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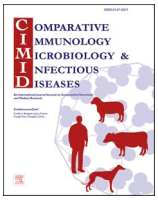

High diversity, novel genotypes, and vertical transmission of hemotropic *Mycoplasma* in micromammals

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ABSTRACT

Hemotropic mycoplasmas (hemoplasmas) are emerging zoonotic pathogens. Micromammals have received little attention as hosts for hemoplasmas despite their ubiquitous presence, high population abundances, and close association with humans. A PCR protocol targeting a fragment of the 16 S rRNA gene and direct sequencing in blood samples of 189 adult specimens and 35 fetuses belonging to three species of Eulipotyphla (shrews) and seven species of Rodentia, captured in three ecologically diverse habitats in North-Eastern Spain (Steppe, High Mountain, Mediterranean) yielded and occurrence of 26%, including 36% of 39 shrews and 23% of 150 rodents. Sequencing revealed the presence of 14 nucleotide sequence types (ntST) among the 56 readable sequences. In general, each ntST was associated with a given host species, although in some cases, the same ntST was sequenced in different species (chiefly rodents). Most ntST were closely related to rodent and/or bat hemoplasmas, but one was identical with *Mycoplasma haemocanis/haemofelis*, and others can be considered novel genotypes. High sequence diversity was detected in rodents, whereas in the white-toothed shrew (*Crocidura russula*), 9/11 sequences from two distant areas were identical. Phylogenetic and network analyses classified our sequences in different clades including hemoplasmas of rodents, carnivores, bats, and humans. Twelve of the fetuses (34.2%) of 9/12 litters (75.0%) of shrews and rodents were hemoplasma-positive, indicating frequent vertical transmission. Our study contributes to expanding our knowledge about the distribution, diversity, and transmission of hemoplasmas.

1. Introduction

Hemotropic mycoplasmas or hemoplasmas are widespread bacteria that attach to the surface of erythrocytes of mammals [1,2], which can cause variable degrees of hemolytic anemia, especially in immunocompromised hosts [1,2]. In the last few years, the host range for hemoplasmas has dramatically increased, and it is rare to find a wild species not infected by one or more species of these bacteria [2].

Despite the abundant populations and worldwide distribution of micromammals (orders Rodentia and Eulipotyphla, the latter formerly known as Insectivora), which are present in all types of environments, including in close association with humans, little attention has been provided to hemoplasma infection in these taxonomic groups. Only a handful of studies in rodents have been published in a few geographical areas. Most of the studies included synanthropic species such as house mice (*Mus musculus*) or rats (*Rattus* spp.) [1,3–8]. Truly wild rodents have been only investigated in Brazil [6,9,10], Switzerland [1], South Africa [11], and Israel [12].

Two hemoplasmas of rodents, both belonging to the *Mycoplasma haemofelis* group, have been established: *M. coccoides* and *M. haemomuris*. Most of the genotypes infecting wild rodents are closely related to these two, except for large-sized rodents, such as capybaras (*Hydrochoerus hydrochaeris*) and hairy dwarf porcupines (*Sphiggurus villosus*), that appear to have their haemoplasmas [13,14]. In addition,

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some poorly characterized, new genotypes have been described [3,7]. In contrast, no study has yet investigated the presence of hemoplasmas in members of the Eulipotyphla, a taxonomic order that includes hedgehogs, shrews, or moles, among others.

There is a need to expand our knowledge of hemoplasma genotypes and other traits of hemoplasma infection in wild rodents for several reasons. In the first place, it would serve as a new piece to complete the complex puzzle of hemotropic *Mycoplasma* phylogeny. In the second place, rodents are abundant and suitable to test different hypotheses regarding transmission and infection dynamics (e.g. [12]). It has also been hypothesized that some rodents could be the natural hosts of some hemoplasmas species infecting domestic cats [1] and wild carnivores [15–17], which might become infected by a prey-to-predator spillover. Finally, it has also relevance from the zoonotic perspective [18–20]. Although no rodent hemoplasma is considered zoonotic to date, often, a new pathogen described in humans is later detected in wildlife, as was the case of the flying fox hemolytic fever caused by *Candidatus* Mycoplasma haematohominis [21–24].

The way hemoplasmas are transmitted is not completely elucidated. Together with vector-borne and direct routes [2], vertical transmission has been suggested as an alternative transmission way in humans and domestic species, although evidence in this sense is limited and controversial [25–30]. An experimental study in wild rodents kept in captivity failed to prove mother-to-offspring transmission, although the sample size in such an experiment was low [12]. No other study has investigated in-utero transmission in wildlife. In consequence, vertical transmission of hemoplasmas calls for further exploration.

