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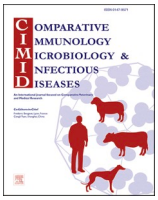

Molecular screening for blood pathogens in synanthropic *Pipistrellus* bats in Spain reveals novel and human-related hemoplasmas

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ABSTRACT

Urbanization profoundly alters natural habitats, creating environments where adaptable species such as bats thrive. In developed countries, cities may act as hotspots for pathogen transmission from bats to humans, yet urban bat pathogens remain understudied in Europe. This study examined vector-borne and zoonotic bacteria and protozoa in soprano (*Pipistrellus pygmaeus*, PPY) and Kuhl's pipistrelles (*Pipistrellus kuhlii*, PKU) inhabiting Zaragoza, Spain. A total of 213 bats (143 urban, 70 rural; including 7 recaptures) were live-trapped between 2022 and 2024, and blood samples were collected. Initial screening of 77 individuals for Anaplasmataceae, *Bartonella*, *Borrelia*, hemotropic *Mycoplasma* (hemoplasmas), and *Leishmania* revealed hemoplasmas as the only haemopathogens present. Sequencing of a 330-bp 16S rRNA fragment confirmed infection, which was subsequently assessed in the full sample. Overall, ten bats (4.69 %) tested positive: eight PPY (two rural, six urban) and two PKU (one rural, one urban). Two hemoplasma genotypes were identified. The first, detected in both species across habitats, showed similarity to sequences from bats in Germany and Chile. The second, found in PPY from both environments, clustered closely with the human hemolytic pathogen *Candidatus* Mycoplasma haematohominis. Extended 16S rRNA (~ 1400 bp) and 23S rRNA (~ 1100 bp) sequences were obtained only for the first genotype, which phylogenetic analyses indicated represents a novel species. Since both samples had 99.8–100 % sequence identity across markers, we propose naming it *Candidatus* Mycoplasma haematopipistrellus sp. nov. Despite the low pathogen diversity observed, results highlight bats as potential ecological bridges for hemoplasma transmission between rural and urban environments.

1. Introduction

Despite significant advances in human and animal health research, much remains unknown about wildlife health, particularly in bats [29]. Bats differ from other disease reservoirs due to their unique and diverse lifestyles, which include flight capability, highly social behaviors, long lifespans, and frequent movement between roosts [21]. These traits create ideal conditions for the transmission and persistence of viruses, bacteria, parasites, and fungi within and between populations, resulting in continuous pathogen exposure [5] that can eventually lead to spillovers into humans. Moreover, it has been suggested that bats' unique metabolism may confer a degree of tolerance to intracellular pathogens [6]. Bats are well-known reservoirs of zoonotic viruses, including those responsible for Ebola, SARS-CoV, SARS-CoV-2, Hendra, and Nipah, all of which have confirmed or probable origins in bats [7,9,12,46]. While viral research has dominated the scientific literature on bat-related diseases, bats are also natural hosts for diverse bacterial clades (see Mühldorfer [40]) and parasites [11,13,48], playing a critical role in the epidemiology and dynamics of numerous veterinary and zoonotic pathogens. For instance, bats have been proposed as ancestral hosts for all mammal-associated *Bartonella* species [33] and even for malaria parasites [31]. *Candidatus* Mycoplasma haematohominis (CMhh), the

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agent responsible for Flying Fox Hemolytic Syndrome in humans, also originates from bats [15,28]. Nevertheless, our understanding of bacterial and parasitic diseases in bats remains limited.

Urbanization profoundly alters habitats, creating novel environments that facilitate the establishment of species capable of adapting to these conditions [32,34], a phenomenon known as "synurbanization" [30]. Certain bat species are among the mammals that adapt exceptionally well to urban environments, utilizing urban forest fragments and buildings as roosts [19]. Such urban living prompts bats to modify their ecology and behavior [47], as well as their geographic ranges and population densities. These changes facilitate interspecific interactions and contribute to the environmental dissemination of pathogens [8]. In Europe, insectivorous bats of the genus *Pipistrellus* are among the most adapted to urban landscapes. Buildings mimic the rocky habitats to which these bats were originally adapted, offering roosting sites, while streetlights provide reliable hunting grounds. Additionally, their echolocation calls and wing morphology are particularly well-suited to navigating and foraging in urban environments [2,47].

