolated.

The phylogenetic relatedness between isolates based on 16S rRNA and *rpoB* sequences is graphically represented by the phylogenetic trees shown in Figure 2 (16S rRNA) and Figure 3 (*rpoB*). As illustrated in these figures, both trees separated slow-growing mycobacteria from rapid-growing mycobacteria. Even though members of the MAC are clearly the most represented mycobacteria in this study and therefore within the trees (even more taking into account that *M. avium* subspecies are not included in the trees), NTM belonging to *M. terrae* complex, *M. simiae* complex and *M. fortuitum* group were also detected, as shown in these figures. However, we have not detected any NTM from *M. abscessus-chelonae* complex, *M. celatum* group or *M. xenopi* group. When outputs of both genes were available, results were mostly concordant. *rpoB* tree was more ramified showing a higher power of discrimination between isolates than the tree for 16S rRNA sequences (e.g., # 116, # 139 or # 353; See Supplementary Table 1).

4. Discussion

The information gained in this study greatly contributes to the body of knowledge on NTM epidemiology, especially to that concerning the Iberian Peninsula (Álvarez et al., 2005; Balseiro et al., 2008; Domingos et al., 2009; Balseiro et al., 2011; Gortázar et al., 2011; Maio et al., 2011; Muñoz-Mendoza et al., 2013; Matos et al., 2013; García-Jiménez et al., 2015; Matos et al., 2016; Lucía Varela-Castro et al., 2020). *Maa*, *Mah* and *M. bouchedurhonense* from the MAC, *M. nonchromogenicum* from the *M. terrae* complex and *M. lentiflavum* from the *M. simiae* complex were the species most frequently isolated. *Maa* causes avian TB in several species of birds (Dhama et al., 2011) and *Mah*, even though considered more environmental and ubiquitous (Turenne et al., 2008; Biet and Boschiroli, 2014), infects

mainly swine (Agdestein et al., 2014), but they can also infect other wild and domestic hosts (Dvorska et al., 2004; Balseiro et al., 2011; Muñoz-Mendoza et al., 2013). We have isolated both or at least one of these subspecies from almost all the hosts. *M. nonchromogenicum* is an environmental mycobacterial species that has been frequently isolated in wildlife and cattle (Biet and Boschiroli, 2014; Rónai et al., 2016). In our study, *M. nonchromogenicum* was one of the most prevalent species, being isolated from cattle and wild boar. *M. lentiflavum* is considered an emerging pathogen for humans causing cervical lymphadenitis (Miqueleiz-Zapatero et al., 2018). It has been previously detected in wild boar, warthog, buffalo, gazelle and cattle, among other hosts (Katale et al., 2014; García-Jiménez et al., 2015), a list of host species extended to the red deer and the roe deer according to our results. Information on *M. bouchedurhonense* is scarce in the literature. It has been reported in an antelope (eland) and a leopard from South Africa (Gcebe and Hlokwe, 2017) as well as from human patients from France and Zambia (Salah et al., 2009; Mwikuma et al., 2015). In our study, its presence in several hosts was noteworthy, including cattle, wild boar, badger, red deer and roe deer. Some of the unclassified mycobacteria detected (*Mycobacterium* sp. GN-9680, TY59 and IEC1808) were previously isolated from human patients (Gitti et al., 2011; Fusco Da Costa et al., 2015), while others were found in cattle and red deer (*Mycobacterium* sp. 8115-1, related to *M. duvalii*) or in wild boar (*Mycobacterium* sp. 34028-3 and 3582, related to *M. triplex*/ *stomatepiae*/ *montefiorense* and to *M. nebraskense*) (Rónai et al., 2016). Most of our isolates were retrieved from wild boar from specific sites, except for *Mycobacterium* sp. 8115-1 and *Mycobacterium* sp. 3582/J16 (the latter related to *M. scrofulaceum*), which were detected only in red deer or in both red deer and wild boar from different sites, respectively. Finally, 12 isolates (4% of the total) did not meet the similarity criteria established for species designation and could belong to novel NTM species or variants not reported yet.

Although most of the NTM species detected throughout this study have been already reported in wild or domestic species (Biet and Boschiroli, 2014; García-Jiménez et al., 2015; Rónai et al., 2016; Gcebe and Hlokwe, 2017), we have greatly contributed to swell the list of potential hosts for different NTM. For instance, we report several NTM not detected before in badger (e.g., *M. florentinum*, *M. interjectum* or *M. septicum*), a host species for which very little is known in Europe with regard to NTM apart from reports on MAC infections (Balseiro et al., 2011). This is also the first report on *M. bouchedurhonense* and *M. nonchromogenicum* detection in cattle and wild boar from Spain. Some NTM were only detected in wildlife (e.g., *M. hiberniae*, *M. kansasii*, *Mycobacterium* sp. 34028-3) or in livestock (e.g., *M. triplex*, *M. senuense*), which could mean they would be exclusively circulating in those animal populations for reasons not assessed in this study. However, *Maa*, *Mah* or *M. nonchromogenicum* were detected in both wild and domestic species, indicating that wildlife and livestock can be infected with the same species and contribute to the environmental spread of such NTM. In addition, these three species together with *M. lentiflavum* were found in different host species from the same geographic location (municipality level). This geographical association was remarkable for wild boar, which was always involved. Despite being the best represented host species in this work, these findings, together with the high NTM prevalence observed in the Basque Country (>10%) make of this wild ungulate a good candidate host for NTM spreading in this region.