The objective of this study was to determine the prevalence of infection and genetic diversity of hemotropic mycoplasmas in a sample of wild rodents and shrews in different habitats of the Iberian Peninsula. We also aimed to understand whether the genetic identity of hemoplasmas is more associated with the host species or the geographic location, by comparing the same species from distant locations; and whether vertical transmission from mothers to offspring occurs.

2. Materials and methods

Micromammals included in this survey were live captured during different studies performed between 2013 and 2020 in four study sites located in three Spanish provinces in the Northeast of the country (Fig. 1). The three areas have contrasting bioclimatic conditions: Ordesa National Park (42°40′N 00°03′E), located at the Pyrenees in the Huesca province (Aragón), is a High Mountain habitat above 2000 m.a.s.l.; Los Monegros (41°49′N 00°45′W), in Zaragoza province (Aragón), is a Steppe with a cold semi-arid climate; and Collserola (41°24′N 02°04′E) and Sant Llorenç del Munt (41°38′N 02°01′E) Natural Parks, located only 25 km apart in Barcelona province (Catalonia), have a typical Mediterranean climate.

Three different Eulipotyphla and six Rodentia species were included in this study (Table 1). Animals were captured using Sherman traps (H. B. Sherman Traps, Inc., Tallahassee, Florida) and transferred from the trap to a plastic bag and weighed using a Pesola scale to the nearest 0.5 g and anaesthetized with a combination of ketamine (Domtor©, Esteve, Barcelona, Spain) and medetomidine (Imalgene©, Merial, Barcelona, Spain) [31]. Rodents were then euthanized and necropsied in detail. A piece of spleen was collected and kept at –20 °C until further analysis. A piece of spleen was also obtained from shrews found dead in the traps. During the necropsy of twelve pregnant females (three shrews and nine mice), the complete uterus was retrieved carefully, placed into a separate vial, and kept at –20 °C. Later, fetuses (n=35, from one to six fetuses per litter) were individually retrieved from the uterus into a dissection hood using a sterilized set of instruments and placed into separate vials.

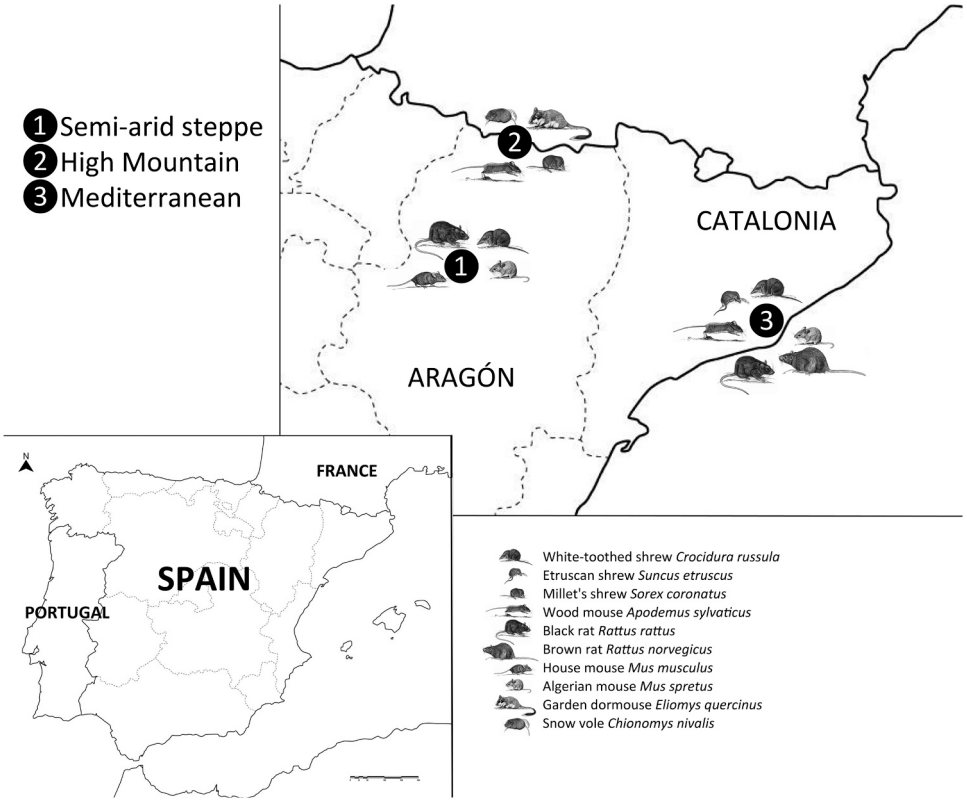

Fig. 1. Map of peninsular Spain showing the study sites in Aragon and Catalonia as well as the micromammal species sampled. Numbers pointed to the three study sites and the habitat type: 1, Los Monegros (Steppe); 2, Ordesa National Park (High Mountain); and 3, Collserola and Sant Llorenç del Munt Parks (Mediterranean). Drawings by Jordi Mateos from the "Atlas y Libro Rojo de los Mamíferos Terrestres de España", edited by Dirección General para la Biodiversidad-SECEMSECEMU, used with authorization.

