

Molecular Variability of House Finch *Mycoplasma gallisepticum* Isolates as Revealed by Sequencing and Restriction Fragment Length Polymorphism Analysis of the *pvpA* Gene

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SUMMARY. *Mycoplasma gallisepticum*, a major pathogen of chickens and turkeys, has caused significant declines in house finch (*Carpodacus mexicanus*) populations in the eastern United States since it was first observed in this species in 1994. There is evidence that *M. gallisepticum* infection is now endemic among eastern house finches, although disease prevalence has declined, suggesting an evolving host–parasite relationship. Studies based on randomly amplified polymorphic DNA (RAPD) have documented the presence of a single, unique RAPD profile in house finch *M. gallisepticum* isolates, suggesting a single point source of origin, which agrees with the known epidemiologic observations. In the present study, we evaluated the molecular variability of 55 house finch isolates as well as 11 chicken and turkey isolates including reference strains of *M. gallisepticum*. Molecular variability was evaluated by polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) analysis and nucleotide sequencing of the *pvpA* gene, which encodes for the putative cytoadhesin protein PvpA. Three different RFLP groups and 16 genotypes were evident from the 55 house finch isolates evaluated. Sequence analysis of *pvpA* gene PCR products showed that although most house finch *M. gallisepticum* isolates clustered more closely to each other, others clustered more closely to either turkey or chicken field isolates. These findings suggest that house finch isolates are more polymorphic than previously recognized by RAPD studies. This feature may allow us to learn more about the molecular evolution and epidemiology of this emerging disease host–parasite relationship.

RESUMEN. Variabilidad molecular de aislamientos de *Mycoplasma gallisepticum* provenientes de pinzones o camachuelos de México revelada mediante la secuenciación y el análisis de la longitud de los fragmentos de restricción del gen *pvpA*.

El *Mycoplasma gallisepticum*, el principal patógeno de pollos y pavos, ha ocasionado una disminución significativa en las poblaciones de pinzones o camachuelos de México (*Carpodacus mexicanus*) en el este de los Estados Unidos desde que fue observada por primera vez en esta especie en 1994. Existe evidencia de que la infección por *M. gallisepticum* es actualmente endémica en el este de los Estados Unidos, aunque la prevalencia de la enfermedad ha disminuido, sugiriendo una evolución en la relación huésped–parásito. Los estudios basados en los fragmentos polimórficos de ADN amplificados al azar han documentado la presencia de un patrón único en los aislamientos de *M. gallisepticum* de pinzones, sugiriendo una fuente de origen única que coincide con observaciones epidemiológicas conocidas. Se evaluó la variabilidad molecular de 55 aislamientos de pinzones y de 11 aislamientos de pollos y pavos incluyendo cepas de referencia de *M. gallisepticum*. Se evaluó la variabilidad molecular mediante la prueba de reacción en cadena por la polimerasa, análisis de la longitud de los fragmentos de restricción y la secuencia de nucleótidos del gen *pvpA*, el cual codifica la proteína citadhesina putativa PvpA. De los 55 aislamientos de pinzones evaluados se evidenciaron tres grupos diferentes mediante el

análisis de la longitud de los fragmentos de restricción y 16 genotipos. El análisis de las secuencias de los productos de la prueba de reacción en cadena por la polimerasa del gen *pvpA* mostraron que aunque la mayoría de los aislamientos de *M. gallisepticum* se agruparon más estrechamente entre ellos, algunos de ellos se agruparon más estrechamente con aislamientos de campo de pavos o de pollos. Estos hallazgos sugieren que los aislamientos de pinzones presentan un mayor polimorfismo que el observado en estudios llevados a cabo mediante los fragmentos polimórficos de ADN amplificados al azar. Esta característica nos permite aprender más acerca de la evolución molecular y epidemiológica de esta enfermedad emergente y de su relación huésped-parásito.

Key words: *Mycoplasma gallisepticum*, house finches, *pvpA* gene, PCR-RFLP, phylogenetic analysis

Abbreviations: CK = chicken; HF = house finch; PCR = polymerase chain reaction; RAPD = randomly amplified polymorphic DNA; RFLP = restriction fragment length polymorphism; TK = turkey

In 1994, *Mycoplasma gallisepticum* conjunctivitis was first reported from house finches in Maryland (11,13). Since then, the disease has spread rapidly throughout the eastern United States and southeastern Canada (3). During the early stages of the epidemic, the mortality rates were very high, and estimates suggest that 20% of the eastern population of house finches died between 1996 and 1997 (14, 16). Since then, however, the prevalence and mortality rates have declined steadily (3,18,19), suggesting a changing host-parasite relationship.