Even though the occurrence of TST-reactor cattle proved to be due to NTM does not seem to be very frequent, in low TB prevalence settings its impact can be considered more relevant because many of these animals are slaughtered with no MTC-infection, causing great concern to both farmers and authorities. The situation is worst if no cause for reactivity is identified for TB-negative reactor cattle. The prevalence of bovine TB in the Basque Country has been falling during last years and it is below 0.1% since 2017 (Varela-Castro et al.,

2021). In our study, all but four cattle were slaughtered for being reactors in annual tuberculin skin testing of the National Eradication Program and came from officially tuberculosis-free herds. Only two of these reactors were TB-positive and represented a new TB outbreak. Thus, the only cause we identified among the remaining slaughtered reactor cattle for their TST response was that they were infected with NTM. TB was already present in the four herds (depopulated or under follow-up after their TB outbreak) to which the remaining non-reactor four animals belonged. Excluding those cases where coinfection with MTC was confirmed, *Maa*, *Mah* and *M. nonchromogenicum* were the NTM most commonly isolated from TST-reactor cattle. All *Maa*, two out of the three *Mah* and all *M. nonchromogenicum* isolates from livestock were retrieved from cows reacting to TST. This is in agreement with the literature in that *Maa* and *Mah* have been pointed out as a cause of interference in the diagnosis of bovine TB (de la Rua-Domenech et al., 2006; Biet and Boschiroli, 2014; Scherrer et al., 2018). This cross-reacting role has also been suggested for *M. nonchromogenicum* (Hughes et al., 1993). *M. scrofulaceum*, *M. triplex* and *M. senuense* were also sporadically isolated from TST-reactor animals not coinfected with MTC. *M. scrofulaceum* is an opportunistic pathogen for animals that has been isolated from farmed and domestic species (e.g., buffaloes, deer, swine and cattle) that reacted to TST, especially to avian PPD (Bercovier and Vincent, 2001). As far as we know from the literature, *M. triplex* and *M. senuense* are also opportunistic pathogens, but despite of having been isolated from wild boar (*M. triplex*) and from pigs and sheep (*M. senuense*) (Muwonge et al., 2012; Zeng et al., 2013; García-Jiménez et al., 2015), detection in reactor animals has not been previously reported. To the best of our knowledge, interferences with the diagnosis of bovine TB have not been experimentally described for any of these two NTM, but in the light of our results their ability to cross react with TST official reagents should not be ruled out. In spite of not having been detected infecting livestock in this study, some NTM species considered to be

able to interfere with bovine TB diagnosis (Biet and Boschiroli, 2014) were detected in wildlife. Specifically, *M. fortuitum* was isolated from wild boar and roe deer, *M. hiberniae* from wild boar and *M. kansasii* from roe deer. Although we did not isolate *M. kansasii* from cattle, this species is of great significance in terms of TB diagnosis because it seems to be cross-reactive not only with PPD, but also with defined antigens based on ESAT-6 or CFP-10 (Scherrer et al., 2019). Regarding the isolates belonging to unclassified mycobacteria strains (e.g., *Mycobacterium* sp. GN-9680 or *Mycobacterium* sp. 34028-3) or to unidentified mycobacteria, none of them were related to cross reactions in TST.

Isolating NTM from animal tissues does not necessarily entail active infection or disease in those animals, especially taking into account that whole carcass inspection was not possible in most cases. But most of the species detected in this work could be pathogenic if conditions for infection development are favourable (e.g., immunocompromised animals, high-dose exposure etc.). In fact, in addition to a few strict pathogens (*Maa* and *Map*) (Turenne et al., 2007, 2008) many of these NTM have already been described as opportunistic pathogens of animals and humans (Pereira et al., 2020) and we have associated NTM with pathological changes in many cases. *Maa* and *M. lentiflavum* were the species most frequently isolated from animals showing lesions compatible with TB, including cattle, common buzzard, Iberian wild goat, rabbit, red deer, roe deer and wild boar. *Mah* is frequently isolated from pigs with subclinical infection showing lesions in LNs (Agdestein et al., 2014). In the current study we have recovered *Mah* from lesioned tissues of wild boar. *M. bouchedurhonense* has been previously pointed out as pathogenic for an African antelope after its isolation from an individual with lesions compatible with TB (Gcebe and Hlokwe, 2017). In our study one cow from which *M. bouchedurhonense* was isolated displayed visible lesions. Although not being usually associated with lesions, *M. nonchromogenicum* was isolated from lesioned tissues from wild boar as well as from a cow. Despite having been

detected less frequently, other NTM species from this study seemed to be involved in the lesions observed in some animals: *M. elephantis*, *M. intracellulare*, *M. parascrofulaceum*, *M. paraense*, *M. porcinum*, *M. triplex* and MAC members other than *M. avium* subspecies. The remaining NTM species of this study were apparently not related with the presence of lesions in their hosts. But other authors have previously pointed out to some of those NTM as the cause of lesions or clinical signs in the same or other hosts (*M. alvei*, *M. confluentis*, *M. engbaekii*, *M. fortuitum*, *M. florentinum*, *M. interjectum*, *M. intermedium*, *M. kansasii*, *M. kumamotonense*, *M. nebraskense*, *M. scrofulaceum*, *M. senuense* and *M. septicum*) (Bercovier and Vincent, 2001; Kik et al., 2010; Zeng et al., 2013; Biet and Boschiroli, 2014; Katale et al., 2014; Gcebe and Hlokwe, 2017; Ghielmetti et al., 2018; Timm et al., 2019; Hernández-Jarguín et al., 2020; Ghielmetti et al., 2021). With regard to the isolates belonging to unclassified mycobacteria strains, only one identified as IEC1808 was cultured from lesioned tissues belonging to a wild boar from CR. None of the isolates belonging to unidentified mycobacteria were cultured from lesioned tissues. On the other hand, culture of some samples displaying lesions did not yield any mycobacterial isolate. This could indicate that other microorganisms might have caused these lesions or that culture failed to grow living mycobacteria from the sample, especially for tissues with necrotic and calcified lesions.