Table 1
Micromammals analysed for the presence of hemotropic mycoplasma DNA in the three studied habitats. Sample size, number of positives, occurrence, and 95% Confidence Interval (only for species over 15 individuals) are shown.

Species	Tested (positive)			Total adults (positive)	Occurrence (95% C.I.)
	High Mountain	**Steppe**	**Mediterranean**		
Eulipotyphla					
White-toothed shrew *Crocidura russula*	0	11 (6)	26 (6)	37 (12)	32.4% (18.0–49.8)
Etruscan shrew *Suncus etruscus*	0	0	1 (1)	1 (1)	-
Millet's shrew *Sorex coronatus*	1 (1)	0	0	1 (1)	-
Total Insectivora				39 (14)	35.9 (21.2–52.8)
Rodentia					
Wood mouse *Apodemus sylvaticus*	6 (1)	0	83 (18)	89 (19)	21.3 (13.4–31.3)
Black rat *Rattus rattus*	0	1 (1)	7 (2)	8 (3)	-
Brown rat *Rattus norvegicus*	0	0	2 (0)	2 (0)	-
House mouse *Mus musculus*	0	1 (1)	0	1 (1)	-
Algerian mouse *Mus spretus*	0	13 (10)	19 (1)	32 (11)	34.4 (18.6–53.2)
Garden dormouse *Eliomys quercinus*	1 (0)	0	0	1 (0)	-
Snow vole *Chionomys nivalis*	17 (1)	0	0	17 (1)	5.88 (0.01–28.7)
Total Rodentia				150 (35)	23.3 (16.8–30.1)
Overall	25 (3)	26 (18)	149 (28)	189 (49)	25.9 (19.8–32.8)

To avoid contamination, different sets of dissection tools were used for each fetus, which were cleaned with 95% ethanol and fire-sterilized after every sample.

A real-time PCR based on Sybr Green chemistry which targets a 391 bp of the 16 S rRNA gene of the *Mycoplasma* spp. [8] was performed. We have shown that this protocol found enough variability to characterize the detected hemoplasmas when compared with the characterization of the entire gene [32]. PCR fragments were sequenced with both primers by Sanger sequencing. We conducted a BLAST search to compare sequenced products with sequences described in GenBank for hemoplasmas. After ClustalW alignment, a selection of evolutionary models was performed, using ModelFinder (IQ-TREE web server) [33]. Then, a phylogenetic analysis was performed by the Maximum Likelihood algorithm based on the Tamura-Nei model. Sequences obtained in the present study showing 100% identity were classified as the same nucleotide sequence type (ntST). All sequence analyses were performed with MEGA software, version X [34]. To infer a host-based clustering of mycoplasms detected in rodents, shrews, chiropterans, primates, carnivores, and ungulates, a phylogenetic network carried on NeighborNet model on Jukes-Cantor distance was performed with the Splitstree4 software (version 4.19) [35].

The new *Mycoplasma* 16 S rRNA gene sequences were submitted to the GenBank© under accession numbers OR661898-OR661911.

Differences in prevalence between orders, species, and habitats were compared using Chi-square or Fisher's exact tests.

3. Results

Forty-nine out of 189 adult individuals resulted positive for hemoplasmal DNA (observed occurrence= 25.9%; 95% Confidence Intervals= 19.8%–32.8%) (Table 1). Overall, pathogen occurrence was not different between Eulipotyphla and Rodentia (36% vs 23%; χ^2=2.8, p>0.5). When comparing the species with enough sample size, the snow voles showed significantly lower occurrence (6%) than Algerian mice (34%) and white-toothed shrews (32%; Fisher's p= 0.037 and 0.007, respectively).

The occurrence was higher in the Steppe (69%) than in the High Mountains (19%; Fisher's p= 0.0001) and the Mediterranean (12%; χ^2=34.6, p<0.001) when species were pooled, but no such differences were observed between the High Mountains and the Mediterranean climate (Fisher's p= 0.42). When comparing the same species captured in different habitats, occurrence was significantly higher in Algerian mice from the Steppe than in the Mediterranean climate (Fisher's p= 0.0001). A similar, non-significant tendency was found for the white-toothed shrews (χ^2=3.4, p=0.06).

