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Investigation of avian louse flies as potential vectors of protozoan and bacterial pathogens of veterinary importance

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Avian louse flies (Hippoboscidae: Ornithomyinae) are blood-feeding parasites with largely unexplored vector potential, although they have been implicated in the transmission of pathogens of medical and veterinary relevance, such as West-Nile virus, *Babesia* and *Trypanosoma* species. We screened 253 specimens of nine Ornithomyinae species for selected bacteria (*Anaplasma*, *Rickettsia*, *Bartonella*, *Borrelia*, and *Ehrlichia* spp.) as well as for trypanosomes and piroplasms. *Anaplasma phagocytophilum* was detected in a single *Ornithomya avicularia* specimen, and *Haematospirillum jordaniae* was identified in three *Ornithomya fringillina* individuals from the same location, representing the first report of these bacteria in avian louse flies. In addition, eight different *Trypanosoma* sequences were obtained, belonging to the *T. corvi/culicavium* and *T. bennetti* groups. Unexpectedly, a sequence resembling *T. theileri* was recovered from an *Ornithoica turdi* specimen. Our findings suggest that the role of louse flies in transmitting the investigated bacterial pathogens is likely minimal, but their involvement in the ecology of *Trypanosoma* species warrants further study. Consistent with previous work, we propose that louse flies could serve as valuable sentinels for monitoring pathogens in wild bird populations.

Louse flies (Diptera: Hippoboscidae) are hematophagous insects with various parasitic strategies. Some of them are wingless, stationary parasites, such as the sheep ked (*Melophagus ovinus*), while others are temporary parasites with decreased ability to fly, for example the ornithophilic *Crataerina hirundinis*. Many species, however, are fully capable of flying, and their robust, durable bodies make them effective bloodsuckers. While a number of louse flies generally feed on mammals, the great majority are ornithophilic[1].

Although several different pathogens have been detected in hippoboscid flies, their vectorial roles are still poorly understood. For instance, it seems very likely that some species, such as *Lipoptena cervi*, play a role in the transmission of *Bartonella schoenbuchensis*[1]. Other *Bartonella* species of unknown pathogenic potential are also commonly found in mammal-associated louse flies, such as *Hippobosca equina*, *Lipoptena cervi*, and *Melophagus ovinus*[2–4]. The vector role of *Hippobosca camelina* in the transmission of *Anaplasma camelii* has also been successfully demonstrated in recent years[5].

In contrast, the vectorial capacity of avian louse flies has been far less studied, probably due to the more challenging nature of sample collection and their limited medical and veterinary relevance for livestock. Current knowledge suggests that they may be involved in the life cycles of flagellated protozoan parasites such as trypanosomes, although their exact ability to transmit these pathogens remains unclear[6].

In the American continent, the RNA of West Nile virus (WNV) was detected in *Icosta americana*, suggesting a potential vector role[7], although this has never been conclusively proven. Recently, a study from Germany

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examined several European species and was unable to detect WNV in any of them; however, they did find a single Usutu virus sequence, as well as a single Sindbis virus sequence, originating from two *Ornithomya avicularia* specimens that had fed on an infected bird. The authors highlighted that louse flies should be considered as potential sentinels of viral pathogens with zoonotic potential[8].

A study from Slovakia investigated the potential vector role of *Ornithomya biloba* and *O. avicularia* in the transmission of bacterial and protozoal pathogens of veterinary importance. The authors reported the DNA of multiple *Babesia* species (including *Babesia canis* and *Babesia venatorum* and *Babesia microti*) in their samples, and suggested that these flies may participate in the transmission of these piroplasms[9]. Although we consider this scenario highly unlikely (among other things, due to the rarity of encounters between the affected species and mammalian hosts) the aim of our study was to screen nine European ornithophilic louse fly species for the presence of bacteria (*Anaplasma*, *Rickettsia*, *Bartonella*, *Borrelia*, and *Ehrlichia* spp.) as well as protozoa (trypanosomes and piroplasms).

Results

Altogether 253 different louse flies were examined during this study. These belonged to nine species. *O. avicularia* (n = 138), *O. biloba* (n = 29), *O. fringillina* (n = 28), *O. chloropus*, (n = 12), *O. turdi* (n = 33), *C. hirundinis* (n = 1), *C. pallida* (n = 8), *Icosta ardeae* (n = 3) and *Ornithophila metallica* (n = 1).

Coinfections with ticks were observed in the case of 11 of the examined flies, (five *O. avicularia* from four hosts, and six *O. turdi* from six hosts) (Table 1). Coinfection with ticks was significantly more common in the case of *O. turdi* (6 in coinfection/ 27 not in coinfection) than in the case of *O. avicularia* (5/133) (P = 0.0076, odds ratio: 0.17). We did not observe tick-coinfection in the cases of the other louse fly species (note, that these flies are very mobile and hard to collect, which may affect the accuracy of such comparisons).