In the present study, the combined use of 16S rRNA and *rpoB* genes was the selected identification approach for NTM isolates other than *M. avium*. 16S rRNA sequencing is considered a standard and reference identification tool for most NTM (Tortoli, 2012) and its combined use with other genetic targets like *rpoB* or *hsp65*, among others, is recommended for a more precise identification at the species or subspecies level (Adékambi and Drancourt, 2004; Simmon et al., 2010). However, we obtained good quality mycobacteria sequences of both genes only for the half of the isolates. Besides, for a quarter of them no sequencing

result was obtained for any of the two genes studied. One of the reasons that contributed to these negative results was the difficulty or impossibility to amplify the targeted genes, maybe due to differences between primer sequences and targeted NTM sequences, the formation of secondary structures, a low quality of extracted DNA or other problems related to PCR amplification. We believe that it is important to report these negative results and thus, to share relevant information on the methodological difficulties that entail working with mycobacteria with other researchers. Approaches based on whole genome sequencing can avoid these issues and be the ultimate tool for identification purposes of unknown mycobacteria isolates. In some cases, we obtained bad or poor-quality sequences that could not be assessed. In others, some electropherograms showed mixed NTM sequences or sequences belonging to microorganisms other than mycobacteria. The liquid culture medium selected (MGIT) may have accounted for the latter two issues. MGIT displays higher sensitivities of detection and offers faster results compared to solid media cultures (Idigoras et al., 2000). However, MGIT does not produce isolated colonies and it is related to higher contamination rates (Idigoras et al., 2000), issues that hinder identification procedures. Using solid media to obtain isolated colonies for DNA extraction would have helped to solve these issues, but unfortunately most of the preserved material was DNA extracted from MGIT cultures.

In conclusion, our findings support that a wide diversity of NTM circulates among domestic and wild hosts in the studied areas, including species potentially pathogenic and causative of interferences in the diagnosis of TB in cattle, being *Maa*, *Mah*, *M. nonchromogenicum* and *M. lentiflavum* above all. Further studies are needed to evaluate the ability of all these NTM to infect different hosts as well as to cross react with the reagents of *in vivo* diagnosis of animal TB. Controlling NTM spread and infection in livestock and

wildlife seems difficult, to say the least. Exploring new alternative or confirmatory diagnostic reagents and tests as well as monitoring the presence of NTM in livestock and wildlife populations would greatly contribute to improving the efficiency of tuberculosis eradication programs.

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6. Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any conflict of interest.

7. Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required since no animal was recruited nor killed specifically for this study.

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Table 1. Primers used for conventional PCR and sequencing.

Target gen	PCR protocol	Sequence (5'-3')	Reference
16S rRNA	A	16S27F: AGAGTTTGATCMTGGCTCAG 16S907R: CCGTCAATTCMTTTRAGTTT	Harmsen et al. 2003
16S rRNA	B	MycGen_F: AGAGTTTGATCCTGGCTCAG MycGen_R: TGCACACAGGCCACAAGGGA	Wilton et al. 1992
rpoB	C	Myco-F: GGCAAGGTCACCCCGAAGGG Myco-R: AGCGGCTGCTGGGTGATCATC	Adekambi et al. 2003

A: 28 cycles of denaturation at 94°C for 45 s, primer annealing at 53°C for 1 min and DNA elongation at 72°C for 90 s. Initial denaturation step of 80°C for 5 min and final elongation step of 72°C for 10 min. B: 40 cycles of denaturation at 94°C for 30 s, primer annealing at 62°C for 30 s and DNA elongation at 75°C for 90 s. Initial denaturation step of 95°C for 5 min and final elongation step of 72°C for 10 min. C: 35 cycles of denaturation at 94°C for 30 s, primer annealing at 64°C for 30 s and DNA elongation at 72°C for 90 s. Initial denaturation step of 95°C for 5 min and final elongation step of 72°C for 10 min.

Table 2. NTM identified per host species. The proportion of isolates out of the total isolates obtained from each host species and their geographic origin is indicated.

