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Genotypic Analyses of *Mycoplasma gallisepticum* Isolates from Songbirds by Random Amplification of Polymorphic DNA and Amplified-fragment Length Polymorphism

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ABSTRACT: *Mycoplasma gallisepticum* (MG) conjunctivitis emerged in 1994 as a disease of free-ranging house finches (*Carpodacus mexicanus*) in North America and has also been isolated from other songbirds with conjunctivitis. Random amplification of polymorphic DNA (RAPD) of house finch and other songbird isolates has suggested that a single 'strain' initiated this outbreak. To explore the possibility of genomic variability among house finch isolates of MG and to evaluate the utility of a second technique for MG genotyping, we selected samples from our archive of reference strains and wild songbird isolates to analyze using both RAPD and amplified-fragment length polymorphism (AFLP); this is a newer technique that has been successfully used to explore the genomic variability of several *Mycoplasma* species. Both RAPD and AFLP results confirmed previous observations that during the initial stages of the MG epidemic in songbirds, isolates from different geographic locations and songbird species had genotypes that appeared to be highly similar, further supporting a single point source of origin. One 2001 isolate from New York was clearly different from the other songbird samples and clustered together with the vaccine and reference strains, indicating that substantial molecular evolution or a separate introduction has occurred.

Key words: AFLP, emerging disease, genotype, house finch, *Mycoplasma gallisepticum*, mycoplasmal conjunctivitis, RAPD.

Recent reports of emerging pathogens point to a growing list of examples where introductions of novel diseases have caused dramatic declines in wild animal populations (Roelke-Parker et al., 1996; Hochachka and Dhondt, 2000; Jensen et al., 2002). An important question concerns the role that genetic change plays in disease emergence and the epidemiology of these recently introduced pathogens (Cleaveland et al., 2001; Altizer et al., 2003; Antia et al., 2003). The evolutionary potential of pathogens, including their short generation times and high population sizes, could lead to molecular and phenotypic changes driven by selection and genetic drift. However, genetic changes associated with emerging wildlife diseases are poorly understood (Schrag and Wiener, 1995), in part because few pathogens have been extensively monitored and sampled during their establishment and spread.

Mycoplasma gallisepticum (MG) conjunctivitis emerged in 1994 as a disease of free-ranging house finches (*Carpodacus mexicanus*) in the mid-Atlantic region of the United States and has since spread to house finches throughout their entire eastern range (Ley et al., 1996; Luttrell et al., 1996; Ley et al., 1997; Dhondt et al., 1998; Hartup et al., 2001a, b). The resulting epidemic of MG conjunctivitis produced an unprecedented decline of eastern house finch populations, and the endemic disease remains associated with repeating seasonal peaks of conjunctivitis and limitation of host populations (Hochachka and Dhondt, 2000; Altizer et al., 2004). MG has also been isolated from other songbirds with conjunctivitis including American goldfinches (*Carduelis tristis*), a blue jay (*Cyanocitta cristata*), purple finches (*Carpodacus purpureus*), evening grosbeaks (*Coccothraustes vespertinus*), and pine grosbeaks (*Pinicola enucleator*) (Fischer et al., 1997; Ley et al., 1997; Hartup et al., 2000; Mikaelian et al.,

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2001). Because conjunctivitis in house finches has been monitored and sampled extensively following initial reports, it is rapidly becoming a model system for understanding emerging infectious diseases in wild avian hosts (Dhondt et al., 1998; Hartup et al., 2001a, b).

Random amplification of polymorphic DNA (RAPD) demonstrated the presence of what appeared to be a single, unique RAPD profile among house finch and other songbird MG isolates, suggesting a single point source of origin and one 'strain' common to the outbreak (Ley et al., 1997). Although genomic variability of MG house finch isolates has recently been identified by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) and nucleotide sequencing of the *pvp*A gene (Pillai et al., 2003), these findings were apparently consistent with the hypothesis that the initial outbreak had a single point source of origin. However, the authors also indicated that house finch MG isolates are more polymorphic than previously recognized by RAPD, and suggested that the observed variability may have resulted from molecular evolution following the initial introduction and spread of disease (Pillai et al., 2003).