Previous epidemiologic studies of this epidemic based on randomly amplified polymorphic DNA (RAPD) analysis (7,12) indicated that *M. gallisepticum* isolates from house finches are distinct from current or historic poultry isolates. All the house finch isolates had identical RAPD patterns, distinct from those of chicken or turkey isolates tested, suggesting a single source of *M. gallisepticum* in house finches (7,12). However, a recent study (12) based on polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis of the *pvpA* gene, which encodes a putative cytoadhesin protein PvpA, found some variability in this segment of the genome, indicating that there may be detectable differences among *M. gallisepticum* isolates from songbirds with this disease (12).

The house finch population on the campus of Auburn University has been evaluated continuously for the prevalence of mycoplasmal conjunctivitis since 1995 (19). Over the course of 6 yr, the prevalence of conjunctivitis in the Auburn University house finch population has decreased from a high of 60% in 1996 to current low levels of about 4% (19). Several isolates of *M. gallisepticum* collected during the course of this investigation from house finches in Auburn provide a unique opportunity to study

genetic changes in *M. gallisepticum* over the course of the epidemic.

In the present study, we used PCR-RFLP and sequencing of the *pvpA* gene to evaluate the genetic variability of *M. gallisepticum* house finch isolates collected from Auburn over the course of the epidemic. We also studied the spatial and temporal variation of *pvpA* genotypes in *M. gallisepticum* house finch isolates from different geographic locations and different time periods during the epidemic. Finally, we compared *pvpA* genotypes of *M. gallisepticum* house finch isolates with those of chicken and turkey field isolates and reference strains.

MATERIALS AND METHODS

Mycoplasma isolates and reference strains.

A total of 66 isolates of *M. gallisepticum* were evaluated; 55 were field isolates from house finches, two were chicken reference strains (R, PG31), one was a chicken vaccine strain (6/85), three were chicken field isolates, and five were turkey field isolates. The origin and biological properties of *M. gallisepticum* reference strains R, PG31, and the atypical field isolate K503 have been described elsewhere (8,21). The properties of vaccine strain 6/85 (Intervet, Inc., Millsboro, DE) have been described earlier (4). The origin of vaccine strain 6/85 has not been fully documented, but there is some evidence that it is closely related to the S6 strain of *M. gallisepticum* on the basis of restriction endonuclease analysis (20).

Forty-one house finch isolates of *M. gallisepticum* were obtained from our repository at Auburn University (18,19). The remaining 14 isolates were obtained from various geographic locations and years starting from 1994. Details of the geographic location, year of isolation, and source of *M. gallisepticum* isolates are presented in Table 1.

Culture procedures and DNA extraction.

Table 1. Location, year of isolation, source, *pvpA* product size, RFLP group, and genotype of *Mycoplasma gallisepticum* isolates.