NTM species (No. of isolates)	Host sp. (No. of isolates/total isolates)	(%)	Region
Maa (48)	Badger (2/23)	8.70%	BC, RI
	Cattle (6/21)	**28.60%**	BC
	Buzzard (2/2)	100%	BC
	Rabbit (2/2)	100%	BA
	Fox (1/3)	33.33%	BC
	Wild goat (1/1)	100%	AR
	American mink (2/2)	100%	RI
	Red deer (2/17)	11.76%	BC, RI
	Roe deer (9/17)	**52.94%**	BC, RI
	Stone marten (1/2)	50%	RI
	Wild boar (20/202)	**9.90%**	AR, BC, CR, RI
Mah (35)	Badger (7/23)	**30.40%**	BC
	Cattle (4/21)	19.05%	BC
	Red deer (1/17)	5.88%	BC
	Roe deer (3/17)	17.64%	BC
	Stone marten (1/2)	50%	BC
	Wild boar (19/202)	9.41%	BC, RI, CR, AR
M. alvei (1)	Wild boar (1/202)	0.50%	CR

NTM species (No. of isolates)	Host sp. (No. of isolates/total isolates)	(%)	Region
M. arosiense (1)	Wild boar (1/202)	0.50%	BC
M. arosiense/colombiense/intracellulare/bouchedurhonense (1)	Wild boar (1/202)	0.50%	CR
M. bohemicum (2)	Wild boar (2/202)	0.99%	BC, RI
M. bouchedurnonense (12)	Badger (2/23)	8.70%	BC
	Cattle (1/21)	4.76%	BC
	Red deer (2/17)	11.76%	BC
	Roe deer (1/17)	5.88%	BC
	Wild boar (6/202)	2.97%	CR, RI
M. bouchedurhonense/intracellulare (1)	Wild boar (1/202)	0.50%	RI
M. colombiense/intracellulare/bouchedurhonense (14)	Badger (1/23)	4.34%	RI
	Red deer (1/17)	5.88%	RI
	Wild boar (12/202)	5.94%	CR, RI
M. colombiense/intracellulare/intracellulare subsp. *yongonense/bouchedurhonense* (2)	Wild boar (2/202)	0.99%	CR, RI
M. confluentis (2)	Wild boar (2/202)	0.99%	BC, RI
M. diernhoferi (1)	Wild boar (1/202)	0.50%	RI
M. elephantis (1)	Wild boar (1/202)	0.50%	AR
M. elephantis/holsaticum (1)	Red deer (1/17)	5.88%	RI
M. engbaekii (2)	Wild boar (2/202)	0.99%	BC
M. europaeum (1)	Wild boar (1/202)	0.50%	BC
M. florentinum (1)	Badger (1/23)	4.34%	BC
M. fortuitum (3)	Roe deer (1/17)	5.88%	BC
	Wild boar (2/202)	0.99%	BC
M. hiberniae (1)	Wild boar (1/202)	0.50%	BC
M. interjectum (6)	Badger (3/23)	13.00%	BC, RI
	Wild boar (3/202)	1.49%	BC, CI, RI
M. intermedium (2)	Wild boar (2/202)	0.99%	BC, RI
M. intracellulare (3)	Wild boar (3/202)	1.49%	BC, CR
M. intracellulare subsp. *chimaera*/subsp. *yongonense* (1)	Roe deer (1/17)	5.88%	BC
M. kansasii (1)	Roe deer (1/17)	5.88%	BC
M. kumamotonense (1)	Badger (1/23)	4.34%	BC
M. lentiflavum (10)	Red deer (2/17)	11.76%	AR
	Roe deer (1/17)	5.88%	AR
	Wild boar (7/202)	3.47%	AR, CR, RI
M. nebraskense (1)	Wild boar (1/202)	0.50%	BC
M. nonchromogenicum (12)	Cattle (3/21)	14.29%	BC, GR
	Wild boar (9/202)	4.46%	BC, RI
M. paraense (2)	Wild boar (2/202)	0.99%	CR
M. paraffinicum (1)	Wild boar (1/202)	0.50%	CR
M. parascrofulaceum (6)	Wild boar (6/202)	2.97%	BC
M. peregrinum/arcueilense/montmartrense/lutetiense/septicum (1)	Wild boar (1/202)	0.50%	BC
M. porcinum (1)	Wild boar (1/202)	0.50%	CR
M. scrofulaceum (2)	Cattle (1/21)	4.76%	BC
	Wild boar (1/202)	0.50%	BC
M. senegalense/farcinogenes/houstonense/fortuitum/conceptionense (1)	Fox (1/3)	33.33%	BC
M. senuense (1)	Goat (1/1)	100%	BC
M. seoulense (2)	Wild boar (2/202)	0.99%	BC, CR
M. septicum (3)	Badger (1/23)	4.34%	BC
	Wild boar (2/202)	0.99%	BC, RI
M. triplex (1)	Cattle (1/21)	4.76%	BC
M. vulneris/colombiense/intracellulare/bouchedurhonense (11)	Badger (1/23)	4.34%	BC
	Red deer (1/17)	5.88%	RI
	Wild boar (9/202)	4.46%	CR, RI
Mycobacterium sp. 34028-3 † (5)	Wild boar (5/202)	2.48%	BC, CR, RI
Mycobacterium sp. 3582† (2)	Wild boar (2/202)	0.99%	BC
Mycobacterium sp. 3582/J16† (4)	Red deer (1/17)	5.88%	BC