The DNA of *Bartonella, Rickettsia* and *Borrelia* species, as well as piroplasms were not detected in the examined flies.

A 450-bp-long *groEL* sequence, closest related to that of *Anaplasma phagocytophilum*, was successfully amplified from a single *O. avicularia* specimen (PX378130). The carrier louse fly was collected from a Common Blackbird (*Turdus merula*) in Ócsa, on 28.05.2022. This sequence showed 100% identity (450/450 bp) to several *A. phagocytophilum* sequences from Europe (Fig. 1). Two of these identical sequences were retrieved from Blackbirds: from a skin sample (MW013534) and from a liver sample (PP179228). One sequence was retrieved from an *Ixodes ricinus* larva that was feeding on a Blackbird (JX082323). One similar sequence was retrieved from an *I. ricinus* nymph feeding on an unspecified bird in Italy (KF031393). Finally, a similar sequence was found in a tick, that the author identified as a hybrid of *I. ricinus* and *I. inopinatus* (PP176527). According to our phylogenetic analyses, the latter sequences form a distinct clade separate from those retrieved from mammals.

Based on the 16S rRNA gene, sequences, closest related to that of *Haematospirillum jordaniae* were successfully amplified from three *O. fringillina* specimens (PX376951—PX376953). All of these flies were collected at Gárdony-Dinnyés. One from a Common Reed Warbler on 22.07.2023, and two from Bearded Reedlings (*Panurus biarmicus*) on 09.26.2023. These showed 100% (299/299 bp) identity to MF374623, isolated from Common Reed Warbler (*Acrocephalus scirpaceus*) in Hungary, and to several human (*Homo sapiens*) samples, e.g. OM075117 isolated from human blood in Slovenia, OP315756 isolated from human blood in Argentina, or CP014525 isolated in the USA from a human patient.

Based on their *ssu* genes, five different genotypes of avian *Trypanosoma* species were revealed (Fig. 2) (PX376905- PX376923). According to our phylogenetic analyses, one of these sequences, that was retrieved from an *O. avicularia* specimen) belongs to the *Trypanosoma bennetti* group (group A) of avian trypanosomes, while the other four genotypes belong to the *Trypanosoma corvi/culicavium* group (group B), specifically to the lineages I, IV, V, and to the recently discovered lineaege B14. The louse flies from which we were able to retrieve

Host bird	Louse fly species(number)	Tick species/stage(number)
TUR PHI	*O. avicularia* (2)	*I. ricinus*/nymph (2) *H. concinna*/nymph (2)
SIT EUR	*O. avicularia* (1)	*I. ricinus*/nymph (1)
LUS MEG	O. avicularia (1)	*I. ricinus*/nymph (1)
LOC LUS	*O. avicularia* (1)	*H. concinna*/larva (2) / nymph (3)
TUR MER	*O. turdi* (1)	*I. ricinus*/nymph (1) *H. concinna*/nymph (2)
TUR PHI	*O. turdi* (1)	*I. ricinus*/larva (1)
LOC LUS	*O. turdi* (1)	*H. concinna*/larva (15) / nymph (1)
LAN COL	*O. turdi* (1)	*I. ricinus*/nymph (1) *H. concinna*/larva (4)
LUS MEG	*O. turdi* (1)	*I. ricinus*/larva (1)
NA	*O. turdi* (1)	*I. ricinus*/larva (1)

Table 1. Tick and louse fly co-infections observed during this study. TUR PH I = *Turdus philomelos;* SIT EUR = *Sitta europaea*; LUS MEG = *Luscinia megarhynchos;* TUR MER = *Turdus merula;* LOC LUS = *Locustella luscinioides;* LAN COL = *Lanius collurio;* NA = *no data; O. avicularia* = *Ornithomya avicularia; O. turdi* = *Ornithoica turdi; I. ricinus* = *Ixodes ricinus; H. concinna* = *Haemaphysalis concinna.*

