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► **To cite this version:**

Clotilde Rouxel, Adrien Etienne, Pascal Arné, Cécile Le Barzic, Guillaume Girault, et al.. Anaplasma phagocytophilum in urban and peri-urban passerine birds in Ile-de-France. Ticks and Tick-borne Diseases, 2024, 15 (4), pp.102350. 10.1016/j.ttbdis.2024.102350 . hal-04604193

HAL Id: hal-04604193

<https://hal.science/hal-04604193v1>

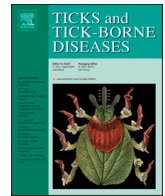
Submitted on 6 Jun 2024

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Original article

Anaplasma phagocytophilum in urban and peri-urban passerine birds in Ile-de-France

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ARTICLE INFO

Keywords:

Passerine

Anaplasma phagocytophilum

MLST

Typing

France

Tick-borne disease

ABSTRACT

Wild animals in general, birds in particular, play a key role in transporting ticks and propagating tick-borne pathogens. Several studies have confirmed the infection of birds with *Anaplasma phagocytophilum*, with overall prevalence varying widely from country to country and/or study to study. This zoonotic bacterium, transmitted mainly by ticks of the genus *Ixodes*, is responsible for granulocytic anaplasmosis in humans (HGA) and domestic animals (cats, dogs, horses). The disease is also called tick-borne fever (TBF) in ruminants. Extremely rare in the USA, TBF is very common in Europe, where it causes economic losses in livestock. Conversely, HGA is well established in the USA whereas only a few less severe cases have been observed in Europe. Current typing techniques support the existence of multiple variants with differences in virulence/pathogenicity and tropism for certain tick and host species. However, epidemiological cycles remain difficult to characterize in Europe. Several studies describe a cycle apparently involving only birds in Europe, but no such study has been conducted in mainland France. Our objectives were to search for *A. phagocytophilum* in passerine birds in the Ile-de-France region and to explore their diversity using *groEL* and *ankA* gene typing and multilocus sequence typing (MLST). Various tissues (spleen, liver, and skin) were collected from cadavers of 680 passerines between March and December 2021. The presence of *A. phagocytophilum* was detected by qPCR Taqman targeting the *msp2* gene. Three blackbirds (*Turdus merula*) were found positive, representing detection rates of 0.4 % in all birds tested and 3.3 % in blackbirds. The higher frequency of detection in blackbirds could be at least partially explained by their lifestyle, as they feed on the ground. Analysis of the results of *groEL* and *ankA* typing and MLST from positive blackbirds support the hypothesis that the avian *A. phagocytophilum* strains in Ile-de-France are distinct from those found in mammals, and that they form their own cluster in Europe.

1. Introduction

Anaplasma phagocytophilum is an intracellular tick-borne bacterium that causes granulocytic anaplasmosis. While this disease affects both humans and domestic animals, it is known as granulocytic anaplasmosis in humans (HGA), and tick-borne fever (TBF) in domestic ruminants (Battilani et al., 2017). HGA is generally characterized by a flu-like syndrome, which is more severe in the USA than in Europe. Conversely, TBF is extremely widespread in Europe, and is characterized in ruminants by hyperthermia, decreased milk production, anorexia, weakness, and abortion. Severe secondary infections can also occur due

to the immunosuppressive effects of *A. phagocytophilum* (Rar et al., 2021). *Anaplasma phagocytophilum* therefore has an important impact on both human and animal health. To better control granulocytic anaplasmosis, it is essential to study and characterize its epidemiological cycles, thus requiring identification of the animal species involved in its maintenance and transmission.

Anaplasma phagocytophilum is transmitted by several tick vectors of the Ixodidae family, mainly *Ixodes ricinus* in Europe. These ticks can feed on many host species and, besides humans, *A. phagocytophilum* has been detected in a wide range of domestic and wild animals, including mammals such as carnivores, ruminants, horses (*Equus caballus*), wild

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<https://doi.org/10.1016/j.ttbdis.2024.102350>

Received in revised form 9 April 2024; Accepted 4 May 2024

Available online 9 May 2024

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boar (*Sus scrofa*), hedgehogs (*Erinaceus* spp.), and rodents, but also birds and reptiles (Rar et al., 2021). Transovarial transmission of *A. phagocytophilum* has not been demonstrated in *I. ricinus* ticks (Stuenkel et al., 2013), so vertebrate reservoirs are needed to maintain it in nature.