Sequencing revealed the presence of 14 ntSTs among the 56 readable sequences from adults (Tables 2 and 3). Sequences showed between 94.9% and 99.2% identity among them and between 94.1% and 100%

Table 2
Nucleotide sequence types (ntST) of hemoplasmas identified in adult micromammals.

ntST	Species (n. of individuals)	Closest ntST (% identity)	Closest Genbank sequence (% identity)
1	Snow vole (1)	ntST2 (97.8%)	*Mycoplasma* sp. KT215621 from wild rodent (98.4%)
2	Wood mouse (4) Black rat (1) Etruscan shrew (1)	ntST14 (99.2%)	*Mycoplasma* sp. KT215621 from wild rodent (97.0%)
3	Wood mouse (1)	ntST13 & ntST6 (97.3%)	*Mycoplasma coccoides* AY171918 from *Mus musculus* (97.0%)
4	Wood mouse (4)	ntST6 (98.7%)	*Mycoplasma* sp. KC863983 from *Mycromis minutus* (99.2%)
5	Wood mouse (2) Algerian mouse (1)	ntST7 (94.1%)	*Mycoplasma* sp. MH383152 from *Ixodes simplex* (100%)
6	Wood mouse (2) Algerian mouse (1)	ntST4 (98.7%)	*Mycoplasma* sp. KC863983 from *Mycromis minutus* (97.8%)
7	Wood mouse (2)	ntST14 (94.9%)	*Mycoplasma* sp. KT215635 from a wild rodent (99.2%)
8	Wood mouse (2) Millet's shrew (1)	ntST3 (92.7%)	*Mycoplasma haemocanis* MZ221174 from *Canis familiaris* (100%)
9	Algerian mouse (2)	ntST1 (97.3%)	*Mycoplasma* sp. KT215621 from a wild rodent (97.0%)
10	White-toothed shrew (9) Algerian mouse (1)	ntST2 (95.7%)	*Mycoplasma* sp. MK353818 from *Natalus stramineus* (96.8%)
11	Algerian mouse (1)	ntST12 (97.3%)	*Mycoplasma* sp. MK353818 from *Natalus stramineus* and *Mycoplasma* sp. AB752303 from *Rattus norvegicus* (93.5%)
12	Algerian mouse (2) House mouse (1) White-toothed shrew (1) Black rat (1)	ntST11 (97.3%)	*Mycoplasma* sp. MN423263 from *Rattus rattus* (94.1%)
13	Algerian mouse (2) White-toothed shrew (1) Black rat (1)	ntST3 (97.3%)	*Mycoplasma coccoides* AY171918 from *Mus musculus* (99.7%)
14	Wood mouse (1)	ntST2 (99.2%)	*Mycoplasma* sp. KT215621 from a wild rodent (97.3%)

Table 3
Nucleotide sequence types (ntST) detected in adult specimens per species and habitat.

Species	High Mountain	Steppe	Mediterranean
White-toothed shrew		ntST-10 (n=4)	ntST-10 (n=5)
		ntST-12 (n=1)	Nonreadable (n=1)
		ntST-13 (n=1)	
Etruscan shrew			ntST-2 (n=1)
Millet's shrew	ntST-8 (n=1)		
Wood mouse	ntST-3 (n=1)		ntST-2 (n=5)
			ntST-3 (n=1)
			ntST-4 (n=4)
			ntST-5 (n=2)
			ntST-6 (n=2)
			ntST-7 (n=2)
			ntST-8 (n=2)
			ntST-14 (n=1)
House mouse		ntST-12 (n=1)	
Algerian mouse		ntST-5 (n=1)	ntST-9 (n=1)
		ntST-6 (n=1)	
		ntST-9 (n=1)	
		ntST-10 (n=1)	
		ntST-11 (n=1)	
		ntST-12 (n=2)	
		ntST-13 (n=2)	
		Non readable (n=1)	
Black rat		ntST-13 (n=1)	ntST-2 (n=1)
			ntST-12 (n=1)
			Nonreadable (n=1)
Snow vole	ntST-1 (n=1)		

identity with already published sequences. In general, these sequences were described in rodents worldwide, with the following few exceptions: ntST-5 was 100% identical to a sequence detected in the bat tick *Ixodes simplex*; ntST-8 also showed 100% identity with the dog hemoplasma *Mycoplasma haemocanis*; and ntST-10 and 11 had, respectively, 95.7% and 97.1% identity with a sequence from a bat (*Eptesicus furinalis*, Genbank® Accession Number MK353816). Besides, ntST-11 had the same identity (97.1%) with a sequence from a rat (*Rattus norvegicus*, Genbank Accession® Number AB752303). The phylogenetic tree classified our sequences in different clades including hemoplasmas of rodents, carnivores, bats, and humans (Fig. 2). High bootstrap values were found for ntSTs-1, 2, 9, and 14 (88% of a rodent cluster), ntST-7 (99% of a rodent cluster), ntST-13 (100%, of a *M. coccoides*, respectively), ntST-11 and 12 (90% of a *Mycoplasma* detected in *I. simplex*) and ntST-8 (100% of *Mycoplasma haemocanis*). ntST-3, 4, 6, and 10 were not properly classified.

