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RESEARCH

Occurrence and relevance of *Mycoplasma sturni* in free ranging barn swallows (*Hirundo rustica*) in Germany

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Abstract
In poultry industry, mycoplasmas cause large economic losses. The pathogenic significance of mycoplasmas varies widely between species. In birds where vocalization is crucial for reproductive success, mycoplasmas seem to be absent. Birds with high mycoplasma prevalence like birds of prey and storks do not rely on their vocalization for reproduction. Corvids with an intermediate prevalence use other strategies besides vocalization for mating. It is hypothesized that mycoplasma prevalence and vocalization used for reproduction is evolutionary related. Barn swallows have two traits relevant for mating: tail feather length and vocalization. An intermediate prevalence is expected. Little is known about mycoplasmas in barn swallows and their role as vector or reservoir for poultry pathogenic mycoplasmas. This study investigates the prevalence of mycoplasmas in barn swallows and their relevance for mycoplasma transmission to poultry. Choanal swabs from 188 healthy barn swallow nestlings of 59 different nests from ten different colonies on farms were examined for mycoplasmas by cultivation and genus-specific PCR including molecular biological differentiation of the species. In total, in 31 of 188 barn swallows (16.49%) and in 14 of the 59 nests (23.73%), mycoplasmas were detected. The occurrence of mycoplasmas per colony ranged from 0 to 50% independent of poultry being kept on the farm. In all positive samples, *Mycoplasma sturni* was identified. *Mycoplasma sturni* seems not to be an obligatory pathogen for barn swallows and occurs with an expected intermediate prevalence in them, so the results support the described hypothesis and underline a minor role of barn swallows for mycoplasma infection in poultry.

Keywords Commensals · Facultative pathogens · Free ranging birds · Wild birds · Mycoplasmas · Prevalence

Introduction

In poultry industry, mycoplasmas are important pathogens, which can cause large economic losses. The role of free ranging birds as reservoir for poultry pathogenic mycoplasma species and in the transmission is a subject of recurrent debate (Bradbury 2005; Michiels et al. 2016; Sawicka-Durkalec et al. 2021). Mycoplasmas can cause various clinical signs, but the most of them are associated with respiratory diseases. There are also mycoplasmas, which occur as commensals in birds. In free ranging healthy birds of prey, the prevalence of mycoplasmas was found to be between 91 and 94% (Lierz et al. 2008) and in storks about 99% (Möller Palau-Ribes et al. 2016). High prevalence of *Mycoplasma* spp. has also been detected in other avian species, including pigeons with a prevalence of 66.7% (Shimizu et al. 1978) and pink pelicans (*Pelecanus onocrotalus*) with 98% (Assunção et al. 2007). There are as well avian species like corvids, in which *Mycoplasma* spp. sometimes are found in healthy birds, but also can be associated with disease (Wellehan et al. 2001; Ziegler et al. 2017). An intermediate prevalence of mycoplasmas in healthy corvids was demonstrated with about 7% (Ziegler et al. 2017). In small Passeriformes, usually no mycoplasmas can be detected in healthy birds (Fischer et al. 2021; Sawicka-Durkalec et al. 2021). A prevalence of 0% was demonstrated in nightingales (*Luscinia megarhynchos*) and blue (*Cyanistes caeruleus*) and great tits (*Parus major*) (Fischer et al. 2021). There is a recently published hypothesis that in bird species using sexually selected song traits as mating signals, like Passeriformes, respiratory infections, like infections with mycoplasmas, could lead to an evolutionary pressure towards the exclusion of pathogens from their respiratory tract. Therefore, those species, like nightingales, blue tits, and great tits,

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would have a very low mycoplasma prevalence, confirmed in the study with 0% (Fischer et al. 2021). Species using other signals than vocalizations for mating, like birds of prey and storks, would not be under the same evolutionary pressure and would still be able to succeed in partner selection and therefore could afford a higher prevalence, which was demonstrated up to 100% (Lierz et al. 2008; Möller Palau-Ribes et al. 2016). Corvids are Passeriformes, but rely besides singing on various other signals for mating, so they would have an intermediate position, which was shown with a prevalence of 7% (Ziegler et al. 2017).

Sawicka-Durkalec et al. (2021) examined 1.141 wild birds from 55 different species for mycoplasmas by PCR and found an association between the prevalence of *Mycoplasma* spp. with feeding habits, movement patterns, and habitat types. The prevalence was found to be highest in species that eat animal-based diets (59.9%); in birds on mixed diets, the prevalence was 52.9%; and in birds feeding on plant material, it was 13%. The prevalence of mycoplasmas in birds living in aquatic environments (46.6%) was higher than in birds living in terrestrial environments (36.8%). Significant differences were also observed in mycoplasma prevalence between migratory (46.8%) and sedentary birds (24.3%).