NTM species (No. of isolates)	Host sp. (No. of isolates/total isolates)	(%)	Region
	Wild boar (3/202)	1.49%	BC
Mycobacterium sp. 8115-1† (3)	Red deer (3/17)	**17.64%**	RI
Mycobacterium sp. GN-9680† (15)	Wild boar (15/202)	7.43%	CR
Mycobacterium sp. IEC1808† (1)	Wild boar (1/202)	0.50%	CR
Mycobacterium sp. TY59† (1)	Wild boar (1/202)	0.50%	BC
Mycobacterium sp. 1 (2)	Wild boar (2/202)	0.99%	CR
Mycobacterium sp. 2 (7)	Wild boar (7/202)	3.47%	CR
Mycobacterium sp. 3 (1)	Wild boar (1/202)	0.50%	BC
Mycobacterium sp. 4 (1)	Wild boar (1/202)	0.50%	BC
Mycobacterium sp. 5 (1)	Cattle (1/21)	4.76%	BU
Mycobacteria with no sequencing result (51)	Badger (4/23)	17.40%	BC
	Cattle (4/21)	19.00%	BC, BU
	Fox (1/3)	33.33%	CR
	Red deer (3/17)	17.65%	BC, RI
	Wild boar (39/202)	19.31%	AR, BC, CR, RI

n.d.=not determined, %=proportion of the specified NTM out of the total number of isolates retrieved from each host species. *Maa=M. avium* subsp. *avium*, *Mah=M. avium* subsp. *hominissuis, Mycobacterium* sp. 1= *Mycobacterium* sp. close to species belonging to *M. avium* complex, *Mycobacterium* sp. 2= *Mycobacterium* sp. close to *M. palustre*/ *lentiflavum/ paraense*, *Mycobacterium* sp. 3= *Mycobacterium* sp. close to *M. scrofulaceum*, *Mycobacterium* sp. 4= *Mycobacterium* sp. close to *M. wolinskyi*. *Mycobacterium* sp. 5= *Mycobacterium* sp. close to *M. chitae*. †: *Mycobacterium* sp. GN-9680 and TY59 are related to *M. avium* complex, *Mycobacterium* sp. 34028-3 to *M. triplex/ stomatepiae/ montefiorense*, *Mycobacterium* sp. IEC1808 to *M. interjectum*, *Mycobacterium* sp. 3582 to *M. nebraskense*, *Mycobacterium* sp. J16 to *M. scrofulaceum* and *Mycobacterium* sp. 8115-1 to *M. duvalii*. See Supplementary Table 1 for more detailed information. The proportion of the NTM species detected more frequently in each animal species is marked in bold letters for those hosts with more than 3 isolates.

Table 3. NTM species isolated from different hosts in the same municipality.

Region	Municipality	NTM species	Hosts
BC	Aiara	*Maa*	Wild boar and cattle
	Gueñes	*Mah*	Wild boar and cattle
	Kortezubi	*Maa*	Wild boar and roe deer
	Kuartango	*M. nonchromogenicum*	Wild boar and cattle
	Vitoria-Gasteiz	*Maa*	Wild boar, badger and roe deer
	Zuia	*Maa*	Wild boar and red deer
		Mah	Wild boar and red deer
AR	Jaca	*M. lentiflavum*	Wild boar and red deer

Maa=M. avium subsp. *avium*, *Mah=M. avium* subsp. *hominissuis*

Table 4. NTM isolated from hosts showing microscopic/gross lesions.

NTM identified (No of animals)	Host	No of animals	Location of lesion	Tissue from which NTM were isolated	*M. bovis* isolation (tissue)	Region
Maa (13)	Buzzard	**1**	Liver/intestines	Liver/intestines	No	BC
	Cattle	1	RPh LN[§]	TBr LN	No	BC
		1	RPh/Med LNs	Med LN	No	BC
		1	RPh[§]/TBr[‡]/Med[‡] LNs	Med LN	Yes (TBr LNs)	BC
	Wild goat	**1**	Lung[‡]	Lung	No	AR
	Rabbit	**2**	Caecal appendix[‡]	Caecal appendix	No	BA
	Wild boar	**4**	Mand LN[‡]	Mand LN	No	AR
		1	Tonsil	Med+TBr LNs	No	CR
		1	Mand LN	Mand LN	No	BC
Mah (3)	Cattle	1	Med LN[§]	RPh LN	Yes (Med and TBr LNs)	BC
	Wild boar	**1**	Mand LN	Mand LN	No	BC
	Wild boar	**1**	Mand LN[‡]	Mand LN	No	AR
M. bouchedurhonense (3)	Cattle	**1**	Tonsil	Tonsil	No	BC
	Wild boar	1	Med LN	Mand LN+Tonsil	Yes (Med+TBr LNs)	CR
		1	Mand LN	Med+TBr LNs	Yes (Mand LN+Tonsil)	CR
M. col/int/bou (3)	Wild boar	**1**	Mand LN	Mand LN+Tonsil/Med+TBr LNs	No	CR
		1	Mand LN	Mand LN+Tonsil	No	CR
		1	Lung[¶]	Mand LN+Tonsil	No	CR
M. elephantis (1)	Wild boar	**1**	Mand LN[‡]	Mand LN	No	AR
M. intracellulare (1)	Wild boar	**1**	Mand LN	Mand+Par LNs pool	No	BC
M. lentiflavum (9)	Red deer	**2**	RPh LN[‡]	RPh LN	No	AR
	Roe deer	**1**	Prescp LN[‡]	Prescp LN	No	AR
	Wild boar	**5**	Mand LN[‡]	Mand LN	No	AR
		1	Mand LN	Med+TBr LNs	No	CR
M. nonchromogenicum (2)	Cattle	**1**	Med LN[§]/Lung	Lung	No	BC
	Wild boar	**1**	Head LNs	Head LNs	No	BC
M. paraense (1)	Wild boar	**1**	Mand LN	Mand LN+Tonsil	No	CR
M. paraffinicum (1)	Wild boar	1	Mand LN	Med+TBr LNs	Yes (Mand LN+Tonsil)	CR
M. porcinum (1)	Wild boar	**1**	Mand/TBr/Med/Lung[¶] /Mes[¶] LNs	Mand LN+Tonsil	Yes (Med+TBr LNs)	CR
M. parascrofulaceum (1)	Wild boar	**1**	Mand LN	Mand LN	No	BC
M. seoulense (1)	Wild boar	1	Mand LN	Med+TBr LNs	No	CR
M. triplex (1)	Cattle	**1**	RPh LN	RPh LN	No	BC
M. sp. IEC1808[†] (1)	Wild boar	**1**	Mand /TBr LNs	Mand LN+Tonsil	Yes (Med+TBr LNs)	CR
Mycobacteria with no sequencing result (5)	Wild boar	**3**	Mand LN	Mand LN	No	AR, BC
		1	Mand LN	Mand LN+Tonsil	No	CR
		1	Mand LN	Med+TBr LNs	No	CR