Network analysis (Fig. 3), in agreement with the phylogenetic tree, revealed at least 9 different clusters classified by the predominant host taxa. The majority of ntSTs (1–4, 6, 7, 9, and 11–13) were classified into three different rodent clusters, whereas ntST5 and 10 were classified in two different chiropteran clusters and ntST8 in an ungulate cluster. Interestingly, our network revealed that hemoplasmas from carnivores were distributed in different clusters from ungulates, rodents, and chiropterans.

The highest diversity was detected in the wood mouse (eight different ntSTs among 19 readable sequences) and the Algerian mouse (seven different ntSTs among nine readable sequences). In contrast, nine out of 11 sequences in the white-toothed shrew belonged to the same ntST. Few cases of shared ntSTs among species were found and always involved either the wood mouse or the Algerian mouse. In the Steppe, ntST-12 was shared among white-toothed shrew, house mouse, and Algerian mouse; and ntST-13 was shared among white-toothed shrew, Algerian mouse, and black rat. In the Mediterranean, ntST-2 was shared among wood mouse, Etruscan shrew, and black rat. When comparing different study areas, ntST-3 was shared between wood mice in High Mountain and Mediterranean areas; ntST-10 among white-toothed shrews in the Steppe and Mediterranean areas; and ntST-12 among a black rat in the Mediterranean area with the above-mentioned species in the Steppe.

Twelve out of 35 fetuses (34.2%) of nine out of 12 litters (75.0%) were positive for hemoplasma (Table 4). Positive fetuses belonged to three different species and were found in both hemoplasma-positive and negative mothers. One positive female shrew had five negative fetuses. When readable sequences were obtained both from the mother and the fetus, these shared the same ntST in one case and did not in two cases. This included one mother with ntST-12 with two positive fetuses, one infected by ntST-12, and the other by ntST-13.

4. Discussion

An increasing number of studies have shown that hemoplasmas are diverse and prevalent in wildlife [2]. However, such a review also identified taxonomic groups that were underrepresented in terms of surveillance, being two of such groups the rodents and the insectivores. That review also reflected that only a handful of studies on the subject have been performed in Europe [2]. The present survey therefore contributes to partially fulfilling these weaknesses with new data about wild rodent hemoplasmas, and with new genetic sequences, some of which may correspond to yet undescribed species.

Previously, only two studies were published about hemoplasmas in European free-ranging rodents. The first one was performed in Switzerland to determine their roles as natural hosts for feline haemoplasmas, with mean prevalences in the wild species with representative sample sizes ranging from 0% to 47% [1]. However, this study used species-specific primers, which could have resulted in the lack of detection of other hemoplasmas. The second study analyzed synanthropic rodents in Hungary, with an overall high prevalence (71%; [3]). Studies elsewhere in the world analyzing blood or spleen samples reported prevalences around or above 50% [7,8,10], except for a study in Chile (8%; [5]). Therefore, the occurrence observed during our survey can be considered between low and intermediate when compared with previous studies [5,7,8,10].

This study is the first to confirm that shrews are also susceptible to hemoplasma infection and with an occurrence comparable with other wildlife. However, sequence diversity was low in this species. Most of its individuals, even from two areas 350 km away, were infected by the same ntST, which was not shared with any other species. This suggests a close association or coevolution between this variant and its host. According to the low level of similarity with the closest sequence (96.8%) and the phylogenetic analysis, this sequence corresponds to a novel genotype. However, the phylogenetic analysis was not able to resolve its classification. Although its closest sequence was found in a bat and the sequence was classified among other bat sequences in the phylogenetic tree, the bootstrap value was low. New sequences from members of the order Eulipotyphla will be needed to solve its phylogenetic classification.

Prevalence was higher in the Steppe habitat when compared with the two other types of habitats, both with all the species pooled but also