However, prevalence studies are still needed to find an explanation for the differences in the prevalence of mycoplasmas in the different free ranging bird populations. For such studies, a mycoplasma culture with a concurrent mycoplasma-genus-specific polymerase chain reaction (PCR) should be used (Lierz et al. 2007; Sawicka-Durkalec et al. 2021). For the identification and taxonomic classification of mycoplasma species, the 16 S ribosomal RNA (rRNA) and the 16-23 S ribosomal RNA intergenic transcribed spacer region (ISR) are useful tools (Brown et al. 2007; Ramírez et al. 2008; Volokhov et al. 2012). An arbitrary similarity value above 97% is proposed for the 16 S rRNA sequence, and an arbitrary similarity value around 95–98% is proposed for the 16-23 S rRNA ISR sequence. Strains with sequence similarity above these values belong most likely to the same species (Volokhov et al. 2012).

Barn swallows belong to the Passeriformes. Male barn swallows have at least two costly traits that are relevant in sociosexual interactions: tail feather length and singing (Saino et al. 2003). The singing of barn swallows relays reliable information about immune system activation and reflects their health status (Dreiss et al. 2008; Galeotti et al. 1997; Saino et al. 1997). Barn swallows that breed earlier in the season and have a good immunocompetence also have higher reproductive success (Saino et al. 2012). Barn swallows live in different terrestrial habitats and climatic zones and are found almost everywhere in the world. There are eight subspecies of barn swallows. Many subspecies such as *Hirundo rustica rustica* are migratory birds, whereas some subspecies such as *Hirundo rustica savignii* and *Hirundo rustica transitiva* are resident. Barn swallows are specialist insect hunters and hunt almost exclusively in the air. Their diet consists mainly of beetles, hymenoptera, and flies (Turner 2010). Additionally, the European subspecies lives in close proximity to human and therefore poultry and might represent a mycoplasma reservoir or at least play a role in the transmission between flocks.

Mycoplasma spp. are found to be a normal part of the intestinal microbiota of free ranging juvenile and adult barn swallows (Ambrosini et al. 2019; Kenzaka et al. 2018; Kenzaka and Tani 2017; Kreisinger et al. 2017; Musitelli et al. 2018; Turjeman et al. 2020). In different studies, a high amount of *Mycoplasmataceae* were demonstrated in feces samples from barn swallows by molecular biological analysis without further differentiation of the mycoplasma species (Ambrosini et al. 2019; Kenzaka et al. 2018; Kenzaka and Tani 2017; Kreisinger et al. 2017; Musitelli et al. 2018; Turjeman et al. 2020). In migratory barn swallows, a higher amount of *Mycoplasma* spp. was found than in resident barn swallows (Turjeman et al. 2020).

According to the available literature, only two studies differentiated the mycoplasma species in barn swallows. *Mycoplasma sturni* and *Mycoplasma moatsii* were isolated from a choanal swab of a barn swallow with no signs of disease in Germany (Klostermann and Lierz 2023), and *M. sturni* was also isolated from conjunctival swabs of four barn swallows with conjunctivitis in North America (Ley et al. 2016). Furthermore, *M. sturni* was isolated from four cliff swallows fledglings (*Petrochelidon pyrrhonota*) in a rehabilitation facility with conjunctivitis and mildly respiratory symptoms. One cliff swallow died, and the others were euthanized due to antibiotic therapy failure. Histopathological examination also revealed *Cryptosporidium* spp. on the conjunctival, nasal, and sinus epithelia (Ley et al. 2012). It could not be determined to what extent the infection with *M. sturni* or the infection with *Cryptosporidium* spp. was responsible for the symptoms.

Interestingly, *M. sturni* was isolated from several other birds of different species with conjunctivitis without an exclusion of co-infections (Forsyth et al. 1996; Frasca et al. 1997; Ley et al. 1998, 2016; Rogers et al. 2019). These species were four European starlings (*Sturnus vulgaris*) (Forsyth et al. 1996; Frasca et al. 1997; Ley et al. 1998, 2016), eight Northern mockingbirds (*Mimus polyglottos*) two blue jays (*Cyanocitta cristata*) (Ley et al. 1998, 2016), three scrub jays (*Aphelocoma californica*) (Rogers et al. 2019), one house finch (*Haemorhous mexicanus*), twenty-one American crows (*Corvus brachyrhynchos*), one American robin (*Turdus migratorius*), one Carolina wren (*Thryothorus ludovicianus*), and three other cliff swallows (Ley et al. 2016).