We have recently observed evidence of genomic variability among MG isolates from house finches using RAPD fingerprinting (D.H. Ley, unpubl. data). However, RAPD fingerprints are prone to variability and are difficult to reproduce and standardize, making interpretation challenging and subjective. To more precisely explore the possibility of genomic variability among songbird isolates of MG we selected archival samples to analyze by RAPD and amplified-fragment length polymorphism (AFLP). The AFLP technique has been successfully used to explore the genomic variability of several *Mycoplasma* species (Kokotovic et al., 1999). An analysis of selected MG isolates using both RAPD and AFLP allows us to compare the congruency of these two methods and evaluate the utility of AFLP for MG genotyping. Also, because AFLP generates a large number of repeatable bands (Vos et al., 1995; Savelkoul et al., 1999), we anticipate being able to use AFLP profiles to quantify the variability and molecular epidemiology of MG isolates from songbirds, especially with respect to host species, time, and geographic location. These analyses should also provide an opportunity to further evaluate whether MG isolates in wild songbirds have resulted from a single vs. multiple sources of introduction.

A total of 16 MG samples were characterized by RAPD and AFLP, including six vaccine and reference strains from domestic poultry and 10 songbird isolates. Vaccine strains included F, 6/85 (Intervet Inc., Millsboro, Delaware, USA), and ts-11 (Select Laboratories, Gainesville, Georgia, USA); reference strains were S6, R, and A5969. MG isolates from wild-captured songbirds showing signs of conjunctivitis were obtained from six birds captured between 1994 and 1996 (one blue jay, one American goldfinch, and four house finches), and five birds captured in 2001, all house finches (Table 1). Mycoplasmas isolated from songbirds by North Carolina State University were from conjunctival swabs cultured in Frey's broth medium with 15% swine serum (Kleven, 1998). *Mycoplasma* colonies on agar plates were identified as MG by direct immunofluorescence (Kleven, 1998) using fluorescein-conjugated rabbit antiserum provided by S. H. Kleven (Department of Avian Medicine, University of Georgia, Athens, Georgia, USA). In preparation for RAPD and AFLP analyses, mycoplasmas were grown in broth cultures for 6–7 days, and DNA was isolated using a DNeasy Tissue Kit (QIAGEN Inc., Valencia, California, USA).

Random amplification of polymorphic DNA is a PCR-based method of DNA fingerprinting that results in amplification of 'anonymous' stretches of DNA with short arbitrary primers and visualization of the amplification products by agarose gel electrophoresis. Compared to other cur-

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TABLE 1. *Mycoplasma gallisepticum* vaccine and reference strains from domestic poultry, and songbird isolates analyzed by random amplification of polymorphic DNA and amplified-fragment length polymorphism.

Sample	Strain/isolate identification	Type/host species	Year isolated	State of origin	Sample collected by[a]	MG isolated by[b]
1	ts-11	Vaccine				
2	6/85	Vaccine				
3	F	Vaccine				
4	S6	Reference				
5	A5969	Reference				
6	R	Reference				
7	7994-1	House finch	1994	Virginia	Porter	Ley
8	K4269	House finch	1996	Ohio	Luttrell	Kleven
9	2001.093-16	House finch	2001	Georgia	Davis	Ley
10	13295-2	House finch	1996	North Carolina	Degernes	Ley
11	11394-2	Blue jay	1994	Virginia	Porter	Ley
12[c]	1596-5	Goldfinch	1996	North Carolina	Joyner	Ley
13	K3839	House finch	1996	Maryland	Luttrell	Kleven
14	2001.035-16	House finch	2001	New York	Dhondt	Ley
15	2001.043-13	House finch	2001	Wisconsin	Hartup	Ley
16	2001.047-5	House finch	2001	New York	Dhondt	Ley

[a] Porter = S. Porter, The Wildlife Center of Virginia, Weyers Cave, Virgina, USA; Luttrell = P. Luttrell, Southeastern Cooperative Wildlife Disease Study, Athens, Georgia, USA; Davis = A. K. Davis, Department of Environmental Studies, Emory University, Atlanta, Georgia, USA; Degernes = L. Degernes, Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, USA; Joyner = K. Joyner, Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, USA; Dhondt = A. A. Dhondt, Laboratory of Ornithology, Cornell University, Ithaca, New York, USA; Hartup = B. Hartup, International Crane Foundation, Baraboo, Wisconsin, USA.

