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House Finch (*Haemorhous mexicanus*)–Associated *Mycoplasma gallisepticum* Identified in Lesser Goldfinch (*Spinus psaltria*) and Western Scrub Jay (*Aphelocoma californica*) using Strain-Specific Quantitative PCR

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ABSTRACT: In 1994 *Mycoplasma gallisepticum* was found to be the etiologic agent of House Finch (*Haemorhous mexicanus*) conjunctivitis, a rapidly expanding epidemic caused by a genetically discrete, House Finch–associated strain of *M. gallisepticum* (HFMG). While most prominent in House Finches, HFMG has been reported in other members of the family Fringillidae, including American Goldfinches (*Spinus tristis*), Purple Finches (*Haemorhous purpureus*), Pine Grosbeaks (*Pinicola enucleator*), and Evening Grosbeaks (*Coccothraustes vespertinus*). Herein we report two new potential host species of HFMG strain, the Lesser Goldfinch (*Spinus psaltria*), belonging to the Fringillidae family, and the Western (California) Scrub Jay (*Aphelocoma californica*), belonging to the Corvidae family. The latter is one of only two reports of HFMG being found outside the Fringillidae family, and of these is the only one reported outside of captivity. Furthermore, non-HFMG *M. gallisepticum* was identified in an American Crow (*Corvus brachyrhynchos*), indicating presence of additional strains in wild birds. Strain typing of *M. gallisepticum* isolates was done via HFMG-specific quantitative PCR analysis and validated using random amplified polymorphic DNA analysis. Our results suggested an expanded host range of HFMG strain, and further suggested that the host range of HFMG was not limited to members of the family Fringillidae.

Key words: Corvidae, House Finch, Lesser Goldfinch, *Mycoplasma gallisepticum*, mycoplasmal conjunctivitis, qPCR, RAPD, Western Scrub Jay.

Mycoplasma gallisepticum is a bacterial pathogen primarily associated with acute and chronic respiratory disease in domestic poultry (Raviv and Ley 2013). It was first reported as the etiologic agent of conjunctivitis in House Finches when it was isolated from a North American House Finch (*Haemorhous mexicanus*) in Virginia, US in 1994 (Ley et al. 1996), and this novel House Finch disease spread into a North American epidemic. Bacterial genomic sequencing (Tulman et al. 2012) and multi-locus genetic analysis (Hochachka et al. 2013) revealed the novel epidemic to be caused by a discrete strain of *M. gallisepticum* (the House Finch–associated *Mycoplasma gallisepticum* [HFMG] strain) which likely became established and spread in the House Finch population following a single introduction. The epidemic spread quickly across the eastern states (Dhondt et al. 1998) and eventually throughout the native House Finch range in the western US (Duckworth et al. 2003; Ley et al. 2006). The HFMG strain has been associated with a substantial decrease in the House Finch population, especially in the eastern range (Dhondt et al. 1998, 2005).

House Finches are the wild birds best known to be impacted by *M. gallisepticum*, in particular by the HFMG strain that is found nearly exclusively in finches (Hochachka et al. 2013). However, recent studies have identified *M. gallisepticum* in other wild bird species (Ley et al. 2016). While most reports do not strain type *M. gallisetpictum* from wild birds, HFMG specifically has been reported in American Goldfinch (*Spinus tristis*), Purple Finch (*Haemorhous purpureus*; Hartup et al. 2000), Evening Grosbeak (*Coccothraustes vespertinus*), and Pine Grosbeak (*Pinicola enucleator*; Mikaelian et al. 2001). In addition, a non-HFMG strain (NY2001-var, ADRL 2001.035-16) of *M. gallisepticum* has been

identified in a subclinical House Finch (Cherry et al. 2006; Hochachka et al. 2013), indicating that independent introduction of *M. gallisepticum* into House Finches may occur and that additional strains may be circulating in the wild. To more fully understand its distribution and host range in wild bird populations, it is important to distinguish the genetically unique HFMG strain from other strains of *M. gallisepticum*. Strain typing of *M. gallisepticum* isolates is commonly done using randomly amplified polymorphic DNA (RAPD) fingerprinting, which yields distinct banding profiles when the RAPD products are resolved electrophoretically (Geary et al. 1994; Ley et al. 1997). Pure cultures of *M. gallisepticum* are essential for consistent and accurate strain identification using a RAPD-based technique. Due to the difficulties associated with acquiring pure cultures from wild birds and to make the process of identifying HFMG from wild bird isolates quick, sensitive, and specific, we designed an HFMG-specific quantitative real-time PCR (qPCR) assay that differentiates HFMG from other *M. gallisepticum* strains in wild birds.