However, a *M. sturni* isolate from a wild caught California house finch with conjunctivitis resulted after conjunctival inoculation in transient infection in one of nine other

wild caught house finches, but no signs of disease. So, the isolate was not able to reproduce the disease by experimental infection (Ley et al. 2010).

Furthermore, *M. sturni* was also detected in birds without signs of conjunctivitis, such as from three blackbirds (*Turdus merula*), five rooks (*Corvus frugilegus*), three carrion crows (*Corvus corone*), two magpies (*Pica pica*), and five starlings, which were immature and exhibited various signs of disease, but no conjunctivitis (Pennycott et al. 2005). *M. sturni* was also isolated in one case from an American crow nestling with conjunctivitis in a Wildlife Rehabilitation Center, but later, *M. sturni* was further detected in other nine juvenile American crows and in six American robins without signs of conjunctivitis. All these birds had contact to the bird with the mycoplasma infection. In addition, a European starling was found dead in the aviary with the robins and was positive for *M. sturni* (Wellehan et al. 2001).

In a prevalence study in Germany, *M. sturni* was detected in five of 68 (7%) corvids without clinical signs from a hunting bag and in nine of 29 (31%) corvids admitted to a veterinary clinic also with no signs of conjunctivitis. Tracheal swabs were examined by culturing *Mycoplasma* spp. and by mycoplasma-genus-specific PCR. The occurrence of *M. sturni* in the randomly selected corvids from hunting bags was significantly lower than in the corvids, which were brought to the veterinary clinic (Ziegler et al. 2017). Therefore, *M. sturni* does not appear to be an obligatory pathogen, but its clinical significance is still uncertain.

In the present study, the occurrence of mycoplasmas in the free ranging barn swallow population in central Germany was investigated. It was aimed to assess the role of mycoplasmas in this species and to evaluate its role as a potential reservoir for poultry pathogenic mycoplasmas. In addition, this should be a first step to test the above described hypothesis about a possible connection between the occurrence of mycoplasmas and the influence of vocalization on reproduction. As barn swallows rely on their song to reproduce and have additional traits like the tail feather length, according to this hypothesis, an intermediate mycoplasma prevalence would be expected.

Materials and methods

Sampling

A sample size was determined based on the tables of Cannon and Roe for sample size calculations to substantiate freedom of populations from infectious agents (Cannon 1982). The population was assumed to be infinite. With a desired minimum confidence level of 95% and a prevalence threshold of 5%, an infinite population resulted in a required sample size of 59 (Cannon 1982; Conraths et al. 2011). In this study, nestlings were sampled, because nests are relatively easy to access, minimizing stress to the animals. Each nest was considered a sample, regardless of how many birds were sampled from a nest. This resulted in a sample size of 59 nests. At least three breeding colonies should be sampled in order to completely exclude any local effects that might be expected to be minor, and at least one colony should not be located on a poultry farm.

Finally, ten colonies of barn swallows were chosen for this study. Four of these colonies were on farms with laying hens (Colony: B, D, G, H), two colonies on a farm with backyard poultry (Colony: C, F), one on a farm with geese and backyard poultry (Colony A), and three were on farms without any poultry (Colony: E, I, J). In two of the farms with laying hens recently, *Mycoplasma synoviae* was diagnosed in the poultry flock (Colony: B, G). Two choanal swabs were taken from each 188 barn swallow nestlings in 59 nests (2 to 4 nestlings per nest) from May to July 2022. All of the nestlings were examined, and only healthy animals sampled. They were completely feathered, but not yet shortly before fledging. All birds were placed back into their respective nests within 5 min. One of the swabs from each bird was taken for mycoplasma culture and was immersed immediately in 2 ml AL10 medium (Avian Mycoplasma Liquid Medium; Mycoplasma Experience Ltd, Bletchingley, UK). The other swab was stored at – 80 °C until further investigation by PCR. Sampling of the birds was govermantally approved by the regional council of Gießen with the permission Nr. G 73/2021.

Mycoplasma culture

For cultivation, the liquid media with the swabs was diluted two times. A total of 200 µl of the original suspension was pipetted in a tube with 2 ml AL10. From that first dilution, 200 µl was pipetted in another tube with 2 ml AL10. A total of 25 µL of the original suspension and each dilution were given onto AS1 agar (Avian Mycoplasma Agar & Supplement; Mycoplasma Experience Ltd). This procedure was repeated after 5 days (d) and 10 d of incubation. All media were incubated at 37 °C with 5% CO_2 in a humidified environment for up to 10 d. The liquid medium was examined daily for color change and agar plates for colony growth. If mycoplasma typical colonies were detected, up to three single colony subcultures were performed. The selection criteria were a typical egg fried appearance and that the colonies lay separately from other colonies. These procedures were repeated twice from the respective subculture to ensure pure sub-cultures. Each single colony subculture was stored at – 80 °C until further investigation.