LN=lymph node. *Maa= M. avium* subsp. *avium*, *Mah=M. avium* subsp. *hominissuis*, *M. col/ int/ bou=M. colombiense/ intracellulare/ bouchedurhonense*. Med=mediastinal, Mand=Mandibular, Mes=

Mesenteric, Par=Parotid, Prescp=Prescapular, TBr=Tracheobronquial, RPh=Retropharyngeal. †: *Mycobacterium* sp. IEC1808 is related to *M. interjectum*. ‡: Lesions were both macro and microscopic. §: Lesions were only microscopic. ¶: Not available for culture. The number of animals from which NTM were isolated from the sample with lesions are highlighted in bold and underlined.

Table 5. Causes for slaughter or for laboratory TB diagnosis in NTM-infected livestock.

NTM isolated	Livestock (N)	TST reactor	TB-compatible lesions at slaughter	Cause for slaughter	TB confirmation
M. senuense	Goat (1)	Yes	No	TST-reactor	No
Maa	Cattle (2)	Yes	No	TST-reactor	No
	Cattle (1)	Yes	Yes	TST-reactor	No
	Cattle (1)	Yes	No	TST-reactor	Yes
Mah	Cattle (1)	Yes	No	TST-reactor	Yes
	Cattle (1)	No	No	Depopulation	No
	Cattle (2)	Yes	No	TST-reactor	No
M. nonchromogenicum	Cattle (3)	Yes	No	TST-reactor	No
M. scrofulaceum	Cattle (1)	Yes	No	TST-reactor	No
M. triplex	Cattle (1)	Yes	No	TST-reactor	No
M. bouchedurhonense	Cattle (1)	No	Yes	Depopulation	No
Mycobacterium sp.5	Cattle (1)	No	No	Follow-up	No
Mycobacteria with no sequencing result	Cattle (3)	Yes	No	TST-reactor	No
	Cattle (1)	No	No	Follow-up	No

TST=Tuberculin skin test. *Mycobacterium* sp. 5 is close to *M. chitae*.

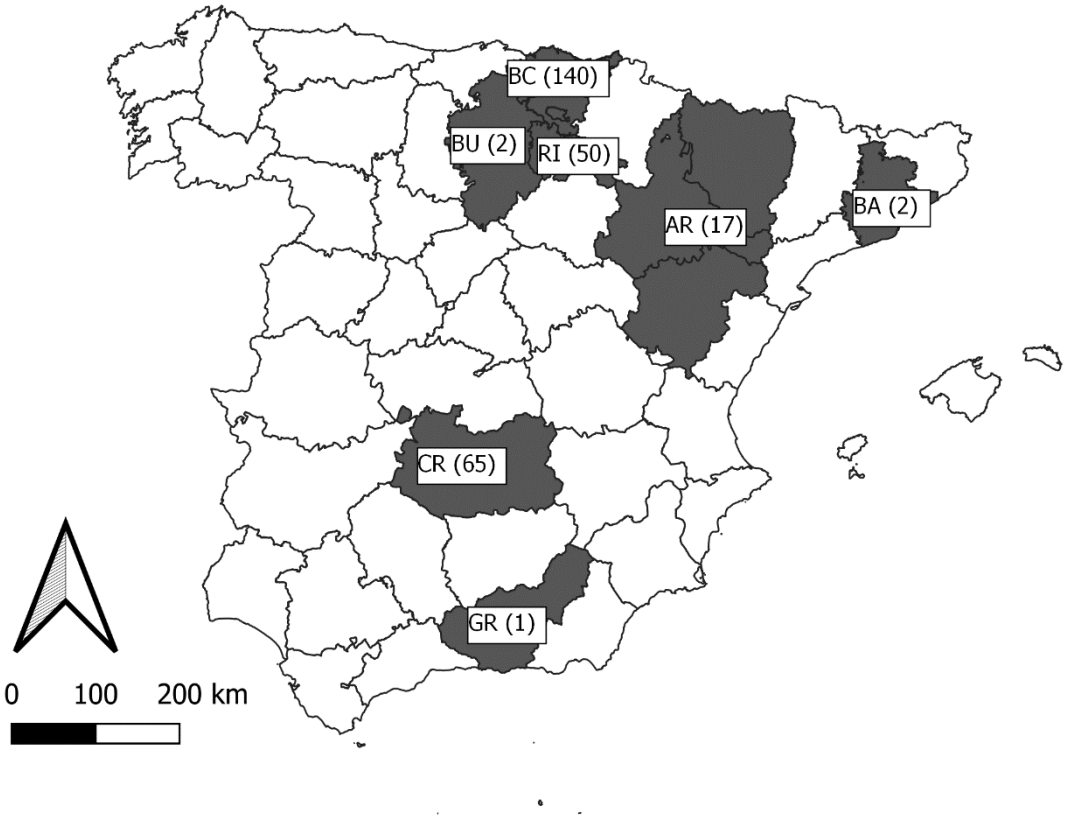

Figure 1. Map showing the sampling sites. The number of animals analysed per site is displayed in brackets.