Serial no.	Isolate no.	Descriptive code ^A	Source ^B	<i>pvpA</i> PCR product size (bp)	RFLP group	Genotype
1	R	CK/GA/63/R	Panangala	660	4	CK1
2	K503	CK/GA/74/503	Panangala	600	2	CK2
3	PG31	CK/GA/64/PG31	Panangala	600	5	CK3
4	1321	HF/AU/99/1	Roberts	600	1	HF1
5	1553	HF/AU/00/1	Roberts	600	1	HF1
6	169125267	HF/AU/00/2	Roberts	600	1	HF1
7	169125226	HF/AU/00/3	Roberts	600	1	HF1
8	169125895	HF/AU/01/1	Roberts	600	1	HF1
9	169125564	HF/AU/01/2	Roberts	600	1	HF10
10	169125454	HF/AU/01/3	Roberts	600	1	HF1
11	169125512	HF/AU/01/4	Roberts	600	1	HF1
12	169125235	HF/AU/01/5	Roberts	600	1	HF1
13	169125566	HF/AU/01/6	Roberts	600	2	HF3
14	169124616	HF/AU/01/7	Roberts	600	1	HF1
15	169125361	HF/AU/01/8	Roberts	600	1	HF1
16	169125466	HF/AU/01/9	Roberts	600	1	HF11
17	169125477	HF/AU/01/10	Roberts	600	1	HF1
18	169125297	HF/AU/01/11	Roberts	600	1	HF9
19	169125436	HF/AU/01/12	Roberts	600	1	HF1
20	174194143	HF/AU/01/13	Roberts	600	1	HF1
21	169124815	HF/AU/01/14	Roberts	600	1	HF1
22	169125631	HF/AU/01/15	Roberts	600	1	HF1
23	169125565	HF/AU/01/16	Roberts	600	1	HF1
24	169125433	HF/AU/01/17	Roberts	600	1	HF1
25	169125553	HF/AU/01/18	Roberts	600	1	HF5
26	169125656	HF/AU/01/19	Roberts	430	3	HF15
27	174194277	HF/AU/01/20	Roberts	600	1	HF1
28	169124371	HF/AU/01/21	Roberts	600	1	HF1
29	169125570	HF/AU/01/22	Roberts	600	1	HF1
30	169125447	HF/AU/01/23	Roberts	600	1	HF1
31	169125822	HF/AU/01/24	Roberts	600	1	HF7
32	169125933	HF/AU/01/25	Roberts	600	1	HF1
33	174194118	HF/AU/01/26	Roberts	600	1	HF1
34	174194119	HF/AU/01/27	Roberts	600	1	HF1
35	418	HF/AU/01/28	Roberts	600	1	HF1
36	174194117	HF/AU/01/29	Roberts	600	1	HF1
37	169125442	HF/AU/01/30	Roberts	600	1	HF1
38	95055063	HF/AU/01/31	Roberts	600	1	HF4
39	174194144	HF/AU/01/32	Roberts	600	1	HF8
40	174194142	HF/AU/01/33	Roberts	600	1	HF1
41	174194298	HF/AU/01/34	Roberts	600	1	HF1
42	169125781	HF/AU/01/35	Roberts	600	2	HF2
43	174194290	HF/AU/01/36	Roberts	600	1	HF1
44	174194193	HF/AU/01/32	Roberts	600	1	HF1
45	K3839	HF/MD/94/1	Luttrell	600	1	HF1
46	S11	HF/VA/94/1	Ley	600	1	HF14
47	K4058	HF/GA/95/1	Luttrell	600	1	HF1
48	K3891	HF/GA/95/2	Luttrell	600	1	HF1
49	K4013	HF/PA/95/1	Luttrell	600	1	HF1
50	K4094	HF/TN/96/1	Luttrell	600	1	HF6
51	K4117	HF/KY/96/1	Luttrell	600	1	HF1
52	K4269	HF/OH/96/1	Luttrell	600	1	HF1

Table 1. Continued.

Serial no.	Isolate no.	Descriptive code ^A	Source ^B	<i>pvpA</i> PCR product size (bp)	RFLP group	Genotype
53	K4369	HF/NB/97/1	Luttrell	600	1	HF1
54	K4376	HF/MO/96/1	Luttrell	600	1	HF12
55	K4409	HF/TX/97/1	Luttrell	430	3	HF16
56	K4548	HF/GA/98/1	Luttrell	600	1	HF1
57	K4593	HF/MD/98/1	Luttrell	600	1	HF13
58	S12	HF/NC/95/1	Ley	600	1	HF1
59	S1	TK/NC/99/1	Ley	650	6	TK1
60	S7	TK/NC/99/2	Ley	430	3	TK2
61	S3	TK/NC/00/1	Ley	600	1	TK3
62	S4	TK/NC/00/2	Ley	600	1	TK4
63	S9	TK/NC/00/3	Ley	600	5	TK5
64	S5	CK/NC/00/1	Ley	600	7	CK4
65	S6	CK/NC/00/2	Ley	600	7	CK5
66	S10	CK/vaccine/6/85	Ley	600	5	CK6

^ADescriptive codes are in the form of avian species/state/year of isolation/number. CK = chicken; TK = turkey; HF = house finch.

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Mycoplasma gallisepticum isolates from different sources were grown to log phase in SP4 medium. Log phase growth was indicated by change in color of the phenol red indicator from red to yellow (6). DNA was extracted from the isolates with Instagene DNA purification matrix (Bio-Rad, Hercules, CA), as described earlier (17).