In developed countries, urban areas provide the highest potential for pathogen transmission from bats to humans. However, the mechanisms and cycles of urban disease transmission in bats remain largely unexplored, with only a few exceptions. For instance, in Australia, an increase in the size of urban bat colonies and changes in roosting behavior due to human disturbances were found to elevate disease transmission risk [44]. Similarly, a high diversity of potentially zoonotic hemoplasmas was identified in bats captured in peri-urban areas of Nigeria [16], while zoonotic pathogens have also been detected in urban bats in South America [10,43]. In Europe, limited information exists on this topic. Mühldorfer et al. [41] reported a high prevalence of infectious agents in bats from urban and suburban settings in Germany. In Spain, the only available study documented the presence of *Leishmania infantum* in common pipistrelles (*Pipistrellus pipistrellus*) from Madrid [4]. However, none of these studies included comparisons with a control group of non-urban populations, leaving unanswered the question of whether urban environments drive higher pathogen prevalence in bats. The objective of this study was to assess the presence of the most significant blood pathogens in a sample of urban pipistrelles and to compare their diversity and prevalence with those observed in a sample of rural conspecifics.

2. Material and methods

2.1. Field methods

Bats were captured in the Autumn of 2022–2024 from two distinct environments: artificial roosts (wooden boxes) installed in trees across three parks and one bridge within Zaragoza city (N Spain, 41°39′06" N, 0°52′58" W), and a rural bat colony inhabiting an abandoned building located 8 km away from the nearest studied urban park (Fig. 1). Urban bats were directly captured from the wooden boxes in the following sites: Parque Labordeta (n = 89), Depósitos de Casablanca (n = 43), Parque Bruil (n = 13) and Puente de la Unión (n = 1). Rural bats (n = 71) were captured using mist nets. Morphological identification of bats was conducted initially, and species confirmation was performed using a previously described PCR protocol [27], which discriminates among *Pipistrellus* spp. This confirmed that 178 individuals were Soprano pipistrelles (*Pipistrellus pygmaeus*, PPY) and 35 were Kuhl's pipistrelles (*P. kuhlii*, PKU) (Table 1). Captured bats were held in individual fabric bags until processing. Handling was performed without anaesthesia. Each bat was weighed to the nearest decigram, and the forearm length was measured to the nearest 0.1 mm. A drop of blood was collected by puncturing the cephalic vein with a 25G needle and applied to FTA™ Nucleic Acid Collection Cards (Whatman, Maidstone, Kent, UK). The samples were air-dried, stored in cryo-vials, and preserved at − 80 °C until further analysis. In 2022, bats were marked with a 1 mm wing punch for temporary identification. In 2023 and 2024, bats were tagged with a transponder injected subcutaneously (Trovan ID-100, Weilerswist, Germany). For this reason, we confirmed that seven individuals (six