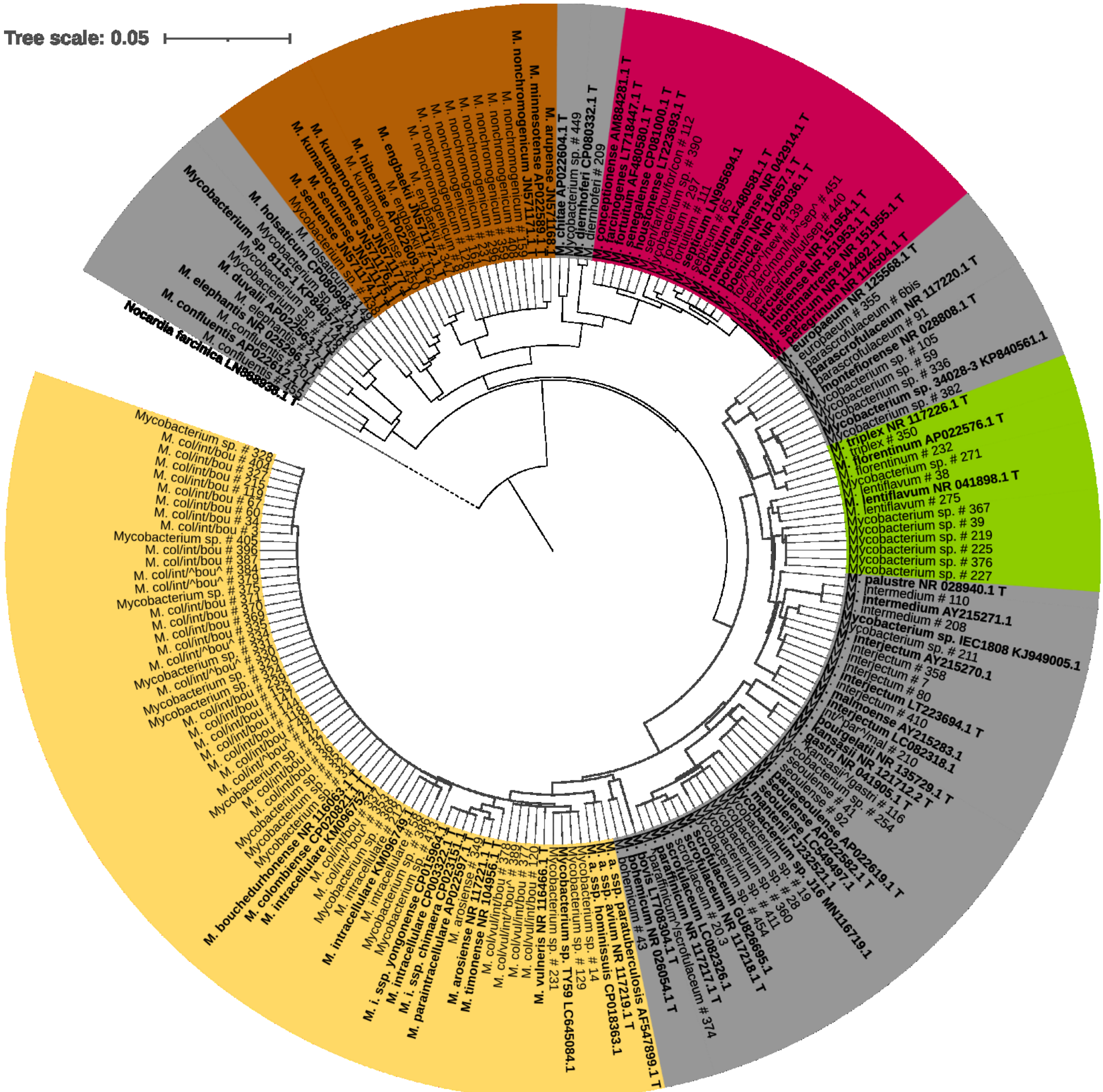

Figure 2. Phylogenetic tree based on 16S rRNA sequences obtained from NTM isolates other than *M. avium*. It was constructed using the neighbour-joining method, the Kimura 2-parameter method and the complete deletion option in MEGA-X 10.2.0 software. Bootstrap test was calculated on 1000 replicates. *Nocardia farcinica* was used as the outgroup. Reference sequences are shown in bold letters. *M. terrae* complex, *M. fortuitum* group, *M. simiae* complex, *M. avium* complex and their related *Mycobacterium* spp. are shown in brown, fuschia, green and yellow respectively

(complex/group designation according to Tortoli et al. 2017). Branches of rapid growers and *M. terrae* complex are drawn thiner than those of slow growers. M. a. ssp.= *M. avium* subspecies, M. i. ssp.= *M. intracellulare* subspecies. Isolates displaying more than one species (because all options had identical 16S rRNA sequence) were abbreviated with a three-letter code: M. col/int/bouch= *M. colombiense/ intracellulare/ bouchedurhonense*, M. col/vul/int/bouch= *M. colombiense/ vulneris/ intracellulare/ bouchedurhonense*, M. int/par/mal= *M. interjectum/ paraense/ malmoense*, M. sen/far/hou/for/con= *M. senegalense/ farcinogenes/ houstonense/ fortuitum/ conceptionense*. M for/por/new= *M. fortuitum, porcinum/ neworleansense*, M. per/arc/mon/lut/sep= *M. peregrinum/ arcueilense/ montmartrense/ lutetiense/ septicum*. When the 16S rRNA sequence was identical for more than one NTM, the species assigend by *rpoB* sequencing (if available) was marked by including its designation between ^ symbols.