PCR and sequencing

The frozen choanal swabs were used for molecular biological analysis. For DNA extraction, each choanal swab was thawed and then soaked and rubbed in 350 µL of PBS (phosphate-buffered saline). A total of 100 µL of this liquid was used for DNA extraction using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For DNA extraction from the third single-colony subcultures, the fluid medium (1 mL) was centrifuged at 4000 × G for 45 min. The remaining pellet was incubated with 180 µL of lysis buffer (ATL buffer, Qiagen) and 20 µL of proteinase K (Qiagen) for 2 h at 56 °C. A total of 100 µL of the liquid was used for DNA extraction using the DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions. The concentration of the DNA of each sample was measured via spectrophotometer (Nanodrop 2000, Thermo Scientific, Wilmington, Delaware, USA) and, if necessary, diluted to 10 ng/µL. The extracted DNA of the swabs and all single-colony subcultures were screened via mycoplasma-genus-specific PCR targeting the 16 S ribosomal RNA gene sequence as described by Kuppeveld et al. (1992) modified by Lierz et al. (2007). Also, an additional PCR, targeting the 16-23 S ribosomal RNA intergenic transcribed spacer region sequence as described by Ramírez et al. (2008), was performed from the DNA of one single-colony subculture from one nestling per nest. Furthermore, the PCR, targeting the 16-23 S rRNA ISR sequence, was performed from the DNA of swabs, in which the mycoplasma-genus-specific PCR detected *Mycoplasma* spp., but no isolate could be obtained by cultivation. A total of 5 µl of each amplified product was mixed with 3 µl loading dye (Thermo Fisher Scientific; Waltham, USA) and was separated by electrophoresis on a 1% agarose gel stained with GelRed™. GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific; Waltham, USA) was used as DNA marker. The amplicons were visualized with ultraviolet light.

The 16 S ribosomal RNA gene sequences from one single-colony subculture per nestling and the 16-23 S rRNA intergenic transcribed spacer region sequences were purified with the GeneJET PCR Purification KIT (Thermo Fisher Scientific). If the morphology of the single-colony subcultures isolated from the same nestling did not definitely match, 16 S ribosomal RNA gene sequences from additional single-colony subcultures were purified. If no single-colony subculture could be obtained, but the mycoplasma-genus-specific PCR from the swab demonstrated a *Mycoplasma* spp. typical PCR product, this PCR product was purified. All purified sequences were sequenced by LGC Genomics (Berlin, Germany). The sequences were viewed with BioEdit Version 7.2.5. (Tom Hall, Ibis Therapeutics, Carlsbad, CA, USA) and analyzed with nucleotide BLAST (Basic Local Alignment Search Tool) by NCBI. The16S rRNA gene sequences from one single-colony subculture per nestling, the sequences of the ten next similar *Mycoplasma sturni* strains according to BLAST search, and the five next similar *Mycoplasma columborale* strain sequences and *Mycoplasma citelli* strain sequences according to BLAST search were adjusted to the same length of 593 bp and included in alignments constructed by Clustal W algorithm (Thompson et al. 1994). Additionally, *Mycoplasma mycoides subsp. mycoides* strain PG1 (GenBank Accession No: NR_074703.2) was included as outgroup in the alignment.

A maximum likelihood tree based on the Hasegawa-Kishino-Yano model (Hasegawa et al. 1985) was generated using the bootstrap method with 1000 bootstrap replications in MEGA Version 7.0.26 (Kumar et al. 2016). Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3609). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Results

In total, *Mycoplasma* spp. were detected in 31 of 188 (16.49%) barn swallow nestlings by PCR or cultivation, respectively. Considering the samples in terms of nests in 14 of 59 (23.73%) nests *Mycoplasma* spp. were detected, counting a nest positive, if *Mycoplasma* spp. from at least one nestling were detected by PCR or cultivation.

Mycoplasma spp. were detected by mycoplasma-genus-specific PCR in 30 of 188 (15.96%) barn swallow nestlings. Isolates of mycoplasmas as single-colony subcultures in the third passage were obtained from 25 of 188 (13.30%) choanal swabs from barn swallow nestlings by cultivation. The isolates grew rapidly and had quite large colonies. The liquid medium showed a color change from red to yellow after 2 days of incubation due to a glucose metabolism of the isolates. In total, 35 swabs (18.62%) showed a heavy contamination by other bacteria or fungi. From six swabs, in which *Mycoplasma* spp. were detected by PCR, no isolates were obtained in the cultivation. Furthermore, from one swab, in which no *Mycoplasma* spp. were detected by PCR, *Mycoplasma* spp. were cultured.