[b] Ley = D. H. Ley, Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, USA; Kleven = S. H. Kleven, Department of Avian Medicine, The University of Georgia, Athens, Georgia, USA.

[c] Sample 12 was not included in the AFLP analysis because results were not obtained at the Genetic Analyzer step, probably because of a sample loading error.

rently available methods of MG strain identification, RAPD fingerprinting is fast, relatively simple to perform, and cost effective, although this method requires a pure culture of each *Mycoplasma* isolate. Limitations related to reproducibility arise because RAPD tests are sensitive to alterations in PCR conditions. Challenges of reproducibility and interpretation can usually be overcome by using one or more additional primer sets to confirm apparent relationships or resolve ambiguous results. Our procedure for RAPD fingerprinting of MG has been published (Ley et al., 1997), and uses the primer sets described by Fan et al. (1995; Fan primers) and Geary et al. (1994; Geary primers).

Amplified-fragment length polymorphism is a selective restriction fragment amplification technique based on the ligation of adapters (linkers and indexers) to a digest of total genomic DNA, followed by a PCR-based amplification with adapter-specific primers (Vos et al., 1995). This allows simultaneous sampling of multiple loci distributed throughout the entire genome, allows the researcher to control the number of bands generated by using increasingly specific primer sets, and can generate consistent and reproducible banding patterns covering a large number of loci with a single amplification (Savelkoul et al., 1999).

Our procedure for AFLP fingerprinting of MG was carried out according to Kokotovic et al. (1999) using a *Bgl*-II / *Mfe*-I restriction enzyme combination. Approximately 600 ng genomic DNA was added to 10 U each of *Bgl*-I and *Mfe*-I (New England Biolabs, Beverly, Massa-

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chusetts, USA), brought to a 20 µl total reaction volume with buffer (10 mM Tris acetate, 10 mM Mg acetate, 50 mM K acetate, 5 mM DDT, and 50 ng/l BSA), and incubated at 37 C for 2 hr. Before the ligation step, adaptors were assembled by mixing equimolar amounts of corresponding oligonucleotides, incubating at 65 C, and then cooling for 15 min at room temperature. Following digestion, ~200 ng of digest was transferred to a new tube containing 2 pmol of the *Bgl*-II adaptor, 20 pmol of the *Mfe*-I adaptor, 1 U of T4 DNA ligase, and 2 µl of 10× T4 ligase buffer. The mixture was then brought to a 20-µl volume (using the same buffer as in the digestion) and left to ligate overnight at room temperature. The following morning the ligation reaction was diluted 10-fold with elution buffer (QIAGEN) and stored at −20 C. In the amplification step, 5 µl of the diluted ligation template DNA was added to 10 µl dNTPs (1mM), 2 µl (65 ng) *Mfe*-I 5′ 6-FAM labeled primer, 2 µl (65 ng) *Bgl*-II primer, 5 µl 10× *Taq* buffer, 5 µl $MgCl_2$ (25 mM), 0.5 µl *Taq* polymerase, and 20.5 µl dH_2O for a total reaction volume of 50 µl. Amplification was performed on a thermocycler by denaturing at 94 C for 3 min, followed by 30 cycles consisting of denaturing at 94 C for 60 sec, primer annealing at 54 C for 60 sec, and extension at 72 C for 90 sec. The last cycle included a final extension at 72 C for 10 min.

In the final step before electrophoresis, 1 µl of the selective amplification product was transferred to a 0.5-ml tube containing 12.0 µl of deionized formamide and 0.5 µl of ABI GeneScan-500 ROX (Applied Biosystems, Foster City, California, USA) size standard, incubated at 95 C for 3–5 min, and then chilled on ice. The samples were then loaded on a 310 Genetic Analyzer (Applied Biosystems) where amplification fragments were detected and initial data collection and preprocessing were performed by using the Genescan analysis software (Applied Biosystems). The preprocessed densitometric curve data were then imported in GelCompar 2.0 (Applied Maths BVBA, St-Martens-Latem, Belgium) where level of similarity among samples was calculated using the band-based Dice similarity coefficient, and clustering of samples was performed using the unweighted pair-group method with arithmetic mean.