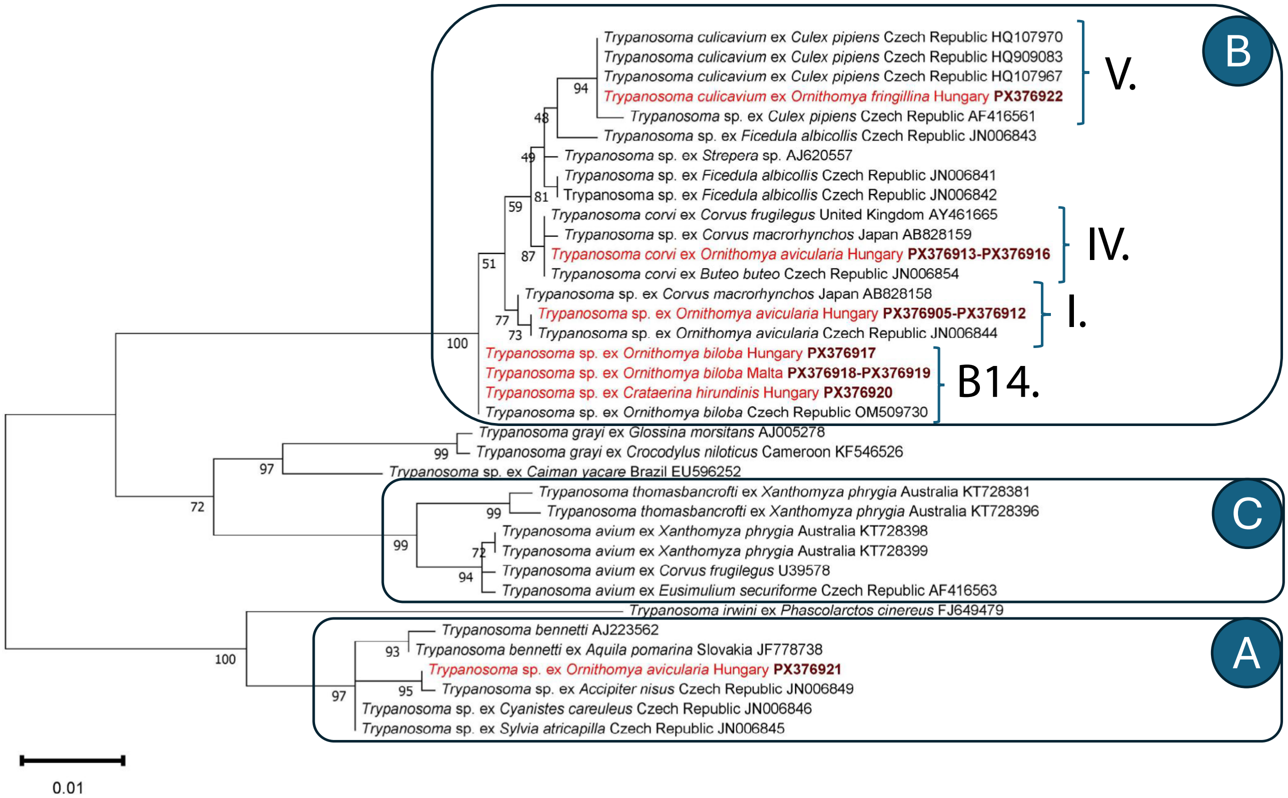

Fig. 1. Phylogenetic tree based on the ssu gene of *Trypanosoma* species. The evolutionary history was inferred using the maximum likelihood method and Tamura 3 (T3) model. (**A**) *Trypanosoma bennetti* group; (**B**) *Trypanosoma corvi/culicavium* group; (C) *Trypanosoma avium/thomasbancrofti* group. Numbers indicate lineages, according to the work of Santolíková et al.[6].

Trypanosoma sequences, their hosts and collection sites are enlisted in Table 2. No sequences belonging to the *Trypanosoma avium/thomasbancrofti* group (group C) were detected. A single sequence of *Trypanosoma* sp. was found in an *O. turdi* specimen; however, this sequence was considerably shorter (470 bp) than the rest of our sequences (~ 820 bp). This showed 100% similarity to several sequences from the *Trypanosoma theileri* group in the GenBank (e.g. OM256597, OM256606, OM256688). This *O. turdi* specimen was collected from an Eurasian Jay (*Garrulus glandarius*) on 17.08.2022 in Csorna, Hungary.

Discussion

In this study, 253 specimens of nine different louse fly species were analysed. Most of these flies were originated from several areas across Hungary. Six *O. biloba and a single O. metallica* were collected in Malta during a previous research (although these were not published)[10], while three *O. avicularia* specimens were originated from Norway. Some flies that were analysed in a different context in a previous study were also used here[11].

While pathogen transmission during co-feeding is a known phenomenon in the case of ticks[12], it is not known whether ticks can infect louse flies via the same route. Based on our results however, tick and louse fly co-infection is relatively rare, we only observed it in the case of two species, *O. avicularia* and *O. turdi* (Table 1), with the latter coinfecting birds with ticks more commonly. Different species of louse flies feeding on the same bird is also uncommon in Central Europe, where co-feeding of louse flies of different species (only *O. avicularia* and *O. turdi*) was observed only on two out of 175 infested birds[11].