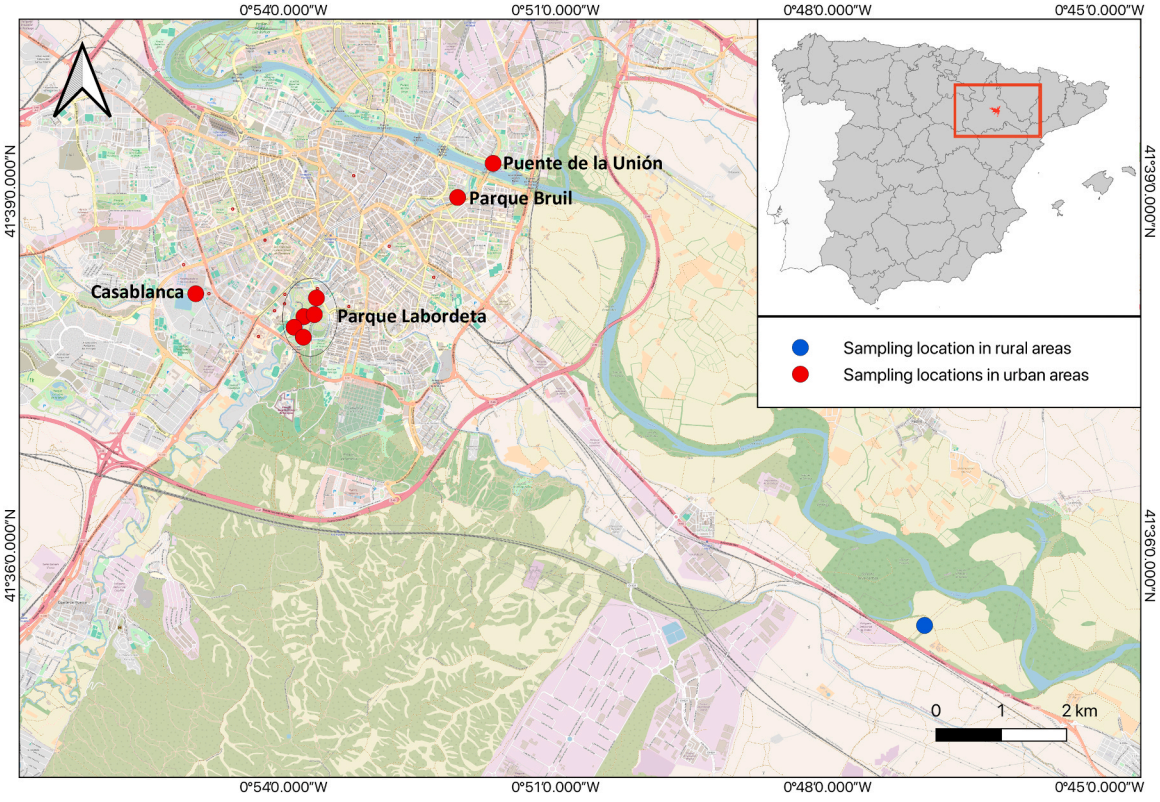

Fig. 1. Map of Spain showing the location of Zaragoza and the distribution of sampling sites within and outside the city.

Table 1
Description of the bats live-trapped in Zaragoza (Spain), 2022–2024. In parenthesis, individuals positive for hemotropic mycoplasmas and the variants detected (*C*Mhp: *C.* M. haematopipistrellus; *C*Mhh: *C.* M. haematohominis-like).

	Pipistrellus kuhlii		*Pipistrellus pygmaeus*		Overall
	Males	Females	Males	Females	
Urban	7 (1 *C*Mhp)	9	42 (1 *C*Mhp, *C*Mhh)	85 (4 *C*Mhp)	143 (7)
Rural	7	12 (1 *C*Mhp)	27	24 (1 *C*Mhp, 1 *C*Mhh)	70 (3)
Overall	14 (1)	21 (1)	69 (2)	109 (6)	213 (10)

urban and one rural PPY) captured in 2023 were recaptured in 2024 but we cannot know the recaptures that occurred between 2022 and 2023-2024. All bats were then immediately released at the capture site.

2.2. Molecular methods

DNA extraction was performed by Chelex® 100 Resin according to the procedure described by Imbert et al. [25]. To verify efficient DNA extraction of each sample, PCR assays targeting a fragment of the β-actin gene (ACTB) from mammals were carried out (Table 2). We first carried out a screening for the most relevant parasites and bacteria found in the blood of bats captured in 2022 with a set of primers and protocols targeting hemotropic *Mycoplasma* spp. (hemoplasmas), Anaplasmataceae, *Bartonella* spp., *Borrelia* spp., and *Leishmania* spp. (Table 2). Due to budget limitations, since only hemoplasmas were detected in these samples (see below), samples from 2023 and 2024 were analyzed only for hemoplasmas. Negative and positive controls were included in all the PCR assays performed. The PCR amplicons obtained with the expected size were sequenced by Sanger in forward and reverse senses. The nucleotide sequences were analyzed using BioEdit v.7.2.6 software. A BLAST search (https://blast.ncbi.nlm.nih.gov) was conducted to compare the sequenced products with hemoplasma sequences available in GenBank®. Following sequence alignment using MUSCLE, a phylogenetic analysis was performed using the Maximum Likelihood algorithm. The optimal models for phylogenetic analysis were selected using the "Models" option in MEGA software. All sequence analyses were carried out with MEGA version 11 [50]. To propose novel *Candidatus* species, we used multilocus data and required that the same genotype be identified in at least two samples based on 16S rRNA and 23S rRNA sequences [51].

The sequence data obtained from this study have been deposited in the GenBank® database (https://www.ncbi.nlm.nih.gov/genbank) under the accession numbers PX271157–PX271162 and PX277289–PX277292 for the 16S rRNA gene, and PX259640 and PX259641 for the 23S rRNA gene.

2.3. Statistical analysis

Given the low occurrence of pathogens, multivariate analyses could not be performed. Instead, Fisher's exact test was used to compare occurrences between species, sex groups, and origins.

3. Results

Ten bats (4.69 %, 95 % Confidence Intervals= 2.27–8.46 %) tested positive for hemoplasmas using the 16S rRNA screening protocol. Among these, eight were PPY (4.5 %, 2.0–8.7; two rural, six urban) and two were PKU (5.7 %, 0.07–19.0; one rural, one urban) (Table 1). No differences in occurrence were found between species, sex groups, and origins (in all cases, Fisher's $p > 0.05$). Among the seven recaptured individuals, all bats tested negative in 2023, while three of them tested positive in 2024.