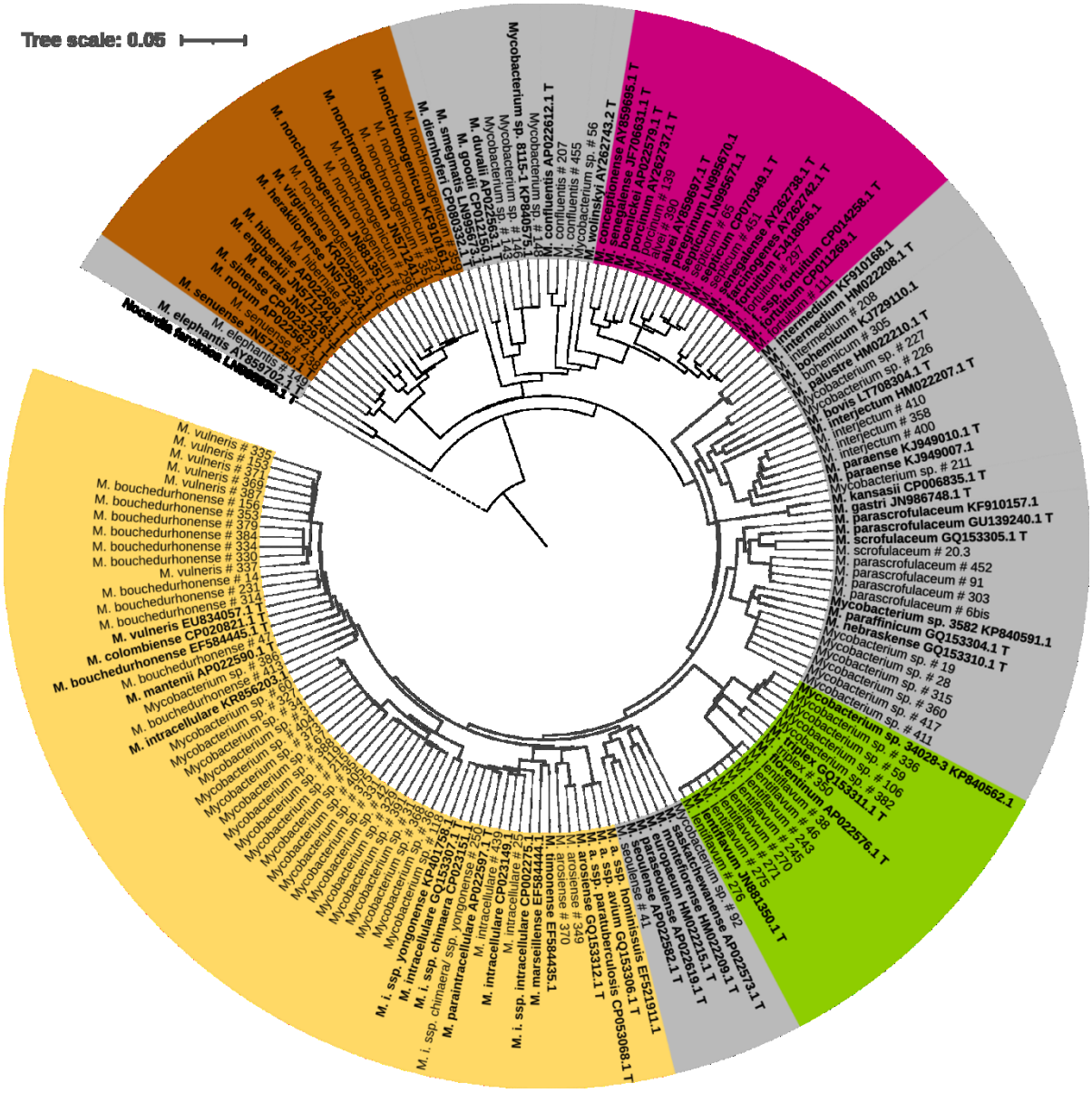

Figure 3. Phylogenetic tree based on *rpoB* sequences obtained from NTM isolates other than *M. avium*. It was constructed using the neighbour-joining method, the Kimura 2-parameter method and the complete deletion option in MEGA-X 10.2.0 software. Bootstrap test was calculated on 1000 replicates. *Nocardia farcinica* was used as the outgroup. Reference sequences are shown in bold letters. *M. terrae* complex is shown in brown, *M. fortuitum* group is shown in fuchsia, *M. simiae* complex and related *Mycobacterium* spp. are shown in green and *M. avium* complex and related *Mycobacterium* spp. are shown in yellow (complex/group designation according to Tortoli et al. 2017). Branches of rapid growers and *M. terrae* complex are drawn thiner than those of slow growers. M. a. ssp.= *M. avium* subspecies, M. i. ssp.= *M. intracellulare* subspecies, M. f. ssp.= *M. fortuitum* subspecies.