Based on our results we suspect that the role of avian louse flies in the transmission of pathogenic *Bartonella, Rickettsia, Anaplasma*, *Borrelia* species*,* as well as piroplasms is either non-existent or minimal in the evaluated region. This is partially in contrast to previous results[9], where the authors examined eight *O. biloba* and 12 *O. avicularia* specimens, and found three different *Babesia* species in the latter. Based on the fact, that this study was performed in a neighbouring country of Hungary (Slovakia), and the fact that we were unable to find any piroplasms despite analysing eleven times more *O. avicularia*, and the fact that the latter species seldom feeds on mammalian host[13] suggest that these previous findings were accidental. We find it unlikely, that members of the subfamily *Ornithomyinae* would play a role in the transmission of piroplasms.

We were able to retrieve a sequence, closest related to that of *A. phagocytophilum* from an *O. avicularia* specimen collected from a Blackbird. Interestingly, this sequence formed a distinct clade in the *A. phagocytophilum* group, with low support, confirming the results of Baráková et al.[14], Lesiczka et al.[15] and Rouxel et al.[16]. The sequences belonging to this clade, are related to Blackbirds (liver and skin samples: PP179228 and MW013534)[15,16], or ticks feeding on Blackbirds (JX082323) and on a non-defined avian host (KF031393)[14]. A single sequence

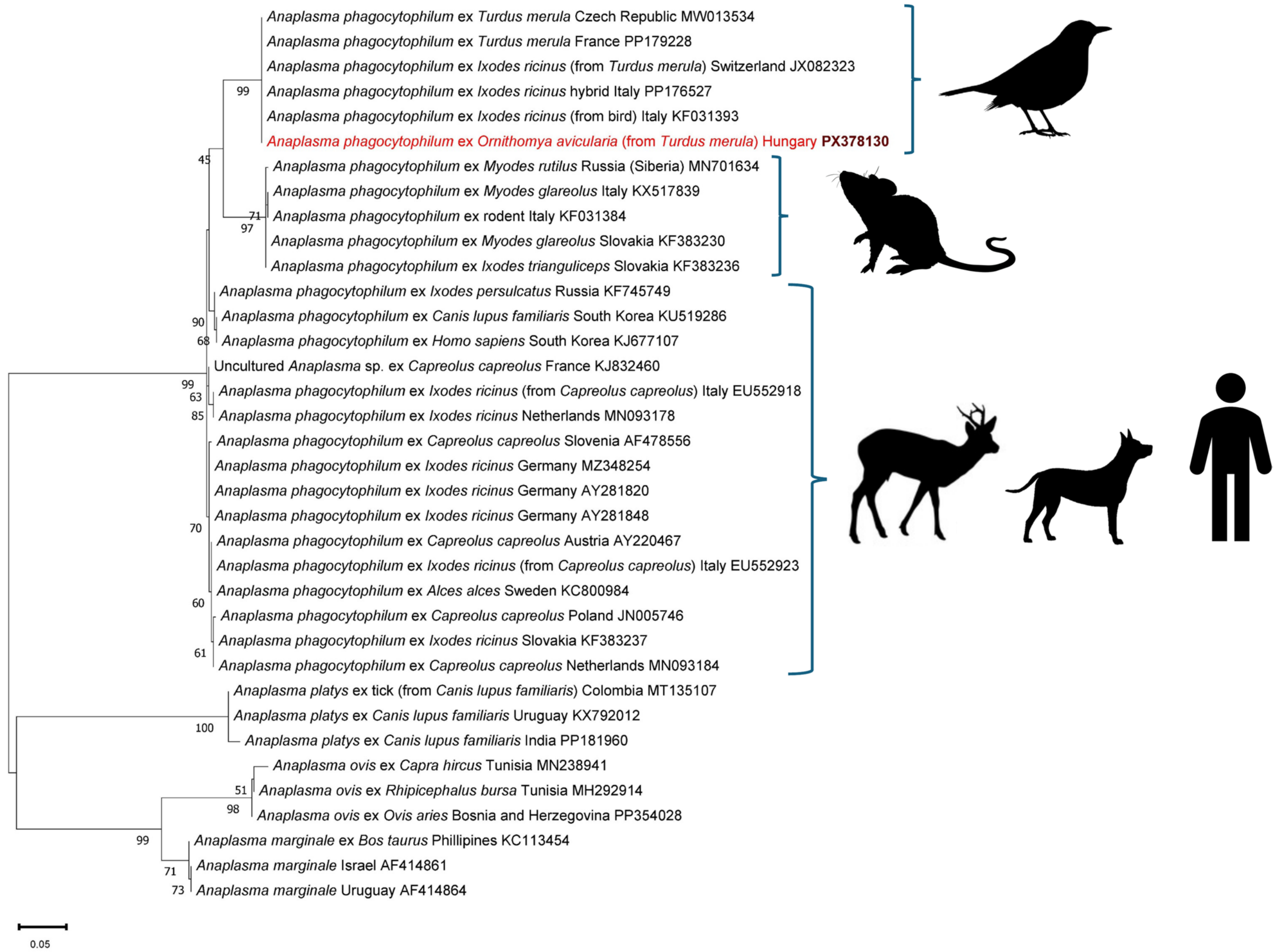

Fig. 2. Phylogenetic tree based on the GroEl gene of *Anaplasma* species. The evolutionary history was inferred using the maximum likelihood method and Tamura-Nei model. The pictograms indicate some species the given clade has been described from so far.