In Europe, different genetic variants of *A. phagocytophilum* have been described by various typing techniques. Single-locus analysis of *groEL*, *ankA* or other genes has identified variants that differ according to animal host, geographic origin or pathogenicity / virulence (Battilani et al., 2017; Hoffman et al., 2020; Rar et al., 2021). Regardless of the gene used for typing, variant clusters remain identical for a few host species, though contradictory results are frequently observed (Battilani et al., 2017). A more discriminating multiple-locus analysis by multi-locus sequence typing (MLST) has shed light on the epidemiological cycles but has so far failed to identify the reservoir(s) for the zoonotic and ruminant variant in Europe (Chastagner et al., 2014; Huhn et al., 2014; Lagrée et al., 2018; Langenwalder et al., 2019; Tegtmeyer et al., 2019). These difficulties may be attributed to the fact that many European countries have only recently shown more interest in granulocytic anaplasmosis. Sampling may also prove difficult, particularly from wild animals, but it is vital because wildlife may play a role in the epidemiological cycles of strains infecting humans or ruminants. To date, the studies based on the *ankA* and *groEL* genes and/or on MLST suggest that strains from certain domestic animals (dogs (*Canis lupus familiaris*), horses) and wildlife such as wild boar, hedgehogs, and red foxes (*Vulpes vulpes*) belong to the same group as strains with zoonotic potential (Fröhlich et al., 2023; Rar et al., 2021). Red deer (*Cervus elaphus*) could play a reservoir role for ruminants, but cattle (*Bos taurus*) and sheep (*Ovis aries*) could also be reservoirs for their own strains (Fröhlich et al., 2023; Lagrée et al., 2018; Ogden et al., 2003). Very few typing studies have as yet been performed on birds, despite their important role in the circulation of ticks and tick-borne pathogens within Eurasia, and during spring and fall migration between Eurasia and Africa (Busse et al., 2015; Hoffman et al., 2023). In many European countries, *I. ricinus* is the main tick species found on birds (Hornok et al., 2014; Jahfari et al., 2014; Lommano et al., 2014; Rataud et al., 2022). Several studies in Europe have shown variable infection rates of *A. phagocytophilum* among birds (from 0 % to 33.8 %) (Borşan et al., 2021; de la Fuente et al., 2005; Ebani et al., 2016; Hornok et al., 2014; Jahfari et al., 2014; Lesiczka et al., 2021; Naranjo et al., 2006; Sándor et al., 2017; Skoracki et al., 2006; Skotarczak et al., 2006). In addition, a number of experimental studies have shown that different bird species can transmit *A. phagocytophilum* to ticks (Heylen et al., 2017; Keesing et al., 2012; Rataud et al., 2022). The involvement of birds as reservoir hosts for *A. phagocytophilum* has already been discussed (Daniels et al., 2002; Hornok et al., 2014; Keesing et al., 2012), but appears unlikely for the zoonotic and ruminant strains. The prevalence of *A. phagocytophilum* among birds and ticks feeding on birds is generally low (Hoffman et al., 2020; Honig et al., 2017; Pedersen et al., 2020), and several studies carried out in Europe have revealed genetic variants and epidemiological cycles specific to birds (Hoffman et al., 2020, 2023; Jahfari et al., 2014; Rar et al., 2021). However, a recent study (Lesiczka et al., 2021) targeting the *groEL* gene showed that *A. phagocytophilum* samples from blackbirds (*Turdus merula*) fitted into two clusters previously described by Jahfari et al. (2014): the ecotype IV cluster, which contained only birds, or ecotype I, which grouped a wide range of hosts (wild and domestic animals, and humans). This recent study also revealed that “ecotype IV” is not in fact specific to birds, as proven by samples from other mammals (one hedgehog (*Erinaceus roumanicus*) and four Eurasian red squirrels (*Sciurus vulgaris*)). This study thus suggests that birds may be involved in the circulation of zoonotic strains of *A. phagocytophilum*. In addition, even if they are not infected, birds may facilitate the long-distance transport and dispersal of infected ticks and the pathogens they harbor, as well as the transfer of pathogens between ticks via co-feeding (Buczek et al., 2020; Hasle, 2013; Levin and Fish, 2000). According to the limited and conflicting data available, the role of birds in the eco-epidemiological cycles of *A. phagocytophilum* remains to be determined.

Until our study, no epidemiological studies of *A. phagocytophilum* in birds had ever been carried out in mainland France. Moreover, most European studies had been conducted on a small number of birds belonging to a few species (de la Fuente et al., 2005; Hornok et al., 2014; Jahfari et al., 2014; Skoracki et al., 2006; Skotarczak et al., 2006). The aim of the present study was to investigate the presence of *A. phagocytophilum* in birds, particularly passerines, from urban and peri-urban areas of Ile-de-France and its neighboring départements (a French administrative unit). We applied the two most commonly used typing techniques for *A. phagocytophilum*, i.e., *groEL* and *ankA* typing on the one hand, and MLST (which is more discriminating) on the other.