***pvpA* PCR-RFLP.** All isolates were first confirmed as *M. gallisepticum* by PCR, as reported earlier, with *M. gallisepticum*-specific primers that amplify a 185-base pair fragment from the 16S rRNA gene (19). *pvpA* PCR-RFLP analysis was conducted as described by Liu *et al.* (12) with few modifications.

All reagents used for PCR were obtained from Life Technologies, Inc. (Rockville, MD). The primers used were A1F (5'GCCAMTCCAACACTCAACAAGCTGA3', located at nucleotide positions 415–437) and A2R (5'GGACGTSCTCCTGGCTGGTTAGC3', located at nucleotide positions 1059–1081). PCR amplification was carried out in 50 µl reaction volumes as follows: 5 µl of 10× PCR buffer without MgCl₂, 1 µl of 10 µM deoxynucleoside triphosphate, 2 µl of 50 mM MgCl₂, 0.5 µl of 10 µM primers, 0.25 µl of Taq DNA polymerase (5 U/µl), 35.75 µl of distilled water, and 5 µl of template DNA. All amplification reactions were performed in a PCR Express thermal cycler (Hybaid, Franklin, MA) as follows: 94 C for 3 min, followed by 40 cycles of 94 C for 30 sec, 55 C for 30 sec, and 72 C for 1 min, followed by a final elongation step of 72 C for 10 min.

The PCR products were detected by electrophoresis

in 2% agarose gels containing 0.6 ng/ml of ethidium bromide. Agarose gel electrophoresis was run for 3 hr at 100 V, and the gels were photographed under ultraviolet illumination by a digital image analysis system (ChemImager, Alpha Innotech Corporation, San Leandro, CA).

The PCR products from various isolates and reference strains of *M. gallisepticum* were digested with restriction endonucleases *Pvu*II, *Scr*FI, and *Acl*I (New England Biolabs, Inc., Beverly, MA) as described earlier (12). Restriction digests were performed in 30-µl reaction mixtures containing 3 µl of 10× buffer, 2 µl of restriction enzyme, 5 µl of distilled water, and 20 µl of PCR product template. Restriction digest mixtures were incubated at 37 C for 3 hr. Digested PCR products were electrophoresed on a 4% agarose gel containing 0.6 ng/ml of ethidium bromide at a constant voltage of 85 V for 6 hr. Gels were photographed under ultraviolet illumination as described earlier for *pvpA* PCR.

Sequence analysis of *pvpA* PCR products.

Amplified *pvpA* PCR products from various isolates and reference strains of *M. gallisepticum* were purified from agarose gels with a QIAquick gel extraction kit (Qiagen Inc., Valencia, CA). Purified gel products were sequenced at the Auburn University Genomics and Sequencing Laboratory. Automated sequencing was done with the Big Dye Terminator cycle sequencing kit with an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Multiple alignment of DNA sequences was performed with Gene Doc (www.