was retrieved from a tick collected from the environment. Since we found this sequence in only a single *O. avicularia,* it cannot be proven that louse flies serve as biological vectors of this pathogen. At the same time, based on our results, it is strongly suspected that a clade of *A. phagocytophilum* exists that is related to Blackbirds (and potentially other birds as well). A recent study shed light upon the fact that Blackbirds yielded the highest number of *A. phagocytophilum* infested ticks in the examined population in Sweden[17]. However, in the same study, only 0.9% of the ticks collected from migratory birds were infected. In a study conducted in France, 680 birds of 11 eleven species were analysed for the presence of *A. phagocytophilum*, and only Blackbirds were found to be infested (three out of 91 birds)[16]. This sequence is identical to the *A. phagocytophilum* sequence of this study. In South-Korea 7/40 (17.5%) birds belonging to the Turdidae family were found to be infested with *A. phagocytophilum*[18]. It has to be stated however, that in Europe, Blackbirds are very frequent hosts of *I. ricinus*, the main vectors of *A. phagocytophilum* in Europe[19,20]. In fact, in Hungary, Blackbirds are the most common hosts of *I. ricinus*[21].

Trypanosoma species, on the other hand, were common in our sample population, similarly to as reported by Santolíková et al., 2022. We were able to detect six different genotypes based on the *SSU* gene (Table 2). Five of these belonged to the avian trypanosomes, namely to the *T. bennetti* group, and to the *T. corvi*/*culicavium* group. No sequences from the *T. avium*/*thomasbankrofti* group were revealed (Fig. 2). However, a single, *T. theileri* sequence was found in an *O. turdi* specimen, which showed 100% identity to those of several members of the *T. theileri* group (TthII) (PX376923), following the results of Brotánková et al. 2022[22]. Members of this lineage are reported to infect bovines and deer, while the vectors can be several Dipteran species, likely mosquitoes[22]. Since this sequence was considerably shorter than the rest of our *Trypanosoma* sequences (470 vs 820 bp) we did not included it in our phylogenetic analysis. The recently discovered *Trypanosoma* lineage B14[6] was revealed from several *O. biloba* specimens from Hungary and Malta, revealing a broad geographical distribution in Europe (Table 2). This result further strengthens the theory that this lineage of the genus *Trypanosoma* may affect mainly the Barn Swallow (*Hirundo rustica*) in Europe, as we detected this protozoon from another stenoxenous parasite of the latter bird species for the first time, namely *C. hirundinis*. It must be mentioned that this lineage has been found in several predator birds in Thailand prior to our study[6]. Similarly to Santolíková et al.[6], the most common

Trypanosoma species/group/lineage	GenBank number	Louse fly species	Avian host	Collection site	Collection date
Trypanosoma sp., group A	**PX376921**	*O. avicularia*	TUR MER	Ócsa, Hungary	28.05.2022
Trypanosoma sp., group B, lineage I	**PX376905**	*O. avicularia*	PIC VIR	Ócsa, Hungary	08.06.2017
Trypanosoma sp., group B, lineage I	**PX376906**	*O. avicularia*	NA	Ócsa, Hungary	04.07.2017
Trypanosoma sp., group B, lineage I	**PX376907**	*O. avicularia*	TUR PHI	Ócsa, Hungary	01.07.2018
Trypanosoma sp., group B, lineage I	**PX376908**	*O. avicularia*	STR ALU	Ócsa, Hungary	01.07.2018
Trypanosoma sp., group B, lineage I	**PX376909**	*O. avicularia*	COR NIX	Barbacs, Hungary	11.07.2022
Trypanosoma sp., group B, lineage I	**PX376910**	*O. avicularia*	PAR MAJ	Veszprém, Hungary	28.06.2022
Trypanosoma sp., group B, lineage I	**PX376911**	*O. avicularia*	ASI OTU	Fehér tó, Hungary	25.07.2023
Trypanosoma sp., group B, lineage I	**PX376912**	*O. avicularia*	ACC GEN	Székesfehérvár, Hungary	13.07.2022
Trypanosoma corvi, group B, lineage IV	**PX376913**	*O. avicularia*	TUR PHI	Ócsa, Hungary	06.07.2018
Trypanosoma corvi, group B, lineage IV	**PX376914**	*O. avicularia*	LOC LUS	Ócsa, Hungary	10.07.2022
Trypanosoma corvi, group B, lineage IV	**PX376915**	*O. avicularia*	NA	NA	NA
Trypanosoma corvi, group B, lineage IV	**PX376916**	*O. avicularia*	ACC GEN	Székesfehérvár, Hungary	13.07.2022
Trypanosoma culicavium, group B, lineage V	**PX376922**	*O. fringillina*	ACR SCI	Dinnyés, Hungary	22.07.2023
Trypanosoma sp., group B, lineage B14	**PX376917**	*O. biloba*	HIR RUS	Ócsa, Hungary	26.09.2017
Trypanosoma sp., group B, lineage B14	**PX376918**	*O. biloba*	HIR RUS	Ghadira, Malta	02.10.2019
Trypanosoma sp., group B, lineage B14	**PX376919**	*O. biloba*	HIR RUS	Buskett, Malta	25.09.2020
Trypanosoma sp., group B, lineage B14	**PX376920**	*C. hirundinis*	HIR RUS	Dinnyés, Hungary	09.09.2023
Trypanosoma sp. Trypanosoma theileri group, lineage TthII	**PX376923**	*O. turdi*	GAR GLA	Csorna, Hungary	17.08.2022