Mycoplasmas were detected in all nestlings in eight positive nests. In five nests, only in one nestling mycoplasmas were detected, and in one nest in three from four nestlings mycoplasmas were found. Table 1 shows the occurrence of positive nests in relation to the different barn swallow colonies. The occurrence of mycoplasmas per colony varied

Table 1 The occurrence of mycoplasma positive nests in relation to the different colonies

Population	Poultry on farm	Sampled nest sites	Positive nest sites	Percentage
A	Backyard poultry and geese	5	1	20.00%
B	Laying hens positive for *M. synoviae*	9	0	0.00%
C	Backyard poultry	4	0	0.00%
D	Laying hens	6	1	16.67%
E	Without poultry	7	1	14.29%
F	Backyard poultry	4	2	50.00%
G	Laying hens positive for *M. synoviae*	16	8	50.00%
H	Laying hens	3	1	33.33%
I	Without poultry	1	0	0.00%
J	Without poultry	4	0	0.00%
Total		59	14	23.73%

between 0 and 50% independent of whether the population was on a farm with poultry or without poultry.

All amplicons of the partial 16 S rRNA gene from single-colony subcultures of the same nestling were identical.

The amplicon of the partial 16 S rRNA gene (length between 920 and 946 bp) from each single-colony subculture and from five swabs, from which no isolates were obtained, had a query coverage between 99 and 100% and a similarity between 98.20 and 99.05% with the 16 S ribosomal RNA sequence of *Mycoplasma sturni* (GenBank Accession No: NR_025968.1). The next similar mycoplasma species were *Mycoplasma columborale* (NR_025179.1) with a query coverage between 99 and 100% and a similarity between 95.63 and 96.48% and *Mycoplasma citelli* (NR_025178.1) with a query coverage between 99 and 100% and a similarity between 94.66 and 95.43%. The amplicon of the partial 16 S rRNA gene sequence from one swab had overlaying signals, and it was just a product with 570 bp sequenced. This sequence was therefore excluded from the phylogenetic tree due to the poor quality of the sequencing product. Nonetheless, it still had a query coverage of 99% and a similarity of 97.54% with the 16 S rRNA of *Mycoplasma sturni* (GenBank Accession No: NR_025968.1). The next similar mycoplasma species were *Mycoplasma columborale* (GenBank Accession No: NR_025179.1) with a query coverage of 100% and similarity of 93.55% and *Mycoplasma edwardii* (GenBank Accession No: NR_104953.1) with a query coverage of 99% and a similarity of 91.93%. The PCR targeting the 16 S rRNA sequence from one swab was negative for *Mycoplasma* spp., but three isolates could be obtained by cultivation. This sample was named Bs50-2. The PCR targeting the 16 S rRNA sequence from these isolates demonstrated a *Mycoplasma* spp. typical PCR product. The amplicon of the partial 16 S rRNA gene sequence from each single-colony subculture had a query coverage between 99 and 100% and a similarity between 99.03 and 99.04% with the 16 S ribosomal RNA of *Mycoplasma sturni* (GenBank Accession No: NR_025968.1).

The PCR targeting the 16 S rRNA sequence from one swab demonstrated a *Mycoplasma* spp. typical PCR product, but the sequencing product relieved a query coverage of 100% and a similarity of 89.37% with the 16 S rRNA sequence of *Ureaplasma gallorale* (GenBank Accession No: NR_026027.1). The next similar sequences were the 16 S rRNA sequences of *Ureaplasma felinum* (GenBank Accession No: NR_025879.1) and *Ureaplasma urealyticum* (GenBank Accession No: NR_041710.1). So it was regarded as *Ureaplasma* spp., and the swab was counted negative for mycoplasmas and was excluded from the positive PCR results.

The PCR targeting the 16-23 S rRNA ISR sequence from each isolate or used swab respectively also demonstrated a *Mycoplasma* spp. typical PCR amplicon. The 16-23 S rRNA ISR sequences (length between 435 and 439 bp) from all isolates and three swabs had a query coverage of 100% and a similarity between 99.77 and 100.00% with the 16-23 S rRNA ISR sequence of the *Mycoplasma sturni* strain ATCC 51945 (GenBank Accession No: AY766090.1) and had a query coverage between 99 and 100% and a similarity between 99.77 and 100.00% with the *Mycoplasma sturni* strain 234 – 21 Cl5 (GenBank Accession No: ON540700.1). The next similar mycoplasma species was *Mycoplasma columborale* (GenBank Accession No: LR215039.1) with a query coverage of 100% and a similarity between 89.96 and 90.24%. The 16-23 S rRNA ISR sequences from three swabs, including the swab with the sequencing product of the 16 S rRNA with overlaying signals, had a query coverage of 100% and a similarity of 99.26% with the chromosome of the *Pseudomonas poae* strain CAP-2018 (GenBank Accession No: CP034537.1). The next similar sequences were the chromosome of *Pseudomonas azotoformans* strain LMG 21611 (GenBank Accession No: LT629702.1) and *Pseudomonas*