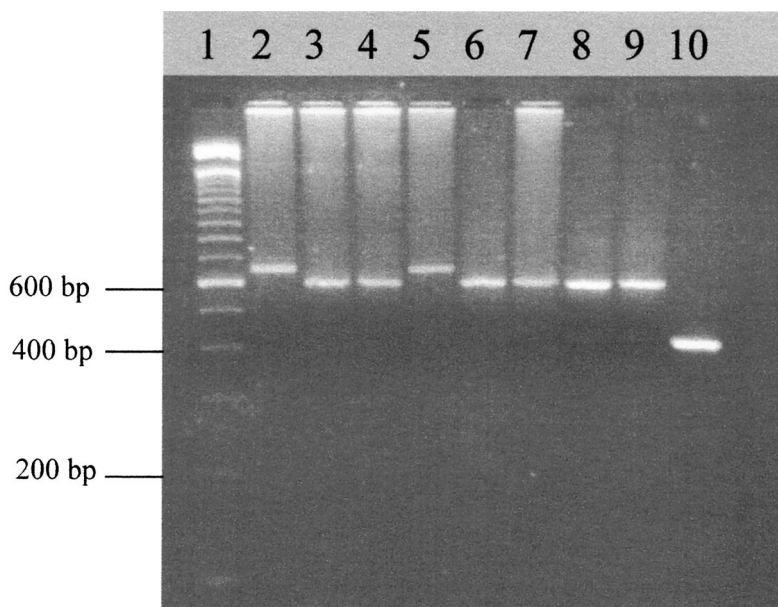


Fig. 1. *pvpA* PCR products of representative *Mycoplasma gallisepticum* isolates showing size variation of amplified products. Lane 1, 100-bp DNA ladder; lane 2, CK/GA/63/R; lane 3, CK/NC/00/1; lane 4, CK/vaccine/6/85; lane 5, TK/NC/99/1; lane 6, CK/GA/74/503; lane 7, CK/GA/64/PG31; lane 8, HF/NC/95/1; lane 9, HF/AU/01/1; lane 10, HF/AU/01/19. Isolate codes are given as species/location/year isolated/isolate number. Ln = lane; CK = chicken; TK = turkey; HF = house finch.

psc.edu/biomed/genedoc). Phylogenetic analysis was performed by the maximum likelihood method with Phylip version 3.5 (www.evolution.genetics.washington.edu/phylip.html). The *pvpA* gene sequence of the R strain (CK/GA/63/R) of *M. gallisepticum* was used as the outgroup for phylogenetic analysis.

RESULTS

***pvpA* PCR.** Amplification of the C-terminus-encoding region of the *pvpA* gene revealed PCR product size polymorphisms among the various reference strains as well as among the field isolates of *M. gallisepticum* (Fig. 1; Table 1). Chicken reference strains and field isolates had product sizes ranging from 600 to 660 base pairs, whereas turkey field isolates had product sizes ranging from 430 to 650 base pairs. All of the house finch isolates had product sizes of 600 base pairs except two isolates (one from Auburn-HF/AU/01/19, and one from Texas-HF/TX/97/1) that had product sizes of 430 base pairs (Table 1).

RFLP. Digestion of *pvpA* PCR products from 66 *M. gallisepticum* isolates (two reference, one vaccine, three chicken field isolates, five turkey field isolates,

and 55 song bird field isolates) with *AccI*, *PvuII* and *ScrFI* restriction endonucleases resulted in seven distinct RFLP groups on the basis of digestion patterns (Table 2). House finch isolates could be classified into three different RFLP groups (Tables 1, 2). Most of the house finch isolates (51/55, 93%) belonged to RFLP group 1. This group also included two turkey field isolates, TK/NC/00/1 and 2. Of the other four house finch isolates, two isolates (HF/AU/01/6 and HF/AU/01/35) belonged to RFLP group 2 along with an atypical chicken field isolate, K503. The remaining two house finch isolates (HF/AU/01/19 and HF/TX/97/1) belonged to RFLP group 3 along with a turkey field isolate (TK/NC/99/2). All three RFLP patterns were evident within the group of 41 Auburn isolates. Only two RFLP groups (groups 1 and 3) were represented among the remaining 14 house finch isolates from different locations and time periods (Table 1).

Sequence analysis of *pvpA* PCR-amplified products. Sequencing of the *pvpA* PCR-amplified products resulted in 27 distinct genotypes among the 66 reference and field isolates of *M. gallisepticum* that were included in the study (Table 1; Fig. 2). All six chicken isolates and all five turkey isolates were

Table 2. *pvpA* PCR-RFLP pattern combinations of representative house finch, chicken, and turkey field isolates and reference and vaccine strains of *Mycoplasma gallisepticum*.

Isolate	<i>pvpA</i> PCR product size (bp) ^A	Fragment sizes (bp)			RFLP group
		<i>pvuII</i>	<i>AccI</i>	<i>ScrFI</i>	
K3839 (HF/MD/94/1)	600	380 + 170 + 50	Not cut	250 + 220 + 130	1
K503 (CK/GA/74/503)	600	380 + 170 + 50	330 + 280	250 + 220 + 130	2
S7 (TK/NC/99/2)	430	380 + 50	Not cut	250 + 180	3
R1 (CK/GA/63/R)	660	380 + 230 + 50	320 + 225 + 115	250 + 235 + 175	4
PG31 (CK/GA/64/PG31)	600	380 + 130 + 50 + 40	Not cut	350 + 250	5
S1 (TK/NC/99/1)	650	380 + 220 + 50	335 + 315	250 + 230 + 170	6
S5 (CK/NC/00/1)	600	380 + 220	Not cut	250 + 220 + 130	7

^ASize in base pairs estimated from molecular weight markers.

classified into distinct genotypes, CK1–6 and TK1–5, respectively, by sequence alignment and phylogenetic analysis of the *pvpA* gene (Table 1; Fig. 2).