2. Materials and methods

2.1. Study areas and bird sample collection

Passerine cadavers were obtained from the wildlife veterinary hospital of Alfort National Veterinary School (EnvA), France, mainly from March to October 2021 (with only seven passerines collected from November to December 2021), as this is both the breeding season for birds and the peak activity of *I. ricinus* nymphs (Hornok et al., 2014; Skoracki et al., 2006; Skotarczak et al., 2006; Yang et al., 2015). The birds included in this study died after admission or were humanely euthanized due to poor prognosis. Bird cadavers were stored at -20°C until necropsy and tissue collection. The medical record of each bird was consulted to collect information on its developmental stage (juvenile or adult), sex (if available), circumstances of its arrival at the veterinary hospital, area where collected, and cause of death.

Prior to tissue collection, birds were thawed in batches at 4°C either overnight for corvids (carrion crow (*Corvus corone*), rook (*Corvus frugilegus*), and common magpie (*Pica pica*)) or for half a day for smaller passerines. The spleen, a piece of liver and a piece of skin were collected from each bird when possible. Skin was collected from the area bounded distally by the most cranial part of the neck and the auditory canals, the eyes, or the beak. These areas are inaccessible to grooming operations, and are the preferred attachment sites for ticks (Fracasso et al., 2019; Klaus et al., 2016). Samples were stored at -20°C until DNA extraction.

2.2. Ethical approval

This work was approved by the EnvA Animal Care and Use Committee (agreement number: 2021–01–06).

2.3. DNA extraction

Skin, liver, and spleen samples were thawed and DNA was extracted in batches using the NucleoSpin® Tissue kit (Macherey-Nagel, Bethlehem, USA) after overnight proteinase K digestion, according to the manufacturer’s instructions except for the following modifications: after the two lysis steps, the suspension was centrifuged at $11,000 \times g$ for 5 min to remove insoluble particles. The supernatant was then transferred to a new microcentrifuge tube before proceeding with the DNA binding step. DNA was eluted with 100 μl of double-distilled filtered water and stored at -20°C until molecular analysis. For each sample, the DNA concentration and quality were evaluated spectrophotometrically using absorbance ratios of 260/280 nm and 260/230 nm (Nanodrop, Thermo Scientific, Waltham, USA).

For all the PCR/qPCR assays performed, negative and positive controls (molecular biology grade water or DNA from HL-60 cells infected with *A. phagocytophilum* strain NY18, respectively) were included in each run.

2.3.1. 12S rRNA PCR

To test for the presence of amplifiable DNA, a conventional 12S rRNA gene PCR (Humair et al., 2007) was performed on 10 % of the samples from each species. A PCR was performed on all samples when there were

fewer than ten samples per species. Supplementary Table S1 gives the primer sequence and describes the PCR cycles. One hundred nanograms of DNA was added to a PCR mix consisting of 1X Colorless GoTaq® Flexi Buffer, 200 µM dNTP (dATP, dTTP, dCTP, dGTP), 2.5 mM MgCl₂, 0.5 µM of forward and reverse primers, 2.5 U of GoTaq® G2 Hot Start Polymerase (Promega, Madison, WI, USA) and sterile water q.s. 50 µL. Amplicons were visualized on a 2 % agarose gel stained with ethidium bromide under UV light.

2.4. Screening of bird samples by *msp2* Taqman qPCR and confirmation of sample positivity by 16S rRNA nested PCR and sequencing