Random amplification of polymorphic DNA banding patterns using Fan primers of MG vaccines (ts-11, 6/85, F), reference strains (S6, A5969, R), and three house finch isolates are shown in Figure 1. Each vaccine and reference strain has a unique banding pattern and can be easily distinguished from one another and from the house finch isolates. Two of the house finch isolates, (sample/lane numbers 7 and 8, from Virginia and Ohio, respectively), have similar banding patterns, and both were from the initial 2 yr of the epidemic. The isolate in lane 9 was collected in 2001 from a house finch in Georgia and has a distinct banding pattern. These results demonstrate both the ability of RAPD to differentiate among known strains of MG and its potential utility to recognize similarities and differences among field isolates of MG.

Figures 2A and 2B show RAPD banding patterns of selected isolates from three songbird species (house finch, American goldfinch, and blue jay) made from 1994 to 2001 in seven states (Virginia, Maryland, North Carolina, Ohio, New York, Wisconsin, and Georgia). Fingerprints resulting from Geary primers (Fig. 2A) showed very similar patterns among isolates in lanes 7, 8, 10, and 12, all of which were isolated between 1994 and 1996 when the epidemic was spreading rapidly. The house finch isolate in lane 15, collected from Wisconsin in 2001 showed a similar banding pattern to these four isolates. All other isolates appear to have unique banding patterns. Fingerprints resulting from Fan primers (Fig. 2B) showed more diversity among the isolates (only isolates in lanes 11 and 13 appear to

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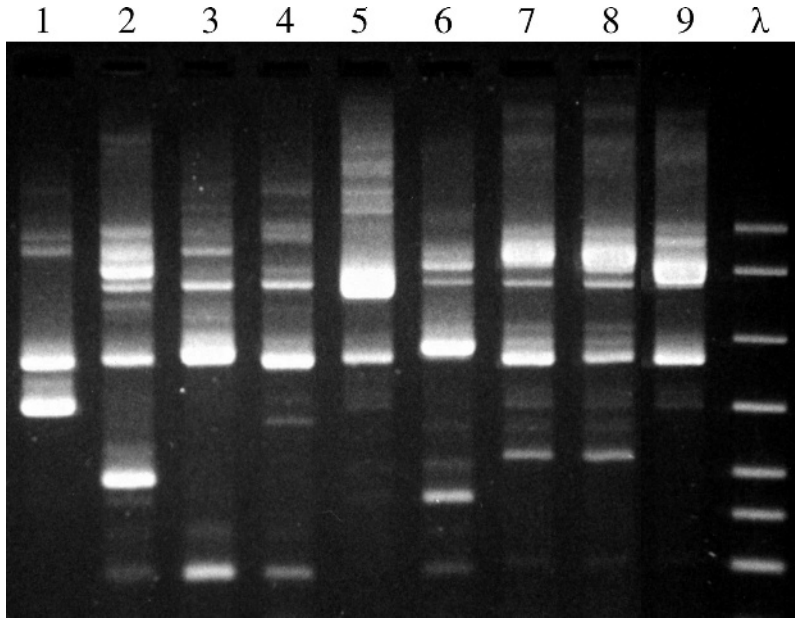


FIGURE 1. Random amplification of polymorphic DNA fingerprints of *Mycoplasma gallisepticum* vaccines, reference strains and house finch isolates using Fan primers. Lane identification is according to sample number in Table 1. λ=DNA ladder.

have identical banding patterns), although some of the pattern differences were subtle. Therefore, Fan primers appear to be more discriminatory than Geary primers in demonstrating genotypic variability among this group of MG isolates. These results indicate that although there may be considerable genotypic homology among MG isolates from songbirds, some variability is also detectable by RAPD. The challenges of making subjective visual interpretations of RAPD banding patterns and the problem of reproducibility are also evident. For example, compare lanes 7–9 in Figure 1 with lanes 7–9 in Figure 2B, for which both sets of fingerprints on the same three samples resulted from Fan primers.