House finch *M. gallisepticum* isolates could be classified into 16 different genotypes (Table 1). Of the 16 genotypes, 12 genotypes were identified within the 41 isolates from Auburn. By comparison, only four additional genotypes were observed among the remaining 14 house finch isolates obtained from various geographic locations and time periods (Table 1).

Although there were 16 genotypes of house finch isolates, most of the house finch isolates (39 of 55, or 71%) belonged to one predominant genotype (HF1) and RFLP group (group 1) represented by the 1995 North Carolina (HF/NC/95/1) house finch isolate. The HF1 genotype was not detected in any of the chicken and turkey isolates studied (Table 1; Fig. 2). Of the remaining 16 house finch isolates, two (HF/AU/01/19, HF/TX/97/1) clustered closer to a turkey field isolate (TK/NC/99/2) and belonged to RFLP group 3 (Table 1; Fig. 2). These isolates shared a 230-bp deletion in the C-terminus-encoding region of the *pvpA* gene (data not shown). Two other isolates (HF/AU/01/6, HF/AU/01/35) clustered closer to an atypical chicken field isolate, K503, and belonged to RFLP group 2. These house finch isolates had an *AccI* restriction site similar to K503 (Table 1; Fig. 2).

The remaining 12 house finch isolates could be classified into 12 distinct genotypes by sequence analysis, although all 12 were classified into RFLP group 1 represented by HF/NC/95/1 (Table 1; Fig. 2). Sequence alignments showed that the differences among these 12 genotypes were small. The nucleotide sequences of these 12 isolates were 98%–99% identical to the *pvpA* sequence of the North Carolina house finch isolate.

Comparison of the amino acid sequences deduced from the *pvpA* nucleotide sequences showed that 53 of 55 house finch isolates had a deletion of about 20 amino acid residues (60 bp) between residue positions 281 and 300 of reference strain R (data not shown). This deletion was shared by all the chicken field isolates and chicken reference strain PG31. All turkey field isolates also shared this deletion except for turkey isolate TK/NC/99/2.

The two house finch isolates (HF/AU/01/19, HF/TX/97/1) and the turkey field isolate (TK/NC/99/2) in RFLP group 3 with smaller *pvpA* PCR products (430 bp) had deletions of 76 amino acids (230 bp) relative to the amino acid sequence of the PvpA protein expressed by chicken reference strain R (GenBank accession no. 224059 [2]). Although both house finch isolates had a 76 amino acid residue deletion, the location of this deletion in the two isolates varied. HF/AU/01/19 had a deletion of 76 amino acids between residue positions 249 and 325. HF/TX/97/1 and TK/NC/99/2 had a similar deletion of 76 amino acids between amino acid positions 276 and 351.

DISCUSSION

In the present study, the genetic variability of a large number of house finch *M. gallisepticum* isolates was studied by PCR-RFLP and sequence analysis of the *pvpA* gene. The 55 house finch isolates of *M. gallisepticum* could be classified into three RFLP types and 16 genotypes by these methods.

The findings reported here extend the results of a previous study by Liu *et al.* (12) that utilized PCR-RFLP and sequencing of the *pvpA* gene to show differences among chicken, turkey, and house finch isolates. In that study, the five house finch isolates