The presence of *A. phagocytophilum* in tissue samples was assessed by a Taqman real-time PCR targeting a 77 bp region of the gene encoding for the major surface protein 2 (*msp2*) (Courtney et al., 2004). The Taqman PCR was performed using 500 ng of extracted DNA, 6 µL of LightCycler 480 Probes Master 2X (Roche, Basel, Switzerland), 0.2 µM of forward and reverse primers, 0.2 µM of probe (Supplementary Table S1) and sterile water q.s. 12 µL. qPCR was performed in a LightCycler 480 thermocycler (Roche, Basel, Switzerland). Thermocycler conditions for the qPCR assays are listed in Supplementary Table S1. Spleen and liver samples from all birds were analyzed by a Taqman qPCR. Only skin samples from birds positive for *A. phagocytophilum* were subsequently tested by a Taqman qPCR. Samples with a C_T value < 33 were considered positive for *A. phagocytophilum* (Courtney et al., 2004). In order to determine the positivity threshold for samples analyzed by qPCR with C_T values between 33 and 39 (Flattery et al., 2022), a nested PCR targeting 16S rRNA was performed (Massung et al., 1998). Each reaction mix consisted of 1X Colorless GoTaq® Flexi Buffer, 1.5 mM MgCl₂, 200 µM dNTP, 0.5 µM of each primer (Supplementary Table S1), 1.25 U GoTaq® G2 Hot Start polymerase (Promega, Wisconsin, USA), and sterile water q.s. 50 µL. Five microliters of DNA samples (40 ng/µL) was used for the first/external PCR and 2 µL of amplified PCR products was used for the second/internal PCR. Thermocycler conditions are listed in Supplementary table S1. All the PCR products were then loaded onto a 1.5 % agarose gel stained with ethidium bromide. After migration, the bands of interest were collected and purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The purified amplification products were sequenced by Sanger sequencing in both directions by Eurofins Genomics (Nantes, France). The DNA sequences obtained were compared with the published sequence databases using the basic local alignment search tool (BLAST) (Altschul et al., 1990).

2.5. Typing of *A. phagocytophilum*-positive samples

Two different methods were used to type *A. phagocytophilum* in positive samples: single-locus analysis targeting *groEL* (Alberti et al., 2005) and *ankA*, and MLST (Chastagner et al., 2014). For MLST, eight segments in the loci *typA*, *msh4*, *pleD*, *recG*, *polA*, *groEL*, and *gyrA*, and the intergenic region *ctrA* (APH 1099–1100), were amplified by PCR. Nested PCRs were performed for all loci, both for single gene typing and MLST, except for *pleD* in MLST (for which only the external PCR was performed directly) (Chastagner et al., 2014). PCR reactions and amplicons purification were conducted according to the protocol of Lagrée et al. (2018).

The primer sequence and thermocycler conditions are shown in Supplementary Table S2. Purified products were sent to Eurofins Genomics (Nantes, France) for bi-directional Sanger sequencing, and results were manually edited in MEGA 11 software (Molecular Evolutionary Genetics Analysis, version 11.0.13 (Tamura et al., 2021)). The ClustalW program was used to align nested PCR product sequences and compare them with GenBank sequences. The amplified segments of the *groEL* and *ankA* genes were compared with the corresponding *groEL* and *ankA* gene sequences deposited in the NCBI database using Nucleotide Blast (Altschul et al., 1990).

2.6. MLST analysis

Sequences obtained for MLST analyses were aligned to *A. phagocytophilum* sequences published in Chastagner et al. (2014) and Lagrée et al. (2018) using MEGA 11. Analyses were performed in BioNumerics 7.6.3 (BioMérieux), using the different genes as separate datasets (i.e., individually) or as a composite dataset (i.e., a combination of genes). The dendrograms were generated after multiple alignments of sequences related to individual genes or composite datasets for the similarity coefficient and using a complete linkage for cluster analysis via BioNumerics. The partial gene sequences obtained in this study were deposited in GenBank (accession numbers PP179222 – PP179232; PP196407 – PP196416; PP210089 – PP210100).

3. Results

3.1. Descriptive epidemiological characteristics of birds

To document the presence of *A. phagocytophilum* in passerines from urban and peri-urban areas of Ile-de-France and neighboring départements, 680 passerine cadavers were collected from EnvA's wildlife veterinary hospital. Most of the passerines collected (87.1 % i.e., 592 birds), were from the Ile-de-France region, 1.2 % (eight birds) from a neighboring département and 0.4 % (three birds) from another département/region. The geographic origin was unknown for 11.3 % (77 birds). Of the birds from Ile-de-France, 26 % (154 birds) were from the Val-de-Marne département, where EnvA is located (Fig. 1). Almost all the passerines (99 %) were collected from March to October, a period characterized by a strong influx of juveniles, which represented 79.9 % of the collected birds (i.e., 543). Eleven species of passerines were collected: the common blackbird (*Turdus merula*), house sparrow (*Passer domesticus*), Eurasian blue tit (*Cyanistes caeruleus*), long-tailed tit (*Aegithalos caudatus*), great tit (*Parus major*), common starling (*Sturnus vulgaris*), European robin (*Erithacus rubecula*), and four corvid species, i.e., the carrion crow, common magpie, rook, and Eurasian jay (*Garrulus glandarius*). The majority of these species are sedentary and all are ground-foraging except for the tits, which are arboreal insectivores. The three most abundant species were the carrion crow ($n = 192$), common magpie ($n = 143$), and common blackbird ($n = 91$). Details are given in Table 1 and Supplementary Table S3 and S4.