Mycoplasma gallisepticum isolates from a variety of wild birds were initially speciated and inventoried as part of the Avian Diagnostic Research Laboratory collection at North Carolina State University. Conjunctival swabs were taken from both eyes, inoculated into mycoplasma transport media that was then stored at 4 C and shipped to the laboratory overnight on cold packs. For analysis, 0.5 mL of sample in transport medium was inoculated into either 10 mL of Frey's medium supplemented with 15% swine serum or complete Hayflick's broth and incubated at 37 C until color change indicated growth. Bacterial concentration was estimated using measurement of optical density at 620 nm. Genomic DNA was extracted from up to 2×10^9 bacterial cells resuspended in 20 μL phosphate buffered saline per extraction using a gram-negative bacteria DNA extraction protocol (GeneJET Genomic, Life Technologies, Carlsbad, California, USA). We quantified DNA concentrations using the Qubit dsDNA HS Assay (Invitrogen, Carlsbad, California, USA) to be between 4 ng/μL and 15 ng/μL and diluted with nuclease-free water 1:10 (0.4–1.5 ng/μL) for qPCR analysis.

For HFMG-specific PCR design, reference genome sequences from multiple strains of *M. gallisepticum* were analyzed to identify sites suitable for discrimination of HFMG from other strains. These included published and unpublished data from HFMG (GenBank accession nos. CP003506–CP003513, CA2009, GA2001, NY1998) and non-HFMG strains low-passage R strain (AE015450), F (CP001873), S6 (CP006916), TS-11, 6/85, and NY2001-variant. The following forward and reverse primers were designed following published suggestions (Dieffenbach et al. 1993), here with HFMG-specific bases highlighted in bold text: SG2307, forward, 5′-AGATAATCATAAAC**T**TT**G**A**C**ACG**G**T-3′ and SG2327, reverse, 5′-GATGAACTTT-**T**ATTTAGTTCTTTACT**G**-3′. These targeted the 3′ end of the HFMG thioredoxin reductase locus (HFMG94VAA_3550) to produce an amplicon of 274 base pairs in length. The qPCR reaction master mix per sample consisted of 6.25 μL of Applied Biosystems Power SYBR Green PCR reagent (Roche Molecular Systems, Pleasanton, California, USA), 0.5 μL working stock (10 μM) of each primer, and 3.25 μL of nuclease-free water. Master mix (10 μL) and diluted DNA template (2.5 μL) was added in optical qPCR tubes to reach a reaction volume of 12.5 μL per sample. The qPCR was run on the Bio-Rad CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA) with the following conditions: 94 C for 3 min, followed by 40 cycles of 94 C for 30 sec, 61.5 C for 30 sec, and 72 C for 1 min, with fluorescence measured once every extension. Under these conditions and after analysis using the Bio-Rad CFX Manager (Bio-Rad), known HFMG isolates consistently amplified at low cycle threshold values (18.32–27.65), while known non-HFMG isolates yielded no amplification or late-stage amplification. Melt-curve analysis indicated one peak at 75.5 C in HFMG samples, suggesting primer specificity and a single amplicon. Given data using reference strains, cycle threshold values of less than 30

at a constant baseline threshold value were considered positive for HFMG strain-specific amplification under these qPCR conditions. To further validate the qPCR assay as HFMG-specific, a panel of isolates previously typed by RAPD fingerprinting was subjected to qPCR and again typed by RAPD. Results indicated that our qPCR assay was fully congruent with RAPD typing (Table 1 and Fig. 1).

We examined currently untyped *M. gallisepticum* isolates from non–House Finch species to gain insight as to the host range potential of the HFMG. Using qPCR, eight *M. gallisepticum* isolates of unknown type were queried. Results indicated the presence of HFMG from two American Goldfinches, one Purple Finch, one House Finch, two Lesser Goldfinches (*Spinus psaltria*), and one Western Scrub Jay (*Aphelocoma californica*) for a total of seven isolates (Table 1). A sample from an American Crow (*Corvus brachyrhynchos*) tested negative for HFMG. To further validate qPCR results, RAPD was conducted and, again, profiles of the qPCR-positive isolates were consistent with that of HFMG (Fig. 1). Similarly, the RAPD profile of the qPCR-negative American Crow isolate did not match the HFMG profile, or that of any other strain that we tested. Our results are consistent with previous data indicating the predominance of HFMG in wild passerines and the likelihood of a single host introduction as being responsible for the emergence of HFMG, but they also indicated presence of additional *M. gallisepticum* strains in wild birds (Cherry et al. 2006; Hochachka et al. 2013).