Table 2. Trypanosomes found in louse flies. O = *Ornithomya/Ornithoica*; C = *Crataerina*; NA = Unknown; TUR MER = *Turdus merula*; PIC VIR = *Picus viridis*; TUR PHI = *Turdus philomelos*; ATR ALU = *Strix aluco*; COR NIX = *Corvus cornix*; PAR MAJ = *Parus major*; ASI OTU = *Asio otus*; ACC GEN = *Accipiter gentilis*; LOC LUS = *Locustella luscinioides*; ACR SCI = *Acrocephalus scirpaceus*; HIR RUS = *Hirundo rustica;* GAR GLA = *Garrulus glandarius.*

Trypanosoma genotypes isolated from avian louse flies belonged to the *T. corvi*/*culicavium* group, lineages I. and IV. In contrast to the latter study however, no sequences from the recently discovered B13 lineage were found among our samples. On the other hand, a single sequence of *T. culicavium* (lineage V) was found in an *O. fringillina* specimen for the first time. The role of avian louse flies in the transmission of several *Trypanosoma* species is strongly suspected. In the superfamily Hippoboscoidea ("Pupipara"), this would be not unprecedented, as tsetse flies (Glossinidae) are biological vectors of *Trypanosoma* species[1].

Haematospirillum jordaniae was detected in three *O. fringillina* specimens. All these flies were collected at the same sample collection site, at Gárdony-Dinnyés in 2023. Two flies were feeding on Bearded Reedlings, and one on a Common Reed Warbler. Interestingly, Hornok et al. found this bacteria in the blood of the latter bird species in central Hungary, in 2017[23]. While *H. jordaniae* was discovered in 2016[24], it is now considered an emerging pathogen of medical importance[25]. While not much is known about this bacterium, it seems to affect mainly middle aged or senior men, and is related to fresh water[25,26]. For example, in a recent case report, a Slovenian man was supposedly infected in Hungary, while he had cut himself with a reed during fishing activity at a lake[26]. Therefore, it is not surprising that all *H. jordaniae* sequences were detected in louse flies that were feeding on reed-associated birds. Little is known about the pathogenic effect of this bacterium on birds, and further studies are needed to assess the reservoir (or potentially vector) role of *O. fringillina* in relation to this bacterium.

In conclusion, this study presents one of the most extensive pathogen-focused investigations on avian louse flies conducted in Europe to date. A total of 253 individual flies representing nine species were examined for the presence of *Bartonella*, *Borrelia burgdorferi* sensu lato, members of the families Rickettsiaceae and Anaplasmataceae, as well as Piroplasmida and *Trypanosoma* species. Based on our results, we suggest that avian louse flies (Hippoboscidae: Ornithomyinae) play a limited role in the transmission of the examined bacterial pathogens and piroplasms. However, the findings of this study further emphasize the potential role of avian louse flies in the transmission of *Trypanosoma* species. To the best of our knowledge, this is the first report of the blackbird-associated *A. phagocytophilum* strain in an avian louse fly, specifically in *O. avicularia*. In addition, this study reports, for the first time, the presence of the newly described *Trypanosoma* lineage B14 in both Hungary and Malta, as well as in the louse fly *C. hirundinis*. Furthermore, we document for the first time the presence of *T. culicavium* (in *O. fringillina)*, a *Trypanosoma* species from the "bennetti group" (in *O. avicularia)*, and *H. jordaniae* (in three specimens of *O. fringillina)* in hippoboscid flies. A *Trypanosoma* species from the "theileri group" was also identified for the first time in an avian louse fly, *O. turdi*. These findings contribute important new data to our understanding of the pathogen-host associations and vector potential of avian louse flies.