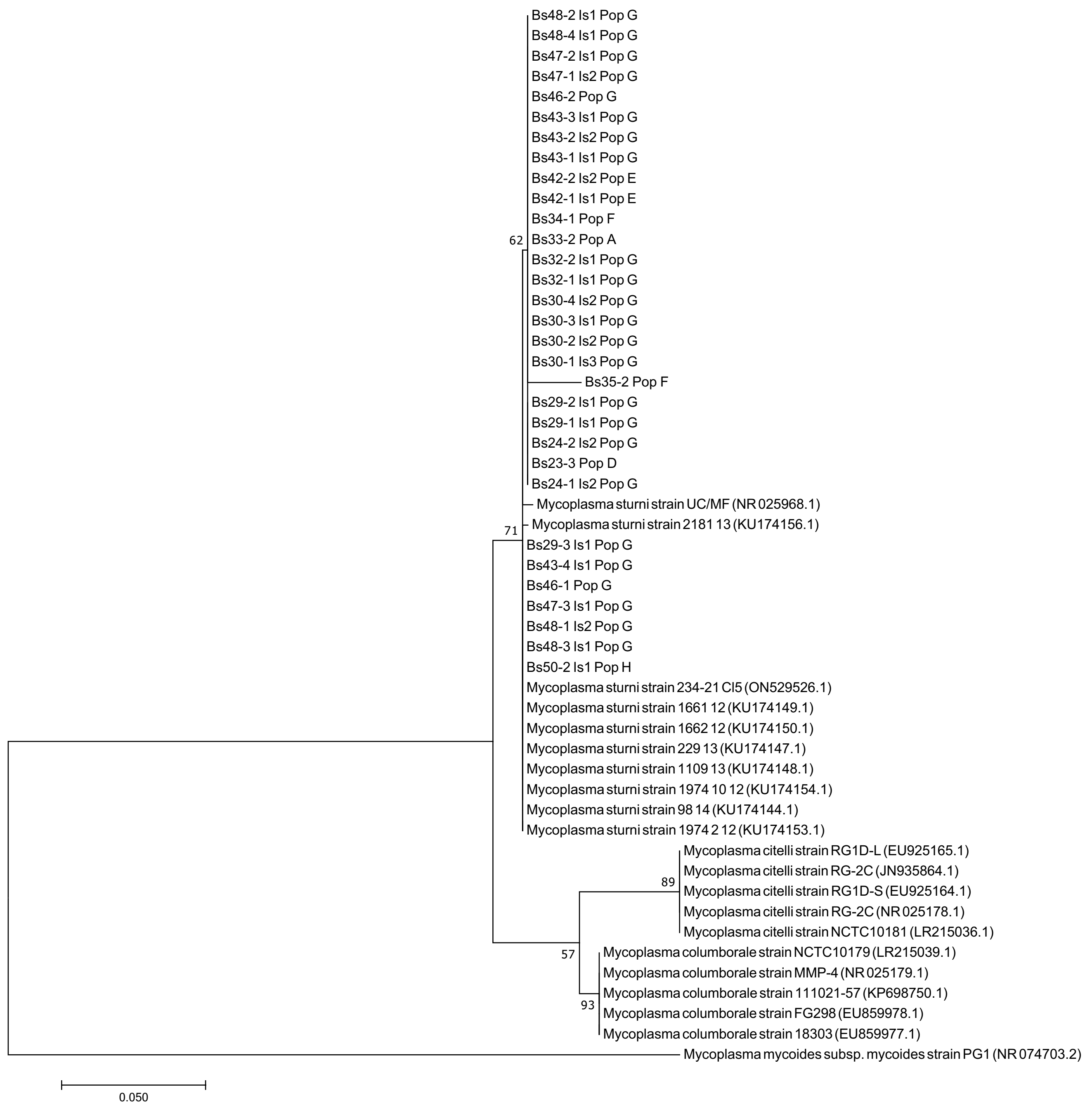

Fig. 1 Phylogenetic tree of 16 S ribosomal RNA sequences of five choanal swabs and of 25 isolates from mycoplasma culture of choanal swabs from barn swallows (*Hirundo rustica*) collected in Hesse, Germany, and of 20 sequences from closely related mycoplasma species. The sequence of *Mycoplasma mycoides subsp. mycoides* SC PG1T was used as an outgroup. The GenBank accession numbers are given in the brackets after the sequences. The phylogenetic tree was generated in MEGA Version 7.0.26. The evolutionary history was inferred by using the maximum likelihood method based on the Hasegawa-Kishino-Yano model. Bootstrap was conducted with 1000 repetitions. The percentage of trees, in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. (Bs, barn swallow; Is, isolate; Pop, population)