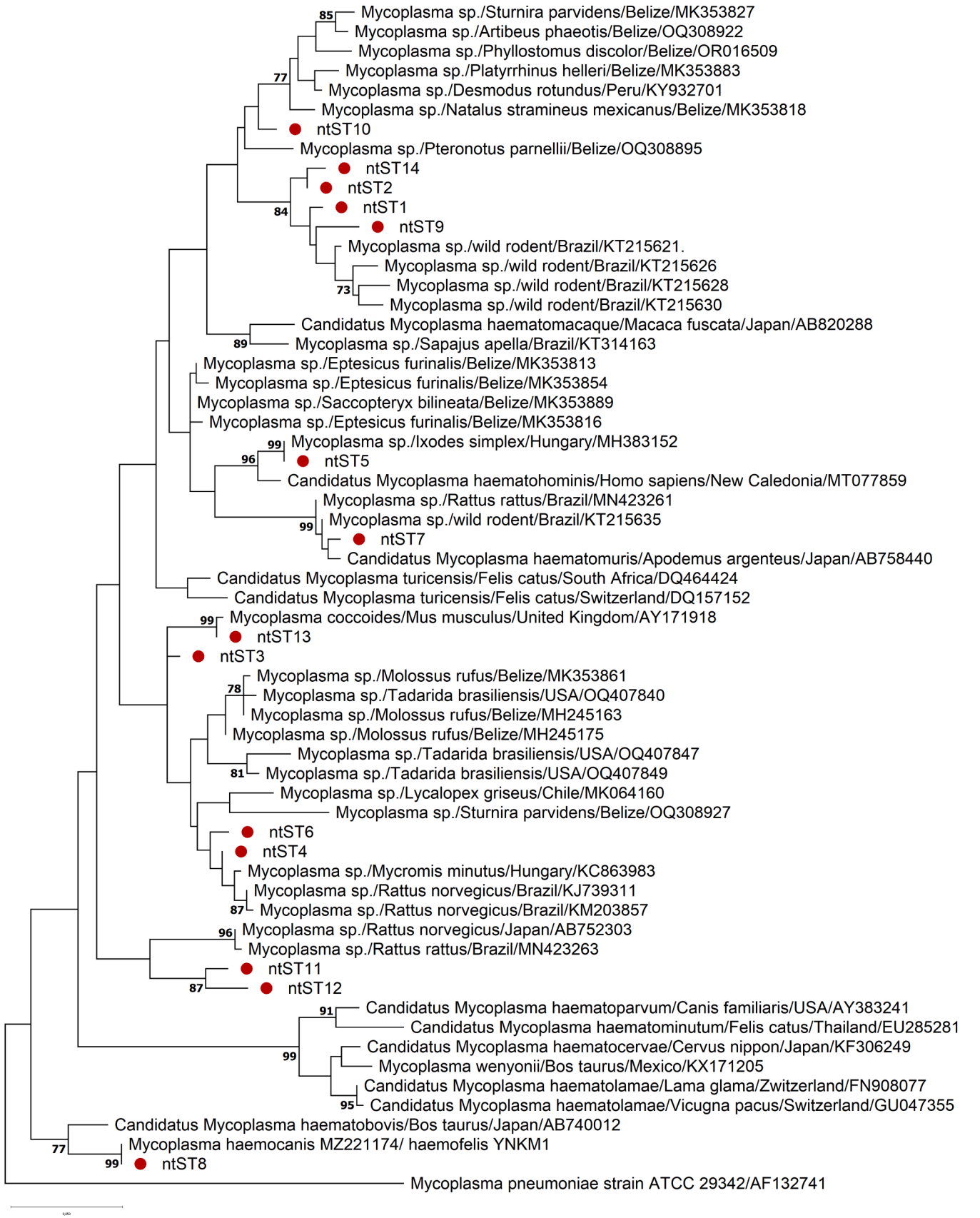

Fig. 2. Maximum-likelihood tree based on the Tamura-Nei model of 60 selected sequences from hemotropic mycoplasmas. The name of the sequence indicates the host species and GenBank accession number. The percentage of trees in which the associated taxa clustered together (bootstrap values) is shown next to the branches. All bootstrap values less than 70 are not shown. The scale bar indicates the p-distance of the branches. *Mycoplasma pneumoniae* (an epitheliotropic species) is included as an outgroup member.