Materials and methods
Sample collection
The collection of louse flies was conducted as described in our previous study, since the majority of those samples were also processed in the present work[11]. In brief, birds were caught and handled for the purpose of ringing, and were handled, identified, and released by professional ringers at multiple locations of Hungary: (1) Tömörd Bird

Ringing Station (coordinates: 47°21′N, 16°39′E), (2) Ócsa Bird Ringing Station (47°19′N, 19°13′E), (3) Bódva Valley Bird Ringing Station (coordinates: 48°27′N, 20°42′E), (4) Fenékpuszta Bird Ringing Station (46°44′N, 17°14′E), (5) Izsák, Lake Kolon Bird Ringing Station (coordinates: 46°46′N, 19°19′E), (6) Dávod, Lake Földvár Bird Ringing Station (coordinates: 46°0′N, 18°51′E), (7) Lake Fehér Ornithology Camp (coordinates: 46°20′N, 20°6′E). Birds were mist -netted by standard ornithological mist-nets (mesh size 16 mm) and were examined for the presence of louse flies, between March and November. Only sporadic data were obtained from the winter months (December, January, February). Sample collection was continuous in Ócsa between 2015 and 2022, while at the other locations it took place only in 2022. Some flies were collected from Hooded Crows (*Corvus cornix*) by licensed hunters as well[11].

In addition to these already published samples, the sample collection continued at the previously described sites and other, new locations in Hungary as well, between March 2023 and November 2024. These new sites were: Gárdony-Dinnyés (47°10′N, 18°33′E), Sumony (45°58′N, 17°53′E) and Patak (48°1′N, 19°8′E). As the aim of this study was to find pathogens in different bird louse species, other specimens from the collection of the Department of Parasitology and Zoology, Veterinary Medicine, Budapest were also analysed. This included louse flies collected occasionally at several other locations in Hungary during bird ringing, or veterinary-related procedures. Some samples from Norway (three specimens of *Ornithomya chloropus*) and from Malta (one *Ornithophila metallica* and six *O. biloba*) were also evaluated. The latter Maltese samples were collected in parallel to our previous study[10].

Ectoparasites were removed with the help of pointed tweezers and were placed and stored in 96% ethanol.

Morphological and molecular analyses

Louse flies were identified to the species level based on morphological keys[13,27].

In this study, louse flies were only analyzed for the presence of pathogens, if phoresy was not detected on them, to avoid potential bias. In the cases of three specimens of *O. biloba* from Malta, the mites that had been present on their wings were accurately removed. In all cases, louse flies were treated individually.

Louse flies were disinfected on their surface with sequential washing for 15 s in detergent, tap water and distilled water. DNA was extracted with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer′s instruction, including an overnight digestion in tissue lysis buffer and Proteinase-K at 56 °C. Extraction controls (tissue lysis buffer) were also processed with the tick/hippoboscid samples to monitor cross-contamination.

The target genes and primers used in this study for the PCR probes are listed in Table 3.

A non-template reaction mixture served as the negative control in all PCR analyses. Extraction controls and negative controls remained PCR negative in all tests. The PCR products were purified and sequenced by Eurofins Biomi Ltd. (Gödöllő, Hungary). The BioEdit program was used for quality control and trimming of sequences. The analyses of assembled sequences were performed with BLASTN via GenBank (https://blast.ncbi.nlm.nih.gov). The sequences obtained in the current study were deposited in the GenBank database and are available under accession numbers: PX376905- PX376923; PX376951- PX376953; PX378130.

Phylogenetic and statistical analyses

Sequences from other studies, used here for phylogenetic analyses, were retrieved from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). The best fitting evolutionary models were chosen with the help of the program IQTREE2 (version 2.4.0)[37].

Analysis of *Trypanosoma* species: The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model[38]. The tree with the highest log likelihood (−2181.24) is shown. The percentage of trees in which the associated taxa clustered together is shown below the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura 3 parameter model, and then selecting the topology with superior log likelihood value. The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 42.22% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 36 nucleotide sequences. There were a total of 829 positions in the final dataset. Evolutionary analyses were conducted in MEGA11[39].

Analysis of *Anaplasma* species: The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model[40]. The tree with the highest log likelihood (−1638.77) is shown. The percentage of trees in which the associated taxa clustered together is shown below the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories (+*G*, parameter = 0.4607)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 36 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. There were a total of 445 positions in the final dataset. Evolutionary analyses were conducted in MEGA11[39].