veronii strain G2 (GenBank Accession No: CP089532.1). In summary, in 14 of 59 nests, *Mycoplasma sturni* was the only mycoplasma species found in this study. Figure 1 shows the phylogenetic tree of the thirty used 16 S rRNA sequences and the closest related *Mycoplasma* spp. (*M. sturni*, *M. columborale* and *M. citelli*) with *M. mycoides subsp. mycoides* as outgroup. The phylogenetic tree

classifies all used sequences from this study as most closely related to *M. sturni*.

Discussion

The present study aimed to evaluate the role of mycoplasmas in barn swallows, to test the hypothesis, that the vocalization and the mycoplasma prevalence is evolutionary related, and the role of barn swallows as a potential reservoir of pathogenic mycoplasmas in poultry. For this purpose, the occurrence of mycoplasmas in the free-living barn swallow population in central Germany was investigated.

We detected in total *Mycoplasma* spp. in 16.49% of free-ranging juvenile barn swallow nestlings in Hesse, Germany. Considering the samples in terms of nests in 23.73% nests *Mycoplasma* spp. were detected. The occurrence of mycoplasmas per barn swallow colony varied between 0 and 50%.

For prevalence studies, a mycoplasma culture with a concurrent mycoplasma-genus-specific PCR is the best method (Lierz et al. 2007; Sawicka-Durkalec et al. 2021). Mycoplasmas can be detected with PCR, even if the samples are contaminated with other bacteria and fungi.

In cultivation, it is very likely that all *Mycoplasma* spp. will be detected, even if they are novel and they are unable to be detected by the genus-specific *Mycoplasma* PCR and, due to enrichment, even in samples with very low levels of mycoplasmas. Therefore, we used both these methods for this study to obtain the best possible results.

The 16 S rRNA sequences and the 16-23 S rRNA ISR sequences of all isolates and additionally of three swabs, where no isolates were obtained, showed a high similarity to *M. sturni.* Also, the isolates from the swab, which was negative in the PCR, showed a very high similarity to *M. sturni*. We assume that the DNA content of *Mycoplasma* spp. on this swab was very low and therefore was not detected by PCR. However, due to the enrichment in the liquid medium, isolates could still be obtained in the cultivation.

The similarity of all 16 S rRNA sequences and all 16-23 S rRNA ISR sequences was above the proposed arbitrary similarity values (Volokhov et al. 2012) compared to the sequences of *M. sturni.* As the high genetic similarity is sufficient for species differentiation, although no serological or biochemical tests were performed, all isolates were classified as *M. sturni*. Biochemical tests are not sufficient to discriminate species, and for serological tests, not all antisera are available. Therefore, we identify the isolates as *M. sturni*, and as well, we diagnose *M. sturni* in the three swabs based on the genetic analysis.

In consideration of the thesis that in highly vocal birds, an evolutionary pressure led to exclusion of mycoplasmas in the respiratory tract (Fischer et al. 2021), barn swallows should have an intermediate prevalence of mycoplasmas, because they rely on singing for mate choice, but the tail length is also an important factor (Saino et al. 2003). Our results show that the prevalence of barn swallows is much lower than that of birds that do not rely on singing for mate choice, such as raptors and storks with a prevalence of almost 100% (Lierz et al. 2008; Möller Palau-Ribes et al. 2016). Furthermore, the prevalence is higher than in nightingales and blue and great tits with a prevalence of 0%, which are birds that use almost exclusively the song for mate choice (Fischer et al. 2021). Corvids and barn swallows are Passeriformes, which both rely besides singing on other signals for mating, and also in both species, the prevalence of mycoplasmas is intermediate with 7% in Corvids (Ziegler et al. 2017) and 16% in barn swallows. Thus, our results do support the hypothesis of an evolutionary influence of mycoplasmas in singing and therefore also on the reproduction. In barn swallows, disadvantages in singing might be compensated in the factor tail length to be still successful in mating. Nonetheless, further studies on different bird species with different mating behavior are needed to prove or disprove this hypothesis. This study provides results on the prevalence of mycoplasmas in one species that can be compared with the results of other species in relation to their mating behavior, so that perhaps in the future an evolutionary link can be established between mycoplasma prevalence and the influence of reproduction.