3.2. Detection of *A. phagocytophilum* in birds

An amplicon was detected for each 12S rRNA gene PCR performed, indicating the presence of amplifiable DNA (data not shown).

In this study, *A. phagocytophilum* DNA was detected in 0.4 % of the birds and 3.3 % of the common blackbirds. Of the 680 birds tested, Taqman qPCR targeting the *msh2* gene was positive for two juvenile blackbirds (#739 and #759) in all organs/tissues tested (spleen, liver, and skin) with a C_T < 33, except for the liver of bird #739 (C_T = 35.55). In addition, an adult female blackbird (#742) was weakly positive by *msh2* qPCR, with a C_T > 34 but < 39 for all three organs tested. In this same blackbird, 16S rRNA PCR amplicons were obtained from the skin and spleen only (data not shown).

Both birds #742 and #759 came from a suburban area in the Essonne département. They had been admitted to the wildlife veterinary hospital in late November with an open fracture and in early August with shock, respectively. No information was available for the third positive bird (#739).

3.2.1. *groEL* and *ankA* typing

groEL sequencing only worked for birds #739 and #759. Both *groEL* amplicons (537 bp) showed 100 % identity with previously published *groEL* sequences of *A. phagocytophilum*, all from birds or ticks collected from birds. This is the case for three sequences published in GenBank, one from a Czech blackbird skin sample (GenBank accession number

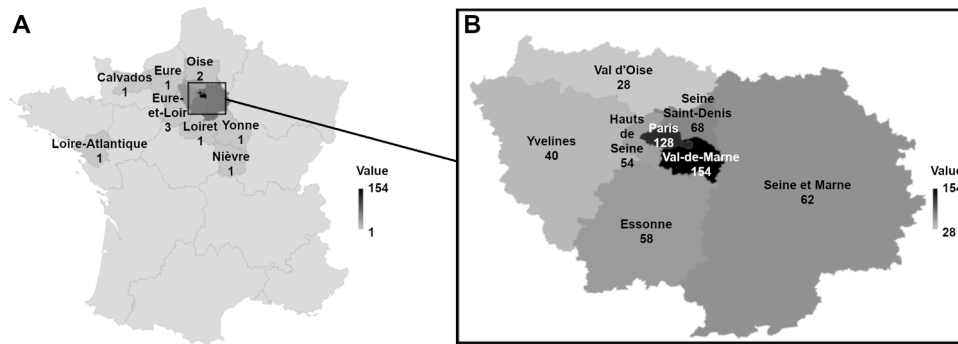


Fig. 1. Geographic distribution of passerines according to collection areas (A: Map of France with location and number of samples outside the département bordering Ile-de-France (IdF); B: Map of Ile-de-France with number and location of samples).

Table 1

Species of passerines from urban and peri-urban areas of Ile-de-France and neighboring départements collected from March to December 2021 and those found to be positive for *A. phagocytophilum* (Aph: *Anaplasma phagocytophilum*).

Species	Common name	Lifestyle & feeding behavior	Tested (n)	Aph positive (n)
<i>Corvus corone</i>	Carrion crow	Sedentary ground-foraging	192	0
<i>Pica pica</i>	Common magpie	Sedentary ground-foraging	143	0
<i>Turdus merula</i>	Common blackbird	Partially migratory Ground-foraging	91	3
<i>Passer domesticus</i>	House sparrow	Sedentary ground-foraging	83	0
<i>Parus major</i>	Great tit	Sedentary arboreal	68	0
<i>Cyanistes caeruleus</i>	Eurasian blue tit	Sedentary arboreal	25	0
<i>Aegithalos caudatus</i>	Long-tailed tit	Sedentary arboreal	1	0
<i>Sturnus vulgaris</i>	Common starling	Partially migratory ground-foraging	37	0
<i>Garrulus glandarius</i>	Eurasian jay	Partially migratory arboreal & ground-foraging	20	0
<i>Erithacus rubecula</i>	European robin	Partially migratory Ground-foraging	17	0
<i>Corvus frugilegus</i>	Rook	Partially migratory ground-foraging	3	0
Total			680	3

MW013534), another from an *I. ricinus* larva feeding on a blackbird collected in Switzerland (GenBank accession number JX082323), and a third from an *I. ricinus* nymph feeding on a bird (species unknown) collected in Italy (GenBank accession number KF031393). These *groEL* sequences were also 100 % identical to the *A. phagocytophilum* sequences published by Jahfari et al. (2014) that came from a blackbird, from *I. ricinus* and *Ixodes frontalis* ticks feeding on blackbirds and from an *I. frontalis* feeding on a great/blue tit.