Fisher's exact tests were used for the comparison of the numbers of *O. avicularia* and *O. turdi* individuals that were co-feeding with ticks (program used: R-program v. 4.3.1)[41].

Citation	Target group	Target gene (amplicon length)	Primers (5′-3′)	Cycling conditions					
				Initial denaturation	Denaturation	Annealing	Extension	Final extension	Number of cycles
[28]	**Piroplasms**	18S rRNS (500 bp)	BJ1 (GTC TTG TAA TTG GAA TGA TGG) BN2 (TAG TTT ATG GTT AGG ACT ACG)	95 °C, 10m	94 °C, 30s	54 °C , 30s	72 °C , 40s	72 °C , 5m	40
[29,30]	***Trypanosoma sp.***	ssu (800–1000 bp)	609F (CAC CCG CGG TAA TTC CAG C) 706Rnew (CTG AGA CTG TAA CCT CAA)	95 °C , 5m	94 °C , 40s	49 °C , 1.5m	72 °C , 1m	72 °C , 5m	40
[31]	**Rickettsiaceae**	gltA (380 bp)	RpCs.877p (GGG GGC CTG CTC ACG GCG G) RpCs.1258n (ATT GCA AAA AGT ACA GTG AAC A)	95 °C , 5m	94 °C , 20s	48 °C , 30s	72 °C , 1m	72 °C , 5m	40
[32,33]	***Bartonella sp.***	16S-23S ITS (600 bp)	Ba325s (CTT CAG ATG ATG ATC CCA AGC CTT CTG GCG) Ba1100as (GAA CCG ACG ACC CCC TGC TTG CAA AGC A)	95 °C , 5m	94 °C , 30s	65 °C , 30s	72 °C , 50s	72 °C , 5m	40
[34]	***Borrelia burgdorferi s.l***	5S-23S IGS (450 bp)	B5Sborseq (GAG TTC GCG GGA GAG TAG GTT ATT GCC) B23Sborseq (TCA GGG TAC TTA GAT GGT TCA CTT CC)	94 °C , 5m	94 °C , 20s	70 °C , 30s (−1°C/ cycle)	72 °C , 30s	72 °C , 7m	10
					94 °C , 20s	60 °C , 30s	72 °C , 30s		40
[35]	**Anaplasmataceae**	16S rRNS (350 bp)	EHR16SD (GGT ACC YAC AGA AGA AGT CC) EHR16SR (TAG CAC TCA TCG TTT ACA GC)	95 °C , 10m	95 °C , 30s	55 °C , 30s	72 °C , 45s	72 °C , 5m	40
This study	**Anaplasmataceae**	16S rRNS (700 bp + 650 bp)	Ana16SF (TTA GTG GCA GAC GGG TGA GTA ATG) Ana16SMR (CTA CCA GGG TAT CTA ATC CTG TTT GC); Ana16SM (GCA AAC AGG ATT AGA TAC CCT GGT AG) Ana16SRR (TGA CGG GCA GTG TGT ACA AGA CCC GAG)	95 °C , 5m; 95 °C , 5m	95 °C , 30s; 95 °C , 30s	57 °C , 30s; 58 °C , 30s	72 °C , 1m; 72 °C , 1m	72 °C , 5m; 72 °C , 5m	40 40
[36]	***Anaplasma phagocytophilum***	GroEL (640 bp)	EphplGroEL(569)F (ATG GTA TGC AGT TTG ATC GC) EphplGroEL(1193)R (TCT ACT CTG TCT TTG CGT TC)	95 °C , 5m	95 °C , 30s	52 °C , 40s	72 °C , 1m	72 °C , 7m	40

Table 3. Oligonucleotide sequences and cycle parameters of PCRs used in this study.

Data availability

The sequences obtained in the current study were deposited in the GenBank database and are available under accession numbers: PX376905- PX376923; PX376951- PX376953; PX378130.

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Reviewer's token

The sequence data have been uploaded to GenBank, where the "release upon publication" option was selected. To facilitate the proper evaluation of the manuscript, we have provided the submission information received from GenBank and uploaded all corresponding sequences in FASTA format as "Related files" accompanying the submission.

Author contributions

GK: Conceptualization, visualization, writing—original draft, writing—review and editing, investigation, formal analysis, data curation, methodology, validation. NT: Writing—review and editing, formal analysis, data curation, methodology, validation. TCS, DK, AP, AB, ÁN, AB, LF, ZL, EAT, AM, ZSK, GK, EdW, BC: Investigation, data curation, writing—review and editing, validation. SH: Conceptualization, visualization, investigation, project administration, resources, supervision, writing—review and editing, validation.

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Declarations

Competing interests
The authors declare no competing interests.

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