The isolation and identification of HFMG in the Lesser Goldfinch and Western Scrub Jay, a member of the family Corvidae, was significant as it suggested novel host species for HFMG. While *M. gallisepticum* in general has been reported in multiple wild bird species, reports of HFMG strain had been limited to House and Purple Finches, American Goldfinches, Pine Grosbeaks, and Evening Grosbeaks, all members of the family Fringillidae (Dhondt et al. 2014). A single Blue Jay (*Cyanocitta cristata*) was reported to be infected with HFMG (Ley et al. 1996) and *M. gallisepticum* has been detected in Western Scrub Jays, but the infections in corvids were all suspected to be obtained nosocomially at rehabilitation facilities housing infected House Finches. The Western Scrub Jay hosting HFMG here had a field description stated as "consistent with ocular mycoplasmosis" including apparent sightlessness, and it was euthanized for humane reasons on accession to the wildlife facility, thus precluding nosocomial infection and suggesting naturally acquired infection by unknown means of transmission. While no tests for viral or protozoal coinfection were conducted, cultures were negative for *Mycoplasma sturni*—the standard differential at North Carolina State University for ocular mycoplasmosis in wild birds. If acquired in the wild, this would be the first report of HFMG naturally found in passerines outside the family Fringillidae and a further indication that HFMG does not exclusively affect birds of Fringillidae. More refined genomic characterization of potential HFMG subgroups may provide further insight into disease transmission and other important epidemiologic questions.

With growing evidence that HFMG can affect multiple species within and outside the Fringillidae, and that feeder behavior plays a role in its transmission (Adelman et al. 2015), more bird species may be affected than is currently appreciated. Additionally, experimental infection with HFMG has been shown to result in clinical disease of varying severity in bird species such as Tufted Titmouse (*Baeolophus bicolor*), House Sparrow (*Passer domesticus*), Zebra Finch (*Taeniopygia guttata*), and Black-capped Chickadee (*Poecile atricapillus*) that are currently believed to be unaffected in the wild (Farmer et al. 2005; Dhondt et al. 2008, 2015). As HFMG is found in increasingly diverse wild bird populations, our understanding of its true host range, and its impact on the spread of the disease, is altered. Our data supported the concept of an expanded HFMG host range, and further highlighted the complexity of *M. gallisepticum* epidemiology in the wild.

TABLE 1. *Mycoplasma gallisepticum* isolates analyzed by qPCR and/or RAPD analysis to be the HFMG strain (+) or other (–).[a]

Host	Location	ADRL no./sample ID	Ct value	HFMG	
				qPCR	RAPD
Samples for primary qPCR assay validation					
Poultry		Rlow	NA	–	–
Poultry		TS-11	NA	–	–
Poultry		6/85	NA	–	–
Poultry		F	NA	–	–
Poultry		S6	NA	–	–
Poultry		A5969	NA	–	NP
House Finch (*Haemorhous mexicanus*)	New York	NY2001-var/2001.036-5	NA	–	+
House Finch	California	CA2006/2006.052-5	19.46	+	+[b]
House Finch	California	CA2008/2008.028-1	20.84	+	+[b]
House Finch	California	CA2009/2009.061-1	21.47	+	+[b]
House Finch	Virginia	VA2013/2013-089-15	21.10	+	+[b]
House Finch	Virginia	VA1994/7994	22.22	+	+
House Finch	Georgia	GA2001/2001.109-15	18.75	+	NP
House Finch	New York	NY1998/1998.025-4	19.02	+	NP
House Finch	North Carolina	NC2006/2006.080-5	21.52	+	+
House Finch	Wisconsin	WI2001/2001.043-13	18.32	+	+
Previously RAPD-typed HFMG isolates used for further qPCR assay validation					
House Finch	California	2010.003-1	20.77	+	+[b]
House Finch	North Carolina	2014.120-1	25.78	+	+[b]
House Finch	California	2015.022-3	21.72	+	+[b]
House Finch	California	2013.151-1	19.90	+	+[b]
House Finch	California	2013.080-1	18.34	+	+[b]
House Finch	California	2013.081-1	20.28	+	+[b]
House Finch	California	2013.103-1	18.47	+	+[b]
Blue Jay (*Cyanocitta cristata*)	Virginia	11394	25.15	+	+[b]
American Goldfinch (*Spinus tristis*)	North Carolina	1596-6	20.49	+	+[c]
American Goldfinch	North Carolina	1696-2	20.71	+	+[c]
Evening Grosbeak (*Coccothraustes vespertinus*)	Canada	1999.012-3	24.59	+	+[d]
Evening Grosbeak	Canada	1999.012-4	23.10	+	+[d]
Purple Finch (*Haemorhous purpureus*)	New York	1998.028-10	21.10	+	+[e]
Samples typed in this study					
American Goldfinch	North Carolina	2006.063-1	27.21	+	+
American Goldfinch	North Carolina	2006.086-1	21.42	+	+
American Crow (*Corvus brachyrhynchos*)	California	2013.062-2	NA	–	–
Purple Finch	Virginia	2013.089-16	22.36	+	+
Lesser Goldfinch (*Spinus psaltria*)	Oregon	2014.022-1	27.65	+	+
Lesser Goldfinch	California	2015.025-1	26.82	+	+
Western Scrub Jay (*Aphelocoma californica*)	California	2015.055-1	23.08	+	+
House Finch	Colorado	2016.083-1	24.28	+	+