Similarly, the *ankA* sequences (686 bp) of the two positive blackbirds #739 and #759 were 100 % identical to *A. phagocytophilum* sequences (GenBank accession numbers MH973606, MH973607) from *I. ricinus* ticks collected from blackbirds (Langenwalder et al., 2020). For bird #742, the *ankA* sequence was 99.9 % identical to that of birds #739 and #759 (A instead of G at position 654).

3.3. Dendrograms and MLST analysis

Dendrograms were constructed by the concatenation of three loci (*msp4*, *recG*, *typA*) and the intergenic region *ctrA* (Fig. 2). There were no ambiguous sites except in the sequence of the *typA* gene at position 237. A dendrogram was constructed using the three possibilities (A, G or R) with similar results (Supplementary Fig. S1). Only the sequences of samples #739 and #759 were analyzed as sample #742's gene amplification, needed for MLST, failed. Eighty-six sequences from the studies of Chastagner et al. (2014) and Lagrée et al. (2018) were included with our samples. An analysis of the dendrogram shows that the two samples we could type are grouped together and distinct from both the cluster of European cattle (*Bos taurus*) strains and the cluster of American strains. In addition, to include as many sequences as possible and thus increase the robustness of our results, a dendrogram based on the concatenation of five loci (*msp4*, *recG*, *gyrA*, *groEL*, and *typA*) and the intergenic region *ctrA* was also constructed (unsuccessful amplification of *pleD* and *polA* genes) (Supplementary Fig. S2). Twenty-three samples were included

and the result was similar to that of the previous dendrogram. The results tend to place the bird variants in a distinct position in relation to the other *A. phagocytophilum* strains.

4. Discussion

Due to their great mobility, birds play an important role in the maintenance and spread of ticks and tick-borne pathogens. Data on *A. phagocytophilum* infection in birds are scarce, and this is the first eco-epidemiological study of *A. phagocytophilum* in birds (as opposed to ticks collected from birds) ever conducted in mainland France.

All the passerines in our study were obtained from EnvA's wildlife hospital, which provided easy, ethical access to birds in Ile-de-France. Therefore, our study sample does not claim to be representative of the avifauna of this region. The presence of *A. phagocytophilum* was detected in 0.4 % of all birds and 3.3 % of blackbirds in this study sample. These detection rates cannot be extrapolated to generate prevalence figures for Ile-de-France. Another study carried out in different areas of France between March and September 2019 and in 2020 (Rataud et al., 2022) documented higher prevalence figures (10.2 % in the total bird population and about 20 % in blackbirds). However, in that study, the proportion of *A. phagocytophilum*-infected birds was estimated from engorged tick larvae on the birds, considering that co-feeding was negligible. The rate of *A. phagocytophilum* detection in our sampled birds was very low, like that found in studies conducted in Hungary (0.8 %) (Hornok et al., 2014) and Romania (0.9 %) (Sándor et al., 2017). Much higher prevalences were found in studies conducted in the Czech Republic (33.8 %) (Lesiczka et al., 2021), Romania (30.3 %) (Borşan et al., 2021), and Spain (21.7 %) (de la Fuente et al., 2005). In contrast, studies in Corsica (France, Mediterranean region) (Defaye et al., 2023), Poland (Skoracki et al., 2006; Skotarczak et al., 2006), and Italy (Ebani et al., 2016) did not detect any *A. phagocytophilum*-positive birds. These differences in the level of infection between studies could be influenced by