Sequencing yielded ten readable sequences classified into two putative genotypes. The first group (genotype 1) consisted of seven sequences with 99 % identity among them, detected in one rural PPY, one rural PKU, and five urban PPY from three different parks. These sequences exhibited the highest similarity (98 %) to a *Mycoplasma* sp. isolated from a *Nyctalus noctula* bat in Germany (LR699020). The second group (genotype 2) included two sequences with 98 % identity between them, originating from one rural female and one urban male PPY. These sequences demonstrated the highest identity (99 %) with a *Mycoplasma* sp. from a *Miniopterus schreibersii* bat in Spain (KM538695) and 97 % identity with *C*Mhh (GU562823). Phylogenetic analysis of the short 16S rRNA fragment (~ 330 bp) revealed that sequences belonging to genotype 1 clustered with those from bats in Germany and Chile, forming a clade that included *Mycoplasma* sequences from rodents, bats, and wild carnivores in Europe and the Americas, albeit with low bootstrap support. In contrast, genotype 2 clustered with cave bats from Spain within the *C*Mhh clade (Fig. 2).

Only two nearly complete 16S rRNA gene sequences were

Table 2
Primers pairs used in this study.

Organisms	Target gene	Primer sequence (5'→ 3')	Fragment size (bp)	Tm (°C)	Refs.
Mammals	ACTB	F: AGCGCAAGTACTCCGTGTG R: CGGACTCATCGTACTCCTGCTT P: TCGCTGTCCACCTTCCAGCAGATGT	96	58	[53]
Mycoplasma spp.	16S rRNA	F: ATGTTGCTTAATTCGATAATACACGAAA R: ACRGGATTACTAGTGATTCCAACTTCAA	391	60	[38]
		F: AGAGTTTGATCCTGGCTCAG R: ACCGCAGCTGCTGGCACATA	492	57	
		F: GCCCATATTCCTACGGGAAGCAGCAGT R: GTTTGACGGGCGGTGTGTACAAGACC	1029	64	
	23S rRNA	F: GGCTAGSGGTGAAATTCCAATCG R: GTAAAGCTTCAYAGGGTCTTTCCGTC	1276–1302 1151–1182	56 55	[42]
		F: GCCGAAATAGYTTTAGGACTAGCG R: CTGCAGYCGAGACAGTTAAGRG			
Anaplasmataceae	16S rRNA	F: GGTACCYACAGAAGAAGTCC R: TAGCACTCATCGTTTACAGC	345	55	[26]
Bartonella spp.	*rpoB*	F: CGCATTGGCTTACTTCGTATG R: GTAGACTGATTAGAACGCTG	825	53	[45]
Borrelia spp.	*flaB*	F: TAATACGTCAGCCATAAATGC R: GCTCTTTGATCAGTTATCATTC	750	56	[3]
Leishmania spp.	ITS2	F: GCATGCCATATTCTCAGTGTC R: GGCCAACGCGAAGTTGAATTC	372–450	60	[14]

F: Forward; R: Reverse; P: Probe; bp: base pairs; Tm: melting temperature; Y: C/T; R:G/A; S: G/C.