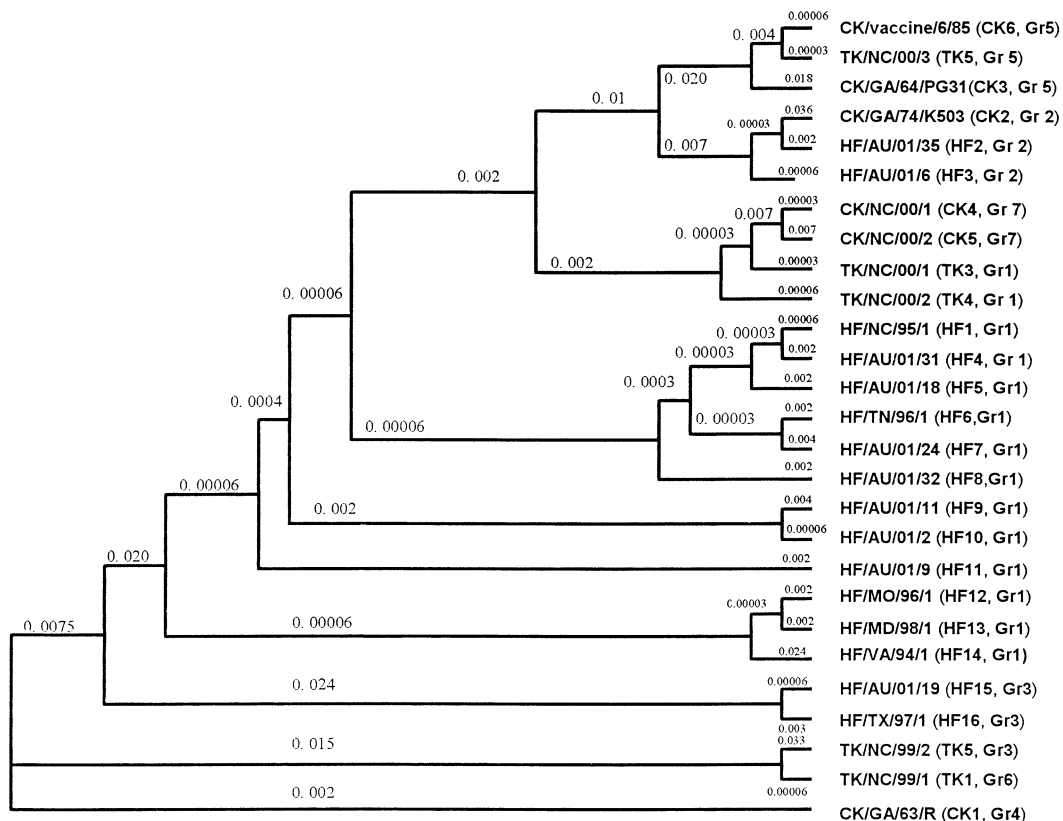


Fig. 2. Phylogenetic analysis of the C-terminus-encoding region of the *pvpA* gene. The dendrogram was constructed with the use of maximum likelihood analysis. In all, 1857 trees were examined. Numbers on branches indicate branch length. Branch lengths were calculated with a transition/transversion ratio of two. Isolate codes are given as species/location/year isolated/isolate number. Numbers in parentheses indicate genotype and RFLP group, respectively. CK = chicken; TK = turkey; HF = house finch.

evaluated were classified into two genotypes and two RFLP groups. However, in the study by Liu *et al.* (12), two house finch isolates (K4094, K4593) could not be distinguished by either RFLP or sequence analysis. In the present study, these two isolates were classified into the same RFLP group, but sequence analysis revealed distinct genotypes, HF 6 and HF 13, for these two isolates.

In the present study, although the majority of house finch *M. gallisepticum* isolates evaluated by *pvpA* sequence analysis clustered more closely to each other, it was evident that a few of the house finch isolates clustered closer to either turkey or chicken isolates. Two house finch isolates from Auburn clustered closer to chicken isolate K503, which is an atypical chicken field isolate from Georgia. Two other house finch isolates, one 1997 Texas isolate and another 2001 Auburn isolate, clustered closer to

a 1999 North Carolina turkey field isolate. By contrast, Liu *et al.* (12) found that the house finch isolates of *M. gallisepticum* clustered more closely with turkey field isolates by phylogenetic analysis, suggesting a closer genetic relationship between house finch and turkey field isolates.

Some of the differences reported here could be due to the target segment of the *pvpA* gene that was evaluated. In the study by Liu *et al.* (12), nested PCR was used to amplify a smaller region of the *pvpA* gene between nucleotide positions 583 and 1079 (496 bp) of reference strain R. In the current study, a larger segment of the *pvpA* gene, between nucleotide positions 415 and 1079 (660 bp), was evaluated. Therefore, the current study would be more likely to detect additional variation between isolates at the *pvpA* locus. Another factor that may contribute to the differences reported here could be the larger number

of house finch isolates evaluated. In the present study, 55 house finch isolates were evaluated as compared with five house finch isolates studied by Liu *et al.* (12).