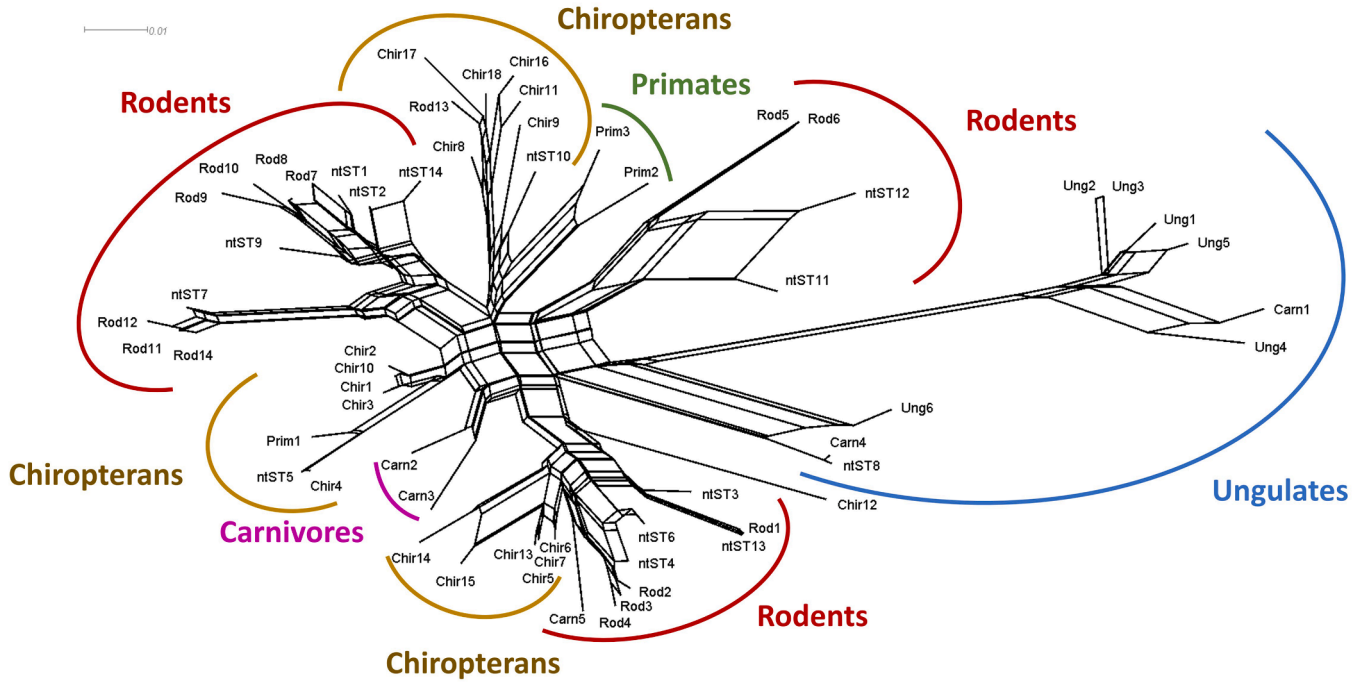

Fig. 3. Phylogenetic network carried on NeighborNet model on Jukes-Cantor distance of 60 sequences obtained from the Genbank and the present study. The sequences obtained in our study are labeled by their nucleotide sequence type (ntST), whereas the label of the sequence obtained in the Genbank is referred to its host taxon (Carn: Carnivore; Chir: Chiroptera; Prim: Primate; Rod: Rodent; Ung: Ungulate).

Table 4
Results from the molecular analysis of fetuses of micromammals for the presence of DNA of hemoplasmas and nucleotide sequence type (ntST) detected in each sample.

Reference	Habitat	Species	Female	Fetuses
20013	Steppe	White-toothed shrew	**Positive**, ntST-10	• 5 negative
20014	Steppe	White-toothed shrew	Negative	• 1 **positive**, ntST-13 • 3 negative
12146	Mediterranean	White-toothed shrew	No sample	• 1 **positive**, ntST-10 • 2 negative
20018	Steppe	Algerian mouse	**Positive**, ntST-6	• 1 **positive**, ntST-13
20001	Steppe	Algerian mouse	**Positive**, ntST-12	• 2 **positive**, ntST-12 & 13 • 2 negative
11254	Mediterranean	Wood mouse	No sample	• 1 **positive**, ntST-5 • 2 negative
12107	Mediterranean	Wood mouse	No sample	• 1 **positive**, ntST-14
12110	Mediterranean	Wood mouse	No sample	• 1 negative
12111	Mediterranean	Wood mouse	No sample	• 1 negative
12170	Mediterranean	Wood mouse	No sample	• 1 **positive**, ntST-14
13076	Mediterranean	Wood mouse	Negative	• 2 **positive**, ntST-5 • 4 negative
13080	Mediterranean	Wood mouse	**Positive**, ntST-2	• 2 **positive**, non-readable • 3 negative

when comparing only the best-represented species. This is in line with a previous observation in Chilean wild foxes, where the prevalence of hemoplasmas was found to be higher in the driest region studied [36]. The Steppe habitat is drier and hotter than the other two studied biotopes but, apparently, this should not benefit the persistence of the bacteria in the host or enhance the transmission of hemoplasmas. Fleas, ticks, and mites were found in animals from all three habitats (data not shown), so differences in arthropod vector prevalence do not seem to be the cause. The very low prevalence found in the snow vole from the High Mountains may be explained by the low abundance of snow vole populations in the study area.

4.1. *Genetic variability and interspecific transmission*

As previously found in wildlife [2], genetic diversity in the sample was considerable, especially in rodents. Some of the wood mice from the Mediterranean area analyzed here were previously analyzed for DNA of *Bartonella* sp. [37], and this species was the one also showing the highest *Bartonella* genotype diversity. The special aggressiveness of this rodent species or a high host population abundance may enhance pathogen transmission, which could explain such diversity. Algerian mice from the Steppe also showed considerable diversity, with seven different ntST among only nine readable sequences. Although we do not have population data on this area, the Algerian mouse was the most frequently captured rodent in the Steppe (data not shown) and is a species typically found in dry climates. In contrast, as previously mentioned, most readable sequences in the white-toothed shrew belonged to the same ntST-10. This is also in alignment with the study by Cevidanes et al. [37], where most of the shrews were infected by the same *Bartonella* genotype. Shrews and rodents are genetically distant taxonomic groups, which probably prevents inter-specific transmission. In fact, most shared ntST were so between rodents, with only one with shrews, probably as a result of isolated spill-over events.