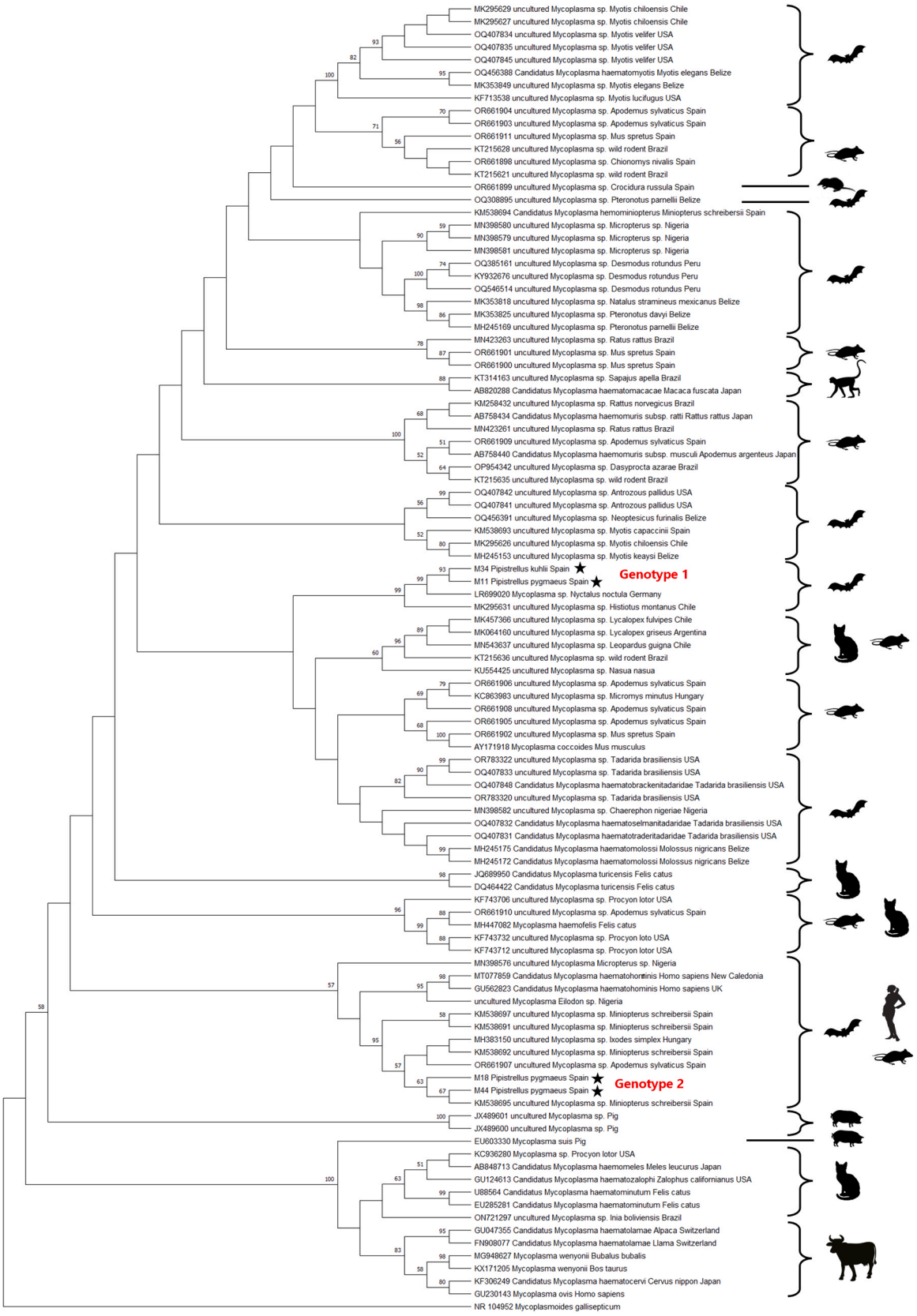

Fig. 2. Maximum-likelihood tree based on partial 16S rRNA (~ 330 bp) gene sequences of hemotropic Mycoplasmas. Numbers represent bootstrap support generated from 1000 replications. The tree was constructed with the Tanura-Nei model (TN93 + G + I). New sequences obtained in the present study are indicated with a star.

successfully obtained from two samples, both corresponding to PPY infected with genotype 1. These sequences, 1300 and 1400 bp in length, were 99.8 % identical to each other and exhibited the highest identity (98.5 %) with the previously mentioned *Nyctalus noctula* sequence (with only 87 % of query cover because that sequence was only 1100 bp in length). In the phylogenetic analysis of the 16S rRNA gene, these sequences clustered with the two abovementioned sequences from Germany and Chile, forming a sister branch to sequences from *Desmodus rotundus* bats from Peru. This cluster was positioned within a larger group of bat sequences, although the bootstrap support for these branches was low (Fig. 3). Similarly, partial 23S rRNA gene sequences were obtained only for the two PPY of the genotype 1. Two sequences, approximately 1100 bp in length and identical to each other, were amplified. These sequences exhibited the highest identity (93 %) with *Candidatus* Mycoplasma haematomolossi from a *Molossus rufus* bat in Belize (OQ518944). In the 23S rRNA phylogram, the consensus sequence for genotype 1 clustered with, but independently of, other bat sequences from the Americas, with high bootstrap value (Fig. 4). Given the 100 % identity of two 23S rRNA sequences and 99.8 % identity of the paired 16S rRNA sequences, we propose the name *Candidatus* Mycoplasma haematopipistrellus sp. nov. to designate this novel hemoplasma species. All bats were negative for all the other tested pathogens.