Sawicka-Durkalec et al. (2021) revealed that *Mycoplasma* spp. are more abundant in birds with animal-based diets (59.9%) than in omnivorous (52.9%) and herbivorous birds (13%), more abundant in birds with aquatic environments (46.6%) as habitant than in those living in terrestrial ones (36.8%), and more abundant in migratory birds (46.8%). Barn swallows are migratory birds, live in terrestrial habitants, are strict insectivore, and are highly vocal birds. In combination, these parameters and the intermediate prevalence (16%) of mycoplasmas in barn swallows seem to fit with the findings of Sawicka-Durkalec et al. (2021) considering the fact that mycoplasma prevalence in highly vocal birds is generally considered to be rather low.

There are large variations in the occurrence of *M. sturni* between the different colonies with a range from 0 to 50%. The colonies were located on farms with poultry, with *M. synoviae* positive laying hens, or even in farms without poultry. One colony on a farm with laying hens positive for *M. synoviae*, and one colony on a farm with backyard poultry had an occurrence of 50% positive nests, but there was also a colony on a farm with laying hens positive for *M. synoviae* and one colony on a farm with backyard poultry with 0% positive nests. Additionally, in a colony on a farm without poultry, mycoplasmas in barn swallows were found with an occurrence of 14.29%. Therefore, it seems that laying hens and other poultry have no influence of the occurrence of mycoplasmas in barn swallow colonies.

M. synoviae was diagnosed in the laying hens from two farms, but in the barn swallows living on those farms, *M. synoviae* was not detected. Additionally, no poultry pathogenic mycoplasmas were detected in any barn swallow at any of the colonies. Therefore, it seems that no transmission of mycoplasmas occurs between barn swallows and poultry. Moreover, barn swallows do not seem to play a role as reservoir for poultry pathogenic mycoplasma species.

In our study, barn swallow nestlings were sampled. The juvenile barn swallows were not yet fledging, so a transmission of mycoplasmas from parents to the offspring seems likely. Due to the transmission of mycoplasma from the parents to the nestlings, the procedure of sampling nestlings represents a good cross-section of the population and thus describes the natural occurrence in the population well.

It is also known that insects can carry mycoplasmas (Gioia et al. 2022). Therefore, a transmission of mycoplasmas through the feeding of insects appears possible as well. In five nests, mycoplasmas were only found in one nestling; this is indicative for the transmission through insects. In this case also, a subsequent transmission of mycoplasmas from the nestlings to the parents during feeding seems possible. However, it still could be a new introduction of mycoplasmas into the nest, and the infection would spread through the nestlings. In the intestinal microbiota of healthy barn swallows, *Mycoplasmataceae* are a common finding (Ambrosini et al. 2019; Kenzaka et al. 2018; Kreisinger et al. 2017), which could as well support the hypothesis about a transmission through flies and feeding. However, more studies are needed to clarify the transmission of mycoplasmas in barn swallow colonies.

Since we detected *M. sturni* in healthy free-ranging juvenile barn swallows, it seems very unlikely that it is an obligatory pathogen in barn swallows. None of the barn swallow nestlings showed any signs of disease, so we speculate that *M. sturni* is apathogenic or a commensal for barn swallows. Some mycoplasma species are secondary pathogens, leading to disease only when additional factors are present (Bradbury 2005; Brown et al. 2011). Additionally, it is well known that some *Mycoplasma* spp. can impair the immune response of their host, leading to other infectious diseases (Bradbury 2005; Brown et al. 2011). A facultative role of *M. sturni* as pathogen for barn swallows cannot be totally excluded by this study, even though there are not any indications of this so far.

In conclusion, our study shows that *M. sturni* occurs frequently in barn swallows with an intermediate prevalence. The hypothesis that mycoplasmas are evolutionary excluded from the microbiota of birds completely relying on singing behavior for mate choice is supported by the findings of this study, but still further studies are needed to prove or disprove this hypothesis. There are large differences in the occurrence of *M. sturni* in different barn swallow colonies, but barn swallows do not appear to play a role as reservoir or in the transmission of poultry pathogenic mycoplasmas. *M. sturni* seems to be most likely commensal, apathogenic, or facultative pathogenic for barn swallows.

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Data availability No datasets were generated or analyzed during the current study.

Declarations

Ethical approval Sampling of the birds was governmentally approved by the regional council of Gießen, Germany, with the permission Nr. G 73/2021. The legal requirements and guidelines in Germany for the care and use of animals have been followed.

Consent to participate All authors contributed to data acquisition and writing of the manuscript.

Consent for publication All authors read and approved the final version.

Competing interests The authors declare no competing interests.

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