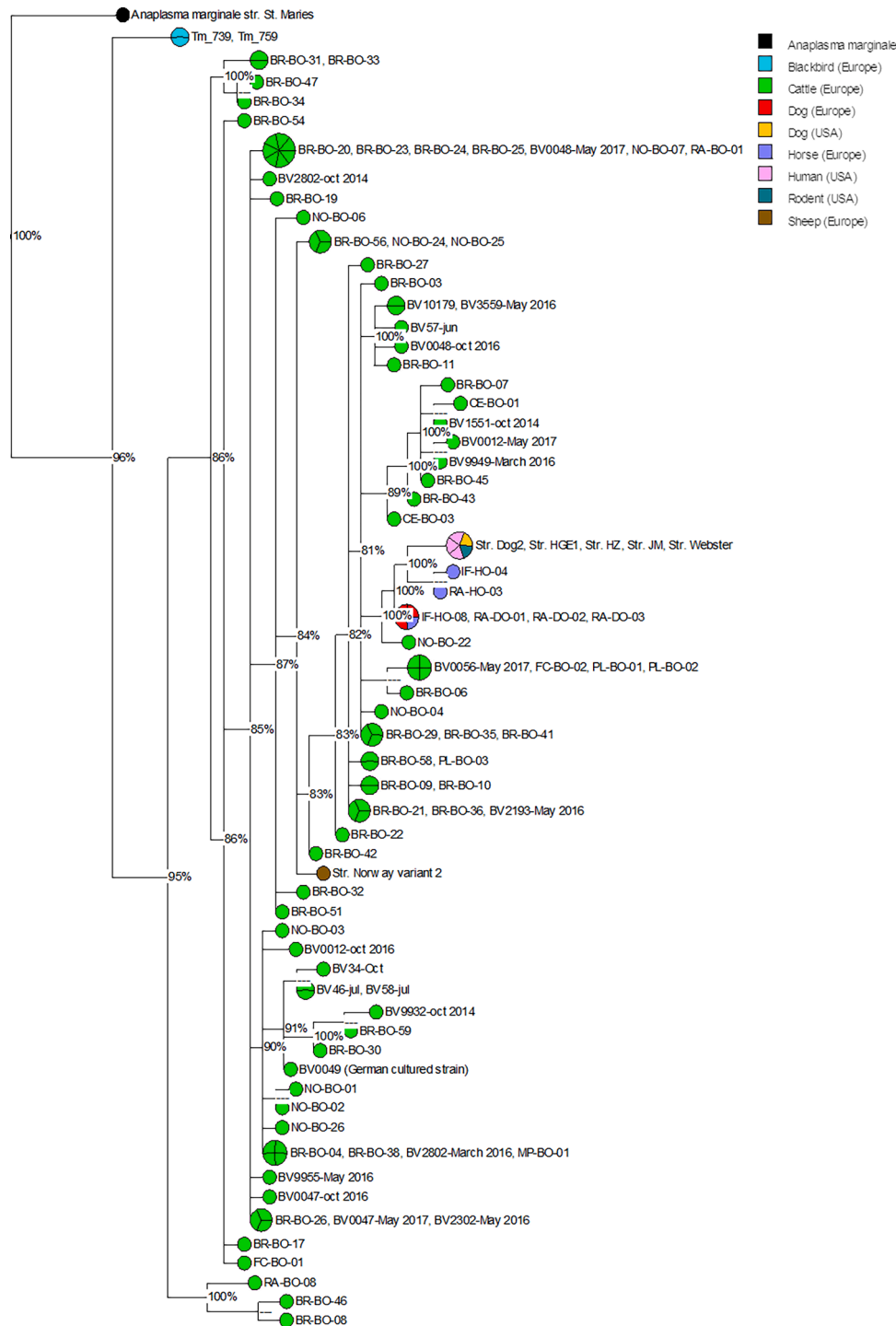


Fig. 2. Dendrogram obtained by concatenating three loci (*msp4*, *recG*, *typA*) and the intergenic region *ctrA* from 95 samples with nucleotide R (A/G) at position 237 of the *typA* gene for blackbird (*Turdus merula*) sequences. Bootstrap values are shown next to the branches. Each circle represents a sequence type. Circles are colored according to host species and geographic origin. Blackbird samples are in light blue. Bird clusters are very distinct from the other strains.

several factors, such as the bird species, sampling period (seasonal or annual variations), geographic area, method used to detect *A. phagocytophilum*, number of birds in the studies, and sampling methods used, which are rarely if ever random (our study included).

Our results are consistent with published data showing that blackbirds and ticks feeding on blackbirds are more often positive for this bacterium than other bird species (de la Fuente et al., 2005; Heylen et al., 2017; Hildebrandt et al., 2010; Jahfari et al., 2014; Lesiczka et al., 2021; Lommano et al., 2014; Palomar et al., 2012; Pedersen et al., 2020;

Rataud et al., 2022). It may be explained by their ground-foraging behavior, as this exposes them to ticks, which are abundant on the ground and in low vegetation (Heylen et al., 2017; Klaus et al., 2016). No other bird species was found positive for this pathogen in our study, despite the large number of birds for some species in our study sample and the fact that several of them are also ground-foraging species (such as the carrion crow and common magpie). Several bird species tested in our study have already been found positive for *A. phagocytophilum* in other studies, including rooks, house sparrows, common magpies,

European robins, Eurasian jays, great tits, and long-tailed tits (Borşan et al., 2021; de la Fuente et al., 2005; Sándor et al., 2017). Although several of the above factors may explain this result, another hypothesis would be that not all—or even very few—bird species are reservoir hosts for *A. phagocytophilum* (Heylen et al., 2017).