4. Discussion

Bats are well-known reservoirs of pathogens, and urban areas are the most likely sites for wildlife-to-human spillover events [8]. In this study, we performed, for the first time, a comparison of potentially zoonotic bacteria and parasites between urban and rural conspecific bats. The study sample exhibited surprisingly low diversity of these pathogens. Although a similar studies performed in Brazil also detected a low diversity of blood pathogens in bats [49], this result was unexpected,

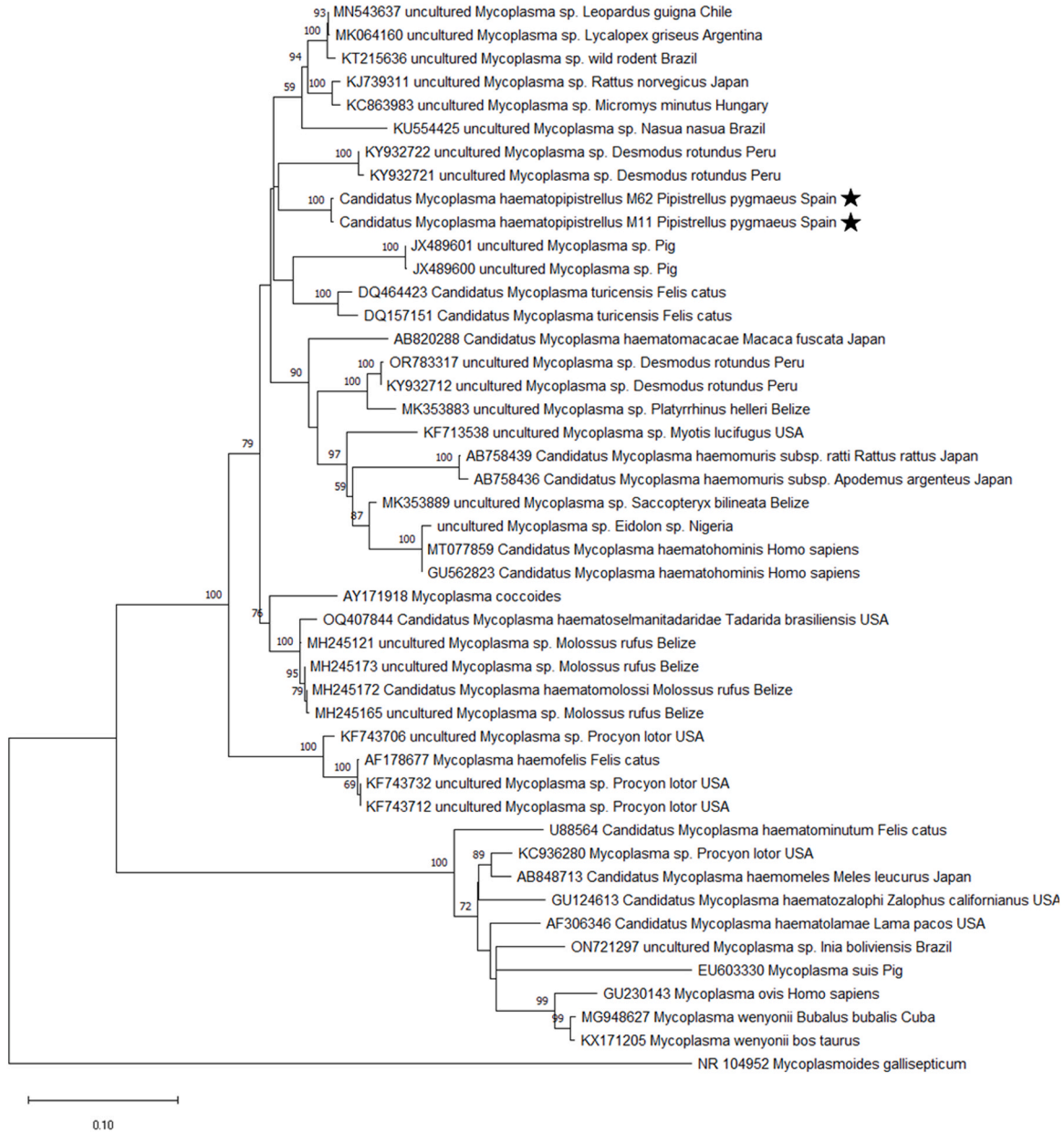

Fig. 3. Maximum-likelihood tree based on partial 16S rRNA (~ 1120 bp) gene sequences of hemotropic Mycoplasmas. Numbers represent bootstrap support generated from 1000 replications. The tree was constructed with the General Time Reversible model (GTR + G + I). New sequences obtained in the present study are indicated with a star.