Amplified-fragment length polymorphism results of MG vaccines (ts-11, 6/85, F), reference strains (S6, A5969, R), and nine songbird isolates (Table 1, except sample 12) are shown in Figure 3. This analysis generated 50–80 bands per sample, which allowed resolution of finer-scale quantitative variation among the samples. All but one of the songbird isolates from 2001 grouped together with the 1994–96 isolates, and similarity values for these eight isolates were high (between 91.5% and 97%). This 'initial epidemic' group clustered at a linkage level of 87%, indicating that they are closely related and are likely the same strain, based on a generalization by Savelkoul et al. (1999) that AFLP patterns with 90–100% homology are probably derived from identical strains. The 90–100% linkage level 'window of similarity' defining essential identical strains as suggested by Savelkoul et al. (1999), is variable depending on the organism and AFLP parameters (restriction enzymes, PCR efficiency, etc.), and must be determined empirically for each genus, species, and strain taxon of interest (Kokotovic et al., 1999). The vaccine and reference strains have similarity values of 72% to 85.5%, confirming that they are most likely distinct strains. These AFLP results confirm previous observations that during the initial stages of the MG epidemic in songbirds, isolates

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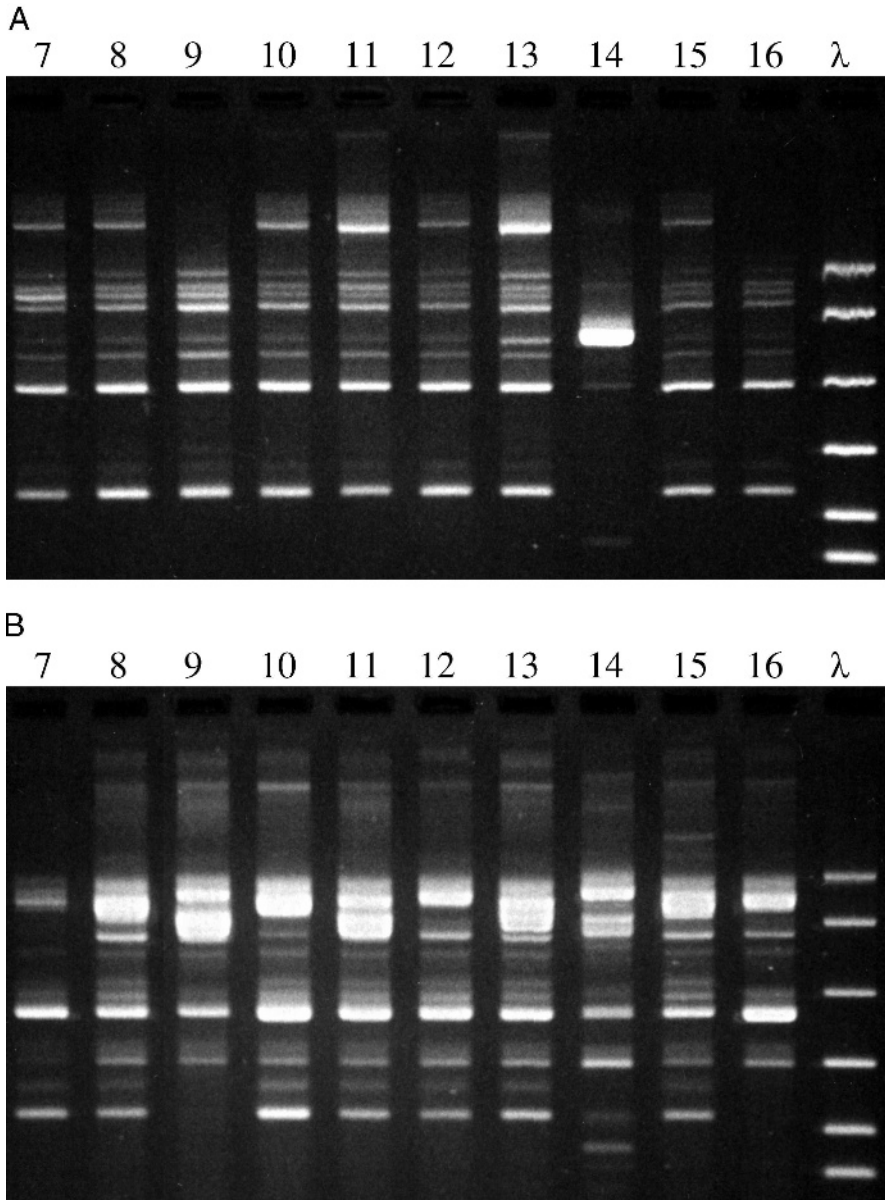