Although some haemoplasma genotypes were shared between micromammal species, most showed host specificity, as previously found in bats [38]. In addition, by comparing the same species from distant locations, we obtained further support for the fact that hemoplasma genotypes are associated with specific hosts. Although in some areas we were able to obtain only a few sequences, in general terms we found that in the three species for which we captured individuals in two different areas, their genetic variants were present in both sites. This was

particularly evident in shrews, with the more frequent haplotype found in individuals captured more than 300 km away. We can conclude in this sense that there is some degree of host-parasite association with eventual interspecific transmission events.

Two wood mice and one Millet's shrew were infected by a sequence showing 100% identity with *Mycoplamsa haemocanis/haemofelis* (these species are not differentiable based on 16 S rRNA sequencing alone), hemoplasmas of domestic and wild carnivores [39]. This might be the result of a spill-over from the carnivore to the rodent, but this hypothesis lacks ecological sense. Instead, this opens the door to the possibility that *M. haemocanis/haemofelis* was originally a rodent pathogen that jumped to carnivores through a prey-to-predator way, as was hypothesized with *M. haemofelis* [1]. In this sense, the network analyses revealed that carnivores are often present in clades of other taxonomic groups. This points to spill-over events via a predation route, as has been hypothesized before [15,39,40].

4.2. Vertical transmission

We have found strong evidence for in-utero transmission of hemoplasmas from females to their offspring. Moreover, we have confirmed this in the three investigated species: one shrew and two mice species. The existence of vertical transmission of hemoplasmas has been often hypothesized, as would have explained the common high prevalence in some of the studied wildlife (e.g. 97% of a bat population sample, [41]) and the lack of differences in prevalence between young and adult individuals found in some studies (e.g. [39]). More than seventy years ago, in-utero transmission was reported in pigs [42]. In humans, hemotropic *Mycoplasma* sp. was found in all 44 umbilical cords of pregnant, positive women tested, as well as in the neonatal peripheral blood samples taken at birth [27]. More recent studies reporting findings supporting potential vertical transmission included the detection of hemoplasmal DNA in pre-suckling newborn alpacas [25,30] and piglets [43], newborn calves [26,29], and dogs [44]. Therefore, we present for the first-time evidence of vertical transmission in wildlife. In contrast to our findings, Cohen et al. [12] failed to detect infected offspring neither from a positive, wild-caught Anderson's gerbil (*Gerbillus andersoni*) female nor from an experimentally infected female or an uninfected female coupled with an experimentally infected male. However, the lack of success of these experiments may be due to the low sample size, since, as we have shown here, not all the litters or all the individuals from a litter of an infected female are positive. A recent study conducted with wildlife showed that transplacental vertical transmission of hemoplasmas was unlikely to occur in bats [45]. Similarly, Hornok et al. [29] only confirmed hemoplasma DNA in 10.5% of calves (*Bos taurus*) (4 out of 38). Hornok et al. [29] suggested that the hemoplasmal genotype was a factor explaining whether vertical transmission takes place or not. We did not find support for the hypothesis, as we found positive fetuses infected with five different ntST that in addition classified separately in the phylogenetic tree.

Another interesting finding derived from our vertical transmission investigation is that fetuses do not always share hemoplasmal genotypes with their mothers. Moreover, one mother had fetuses with two different ntST. The most plausible explanation for this finding is that these females were co-infected by more than one hemoplasma. Indeed, coinfection by hemoplasmas was shown to be frequent whenever investigated [1,15,36,39,46–48]. Finally, some positive fetuses belonged to apparently negative females. This finding could constitute a false negative result.

5. Conclusion

Overall, our study advances our understanding of the distribution, genetic diversity, and transmission of hemotropic mycoplasmas. We present robust evidence that vertical transmission is not only possible but frequent, which is of relevance not only for microbiologists and disease ecologists but also for large and small animal veterinary practitioners. We also showed that shrews are hosts for a novel genotype that warrants further characterization.

CRediT authorship contribution statement

Ruth Rodríguez-Pastor: Writing – review & editing, Investigation. **Jesús Martínez-Padilla:** Writing – review & editing, Resources, Investigation. **Javier Millan:** Writing – original draft, Resources, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Bárbara Martín-Maldonado:** Writing – review & editing, Investigation. **Fernando Esperón:** Writing – original draft, Resources, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare no conflict of interests.

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