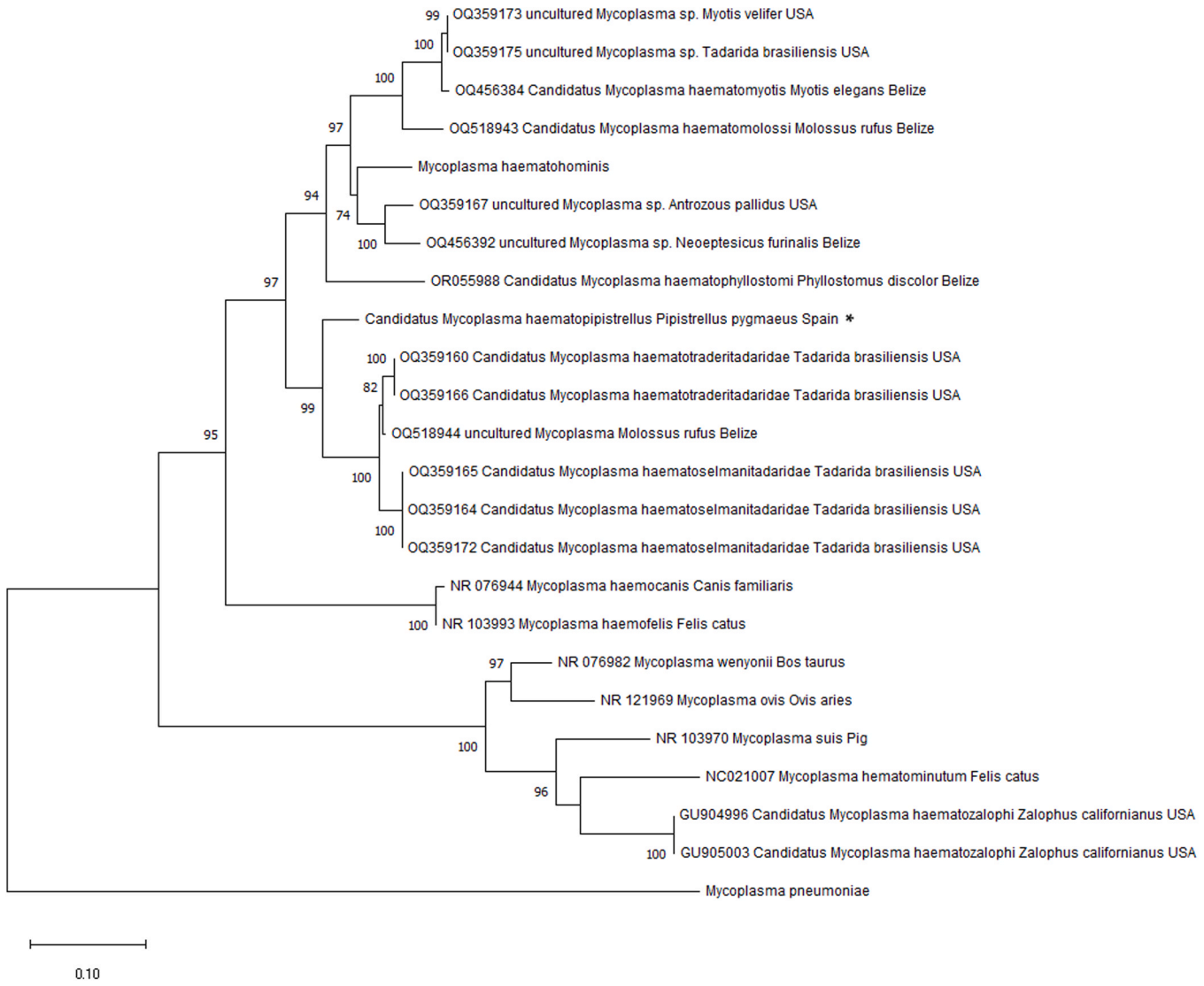

Fig. 4. Maximum-likelihood tree based on partial 23S rRNA (~ 1100 bp) gene sequences of hemotropic Mycoplasmas. Numbers represent bootstrap support generated from 1000 replications. The tree was constructed with the Tanura-Nei model (TN93 + G + I). The new sequence obtained in the present study is indicated with a star.

given that bats are considered natural hosts for many disease agents, such as for *Bartonella* [33]. In addition, the study area is enzootic for some of these agents, such as *Leishmania infantum* [1]. Previous studies in Spain on *L. infantum* have shown no infection in blood samples from cave bats (*Miniopterus schreibersii*) in Barcelona [37], but a relatively high prevalence in spleen samples from common pipistrelles (*Pipistrellus pipistrellus*) in Madrid (14 of 27 bats positive; [4]). The fact that we used blood samples rather than tissue samples (e.g., spleen) may have influenced the detection probability of some of these agents (see Ikeda et al. [24]).