[a] qPCR = quantitative real-time PCR; RAPD = randomly amplified polymorphic DNA; HFMG = House Finch–associated *M. gallisepticum*; ADRL = Avian Diagnostic and Research Laboratory; Ct = cycle threshold; NA = no amplification; NP = not performed.

[b] Performed at North Carolina State University.

[c] Cherry et al. (2006).

[d] Mikaelian et al. (2001).

[e] Hartup et al. (2000).

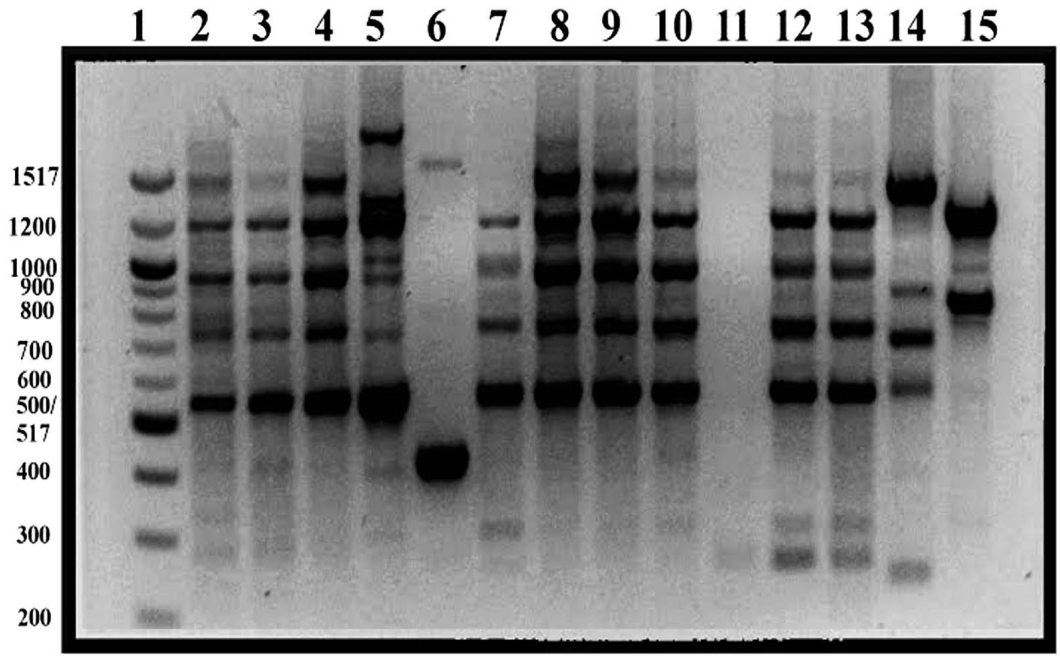

FIGURE 1. Randomly amplified polymorphic DNA profiles of *Mycoplasma gallisepticum* samples typed in this study to be either House Finch (*Haemorhous mexicanus*)–associated *M. gallisepticum* strain (band profile matches index strain VA1994) or other strain. Lane 1: 100-base pair ladder; Lane 2: 2014.022-1 (Lesser Goldfinch [*Spinus psaltria*]); Lane 3: 2015.025-1 (Lesser Goldfinch); Lane 4: 2015.055-1 (Western Scrub Jay [*Aphelocoma californica*]); Lane 5: 2013.062-2 (American Crow [*Corvus brachyrhynchos*]); Lane 6: 2015.069-1 (American Crow); Lane 7: 2016.083-1 (House Finch); Lane 8: 2006.063-1 (American Goldfinch); Lane 9: 2006.086-1 (American Goldfinch [*Spinus tristis*]); Lane 10: 2013.089-16 (Purple Finch [*Haemorhous purpureus*]); Lane 11: no template, negative control; Lane 12: index strain VA1994; Lane 13: WI2001; Lane 14: Rlow; Lane 15: F strain.

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