FIGURE 2. Random amplification of polymorphic DNA fingerprints of *Mycoplasma gallisepticum* isolates from songbirds, 1994–2001. Lane identification is according to sample number in Table 1. A. Geary primers. B. Fan primers. λ=DNA bp ladder.

from different geographic locations and songbird species had closely related genotypes, a result that is consistent with the hypothesis of a single point source of origin (Ley et al., 1997). One 2001 house finch isolate from New York (sample 14; Fig. 3) was clearly different from the other songbird isolates by AFLP and by RAPD fingerprints, especially with Geary primers (Fig. 2A). According to AFLP analysis this sample shared less than 78% of bands in common with the other songbird samples and clustered together with the vaccine and reference strains. This isolate is sufficiently different from the other songbird isolates to be considered a different

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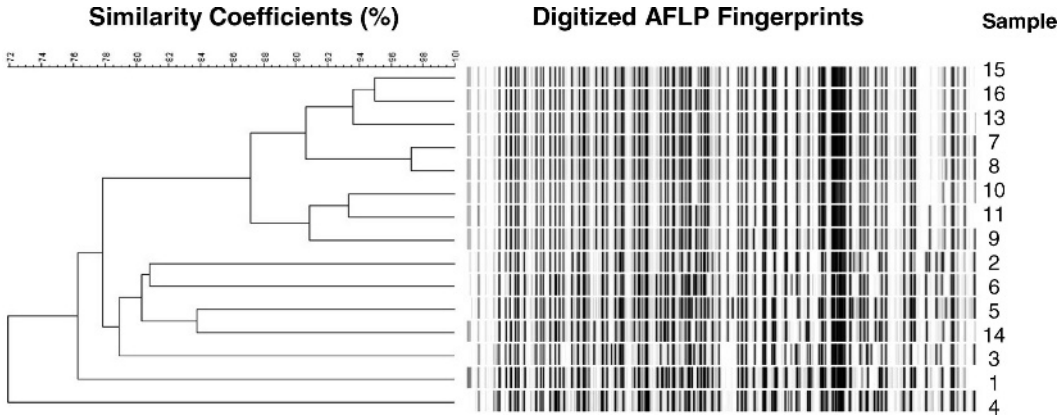


FIGURE 3. Amplified-fragment length polymorphism (AFLP) analysis of *Mycoplasma gallisepticum* vaccines, reference strains, and songbird isolates showing dendrogram, similarity coefficients (%), and digitized AFLP fingerprint. Samples are numbered according to Table 1. Sample 12 was not included in this AFLP analysis because results were not obtained at the Genetic Analyzer step, probably because of a sample loading error. Level of similarity was calculated using the band-based Dice similarity coefficient, and clustering of samples (dendrogram) was performed using the unweighted pair-group method with arithmetic averaging.

strain, which could be the result of substantial molecular evolution from the original strain or evidence that a separate introduction with another strain of MG occurred.

Our results are based on a relatively small number of samples, but in general they agree with recent work demonstrating the occurrence of genotypic differences among MG isolates from songbirds (Pillai et al., 2003). In all cases but one, the relatively low variability identified by AFLP most likely resulted from a genetic drift type of 'molecular evolution' manifested as minor changes occurring in the genome of the original/dominant MG strain. However, one house finch isolate was identified as a different strain compared to the others, which could represent a new introduction to house finches from another 'external' source(s) or a more substantial genetic shift in the original/dominant strain. More extensive analyses of historical and contemporary isolates of MG from house finches and other songbirds, using improved genotyping techniques such as AFLP, could help resolve this and other questions about the epidemiology and molecular evolution of MG conjunctivitis in house finches and other songbirds.

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