Hemotropic mycoplasmas are facultative intracellular bacteria of special interest in bats, given their high prevalence and notable genetic diversity [36]. In Spain, the presence of hemoplasmas was only studied in a population of live-trapped cave bats, with a prevalence of 97 % [38]. The markedly higher occurrence in cave bats compared to the pipistrelles can likely be attributed to the distinct ecology of these species. Cave bats typically live in large colonies of thousands of individuals, which facilitates interspecific transmission, whereas pipistrelles tend to form much smaller groups that are formed only in some situations such as mating. Elsewhere in Europe, a study analysed dead bats from various species in Switzerland and Germany, including approximately 200 *Pipistrellus* spp. that tested negative for hemoplasmas [18]. In light of these findings, the occurrence observed in our study can be regarded as comparatively high. Nevertheless, the use of different types of samples (spleen vs blood) and the use of different PCR protocols might have influence these differences.

Two distinct hemoplasma genotypes were detected. The first genotype, identified through sequencing a short fragment of the 16S rRNA gene, clustered in the clade of *C*Mhh, the causative agent of the flying fox hemorragic fever [15]. Sequences into this clade have been detected not only in flying foxes (*Pteropus* spp.) but also in other species of bats from Spain [38], Nigeria [16], China [52], and Australia [22], in bat ticks from Hungary [23], and in a wood mouse (*Apodemus sylvaticus*) from Spain [39]. It is worth mentioning that this hemoplasma species was recently detected in four immunosuppressed children in Spain [17]. Therefore, this hemoplasma appears to be widespread in bats from Eurasia and Australia, with sporadic spillovers to other species, including humans. This pattern may also reflect host shifts over evolutionary time rather than contemporary cross-species transmission [36]. Unfortunately, further characterization of this genotype was unsuccessful. Nevertheless, regarding the analysis of the 16S rRNA gene, it is worth noting that the topologies of the phylogenetic trees obtained with

both short (~ 300 nt) and long (~ 1100 nt) 16S rRNA sequences were highly similar, a pattern also reported by Di Cataldo et al. [16]. This provides additional support for the placement of this genotype within the *C*Mhh clade.

The second genotype, the most frequently detected in the studied bats, meets the criteria for being considered a novel species, as previously mentioned. Although a closely related fragment of the 16S rRNA gene was reported in a bat from Germany [18] and another from Chile [35], no further molecular characterization was conducted in these studies. While this genotype is not related to *C*Mhh, this does not rule out its zoonotic potential. Many pathogens are initially detected when they cause disease in humans, and their zoonotic origin is only later revealed, as was the case with *C*Mhh [28]. Moreover, *C*Mhh is not the only hemoplasma found infecting humans; human cases of *Mycoplasma ovis*, *M. suis*, *M. haemofelis*, and *Candidatus* M. haematoparvum have been confirmed (see review in [36]).

Interestingly, both variants were found in both urban and rural specimens. This is significant because it suggests that pathogen transmission occurs between urban and rural colonies, indicating that bats are acting as bridges between natural and urban environments, highlighting their potential for circulation across ecological contexts. Urban areas serve as interfaces that represent critical points for cross-species transmission and the emergence of pathogens into new host populations [20]. Our survey provides evidence of pathogen transmission between counterparts inhabiting rural and urban areas. Additionally, one of the genotypes (*C.* M. haematopipistrellus) was shared between species. We observed that, in urban settings, PPY and PKU individuals did not share artificial roosts. However, we cannot be certain whether this was also the case in the abandoned building used by rural individuals. Hemoplasmas can be transmitted through various routes. Bats of different species may transmit the bacterium during aggressive interactions via saliva or blood. Other transmission pathways, such as through vectors, cannot be excluded. The studied pipistrelles hosted mites, fleas, ticks, and batflies (data not shown), all of which may be implicated in transmission.

In summary, we confirmed the presence of potentially zoonotic hemoplasmas in urban bats for the first time, included a genotype with phylogenetic similarity with *C*Mhh. Although the studied populations seem to have limited contact with other zoonotic pathogens, longitudinal studies are essential to better understand the dynamics and impact of these agents in urban bats. It is important to remember that urban pipistrelles provide significant ecosystem services, such as pest control, making the characterization of their parasites and pathogens crucial from a One Health perspective.

CRediT authorship contribution statement

Javier Millán: Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Paula Santibáñez:** Writing – review & editing, Methodology, Investigation.**Luis Vicente Monteagudo:** Writing – review & editing, Project administration, Investigation. **Sofia M Soares:** Writing – review & editing, Investigation. **Alberto Israel:** Visualization, Investigation. **Ruth Rodríguez-Pastor:** Writing – review & editing, Investigation.

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Conflict of Interest

Authors declare no conflict of interests.

Declaration of Competing Interest

The authors have nothing to declare.

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Data availability

The sequence data obtained from this study have been deposited in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank). All sequences are available in GenBank under the accession numbers PX271157–PX271162 and PX277289–PX277292 for the *16S rRNA* gene, and PX259640 and PX259641 for the *23S rRNA* gene.

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