The findings reported here and those of Liu *et al.* (12) differ from previous epidemiologic studies on song bird conjunctivitis that utilized RAPD to differentiate songbird isolates of *M. gallisepticum* from chicken and turkey isolates and vaccine strains (7,12). Whereas RAPD does differentiate songbird *M. gallisepticum* isolates, chicken and turkey field isolates, and vaccine and reference strains, results thus far suggest that RAPD does not distinguish differences among house finch *M. gallisepticum* isolates (9,12,13). Therefore, RAPD may be more appropriate to monitor the appearance of new strains of *M. gallisepticum* in flocks, whereas sequence analysis of targeted genes such as the *pvpA* gene may be more appropriate to study the molecular evolutionary history of *M. gallisepticum* isolates (5).

In the present study, *pvpA* PCR-RFLP analysis detected only three RFLP groups among 55 house finch isolates. By comparison, sequence analysis of the *pvpA* gene PCR-amplified products from the same isolates resulted in 16 genotypes of *M. gallisepticum*. This finding is not surprising because RFLP focuses on small genomic target sites (5–6 bp) specific to the three enzymes used and detects variation only at those target sites. By comparison, sequence analysis targets the complete *pvpA* gene segment (over 400 bp). Thus, PCR-RFLP may not be as useful as *pvpA* gene sequencing to determine molecular evolutionary changes of *M. gallisepticum* in house finches.

In the current study, different sized deletions were observed within the proline-rich carboxy terminal end of the *pvpA* gene of *M. gallisepticum* containing the two direct repeat regions DR1 and DR2 as reported previously (2,12). It is interesting to note that most house finch isolates share a deletion of 20 amino acids between DR1 and DR2 in common with chicken and turkey reference strains, field isolates, and chicken vaccine strain 6/85. Additionally, two house finch isolates, one from Auburn and one from Texas, and a turkey field isolate from North Carolina shared a deletion of 76 amino acids in the carboxy terminal region of the *pvpA* gene similar to deletions reported for turkey vaccine strain F (12). Further, two house finch isolates had an *AccI* restriction site similar to the atypical chicken field isolate K503. These results are consistent with the hypothesis that there was a single point source of infection in house finches, followed by molecular

evolution resulting in variability. Additionally, some parallel molecular evolutionary changes at the *pvpA* locus may have led to epidemiologically unrelated isolates with similarities at the *pvpA* locus. However, the present study evaluated only a single polymorphic locus of *M. gallisepticum*. A more precise molecular evolutionary history of the house finch *M. gallisepticum* isolates will require the sequencing of several other polymorphic loci within the genome of *M. gallisepticum*.

Another factor that needs to be considered is the outgroup chosen for the phylogenetic analysis. In the present study, the outgroup was based on a chronological history, with the oldest reference isolate in the data set serving as the outgroup. It would be more precise to construct dendrograms with an outgroup from nucleotide sequences of adhesion proteins homologous to PvpA from an unrelated mycoplasma species (15). Although the PvpA protein shared more than 50% amino acid homology with the P30 and P32 cytoadhesin proteins from *Mycoplasma pneumoniae* and *Mycoplasma genitalium*, respectively (2), the nucleotide sequences of the genes encoding either protein did not have significant homology with the *pvpA* gene nucleotide sequence.

In this study, no distinct trends were observed for either RFLP profiles or *pvpA* gene sequences with respect to spatial distribution of the *M. gallisepticum* isolates. However, both RFLP and sequence analysis suggested that the 2001 isolates from Auburn were genetically more diverse than isolates from earlier time periods. It will be necessary to study several additional isolates from other geographic locations and time periods to confirm these observations.

The phenotypic characteristics of the various house finch genotypes observed in this study were not elucidated. Because PvpA is a putative adhesion protein and attachment of mycoplasma to tissues is central to virulence (2,12), it is possible that the different house finch genotypes vary in virulence characteristics. Phenotypic assays for virulence such as the tracheal ring inhibition assay (1) or infection studies (19) may help to elucidate virulence characteristics of the various house finch genotypes.

In summary, the current study based on a single polymorphic *pvpA* gene locus has demonstrated three RFLP types and 16 genotypes of *M. gallisepticum* among the 55 house finch isolates evaluated. These findings demonstrate that house finch isolates are more variable than previously recognized by RAPD studies. Although house finch *M. gallisepticum* isolates were genetically distinct

from chicken and turkey isolates, some house finch isolates clustered more closely to turkey field isolates, whereas others clustered more closely to chicken field isolates. These findings are consistent with the hypothesis that the outbreak had a single point source of origin and that molecular evolution has resulted in genotype variability, with perhaps some examples of parallel molecular evolution.

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