Anaplasma phagocytophilum infection was present in all three organs examined (spleen, liver, skin) with the exception of the liver of blackbird #742, indicating a systemic infection of the birds as previously shown in the literature (Jahfari et al., 2014; Skoracki et al., 2006). This is the second study to detect *A. phagocytophilum* in avian skin samples (Lesiczka et al., 2021). Our data confirm that it may be beneficial for future studies to use skin for detecting *A. phagocytophilum*, as it has the dual advantage of being easy to collect and less rapidly autolyzed than the spleen and liver.

The *ankA* and *groEL* amplified segments were 100 % identical to *A. phagocytophilum* sequences belonging to clusters associated with strains that were found in blackbirds and ticks feeding on birds, including blackbirds (cluster 6 for the *ankA* gene, ecotype IV, and cluster 7 for the *groEL* gene) (Jahfari et al., 2014; Rar et al., 2021). Similarly, a bird-specific cluster was detected in our study by MLST using the method of Chastagner et al. (2014). This result is also consistent with those of previous investigations (Langenwalder et al., 2020; Rar et al., 2021) that identified a bird-related MLST “cluster 7” using the MLST method of Huhn et al. (2014). It is particularly interesting to note that in our study, the two techniques tested on the same blackbird samples converged to identify profiles already individually associated with birds in other studies, which further strengthens the likelihood of a cycle independent of that of mammals, involving bird-specific variants (Jahfari et al., 2014; Rar et al., 2021).

The low level of infection by *A. phagocytophilum* in the birds in our study and the fact that European birds appear to be involved in a distinct epidemiological cycle suggest that they are not reservoirs of zoonotic or ruminant strains. However, according to a recent study (targeting *groEL*), blackbirds could be involved in the circulation of several strains of *A. phagocytophilum*, some of which have the same *groEL* signature as variants found in humans, suggesting that they could be zoonotic (Lesiczka et al., 2021). Further epidemiological studies and experimental transmission studies are thus needed. Therefore, it may be useful to use whole-genome sequencing to analyze in greater depth the strains present in birds and to consolidate the credibility of phylogenetic studies based until now on a very limited number of markers (Fröhlich et al., 2023). In addition, it would also be worthwhile to continue studies in other French urban areas, especially as studies have shown that the blackbird population density is increasing, and tends to be higher in urban areas than in rural areas due to more favorable conditions (Lesiczka et al., 2021; Möller et al., 2014). Finally, the impact of *A. phagocytophilum* on avian health is currently unknown and needs to be investigated.

CRedit authorship contribution statement

Clotilde Rouxel: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Adrien Etienne:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pascal Arné:** Writing – review & editing, Resources, Methodology, Investigation, Data curation, Conceptualization. **Cécile Le Barzic:** Writing – review & editing, Resources, Investigation, Data curation. **Guillaume Girault:** Writing – review & editing, Visualization, Software, Resources, Methodology, Formal analysis, Data curation. **Henri-Jean Boulouis:** Writing – review & editing, Resources. **Nadia Haddad:** Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Funding acquisition, Conceptualization. **Anne-Claire Lagrée:** Writing – review & editing, Writing – original

draft, Validation, Resources, Methodology, Funding acquisition, Conceptualization. **Pierre Lucien Deshuillers:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declarations of competing interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

Funding information

This work was supported by the Alfort National Veterinary School (EnvA), the French Agency for Food, Environmental and Occupational Health & Safety (ANSES), and the French National Research Institute for Agriculture, Food and the Environment (INRAE). The BIPAR joint research unit is supported by the French Government’s Investment for the Future program as a Laboratory of Excellence in “Integrative Biology of Emerging Infectious Diseases” (grant No. ANR-10-LABEX-62-IBEID).

Acknowledgments

The authors would like to thank all the veterinarians, staff and volunteers at the University Wildlife Veterinary Hospital (Chuv-FS) in Alfort who facilitated the collection of birds for this study. Our thanks also go to the Anatomopathology department of Biopôle in Alfort for providing access to EnvA’s dissection facilities. We are also grateful to the Alfort wildlife association for supporting the wildlife hospital.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.tbd.2024.102350](https://doi.org/10.1016/j.tbd.2024.102350).

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