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USING AMPLIFIED FRAGMENT LENGTH POLYMORPHISM ANALYSIS TO DIFFERENTIATE ISOLATES OF *PASTEURELLA MULTOCIDA* SEROTYPE 1

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ABSTRACT: Avian cholera, an infectious disease caused by the bacterium *Pasteurella multocida*, kills thousands of North American wild waterfowl annually. *Pasteurella multocida* serotype 1 isolates cultured during a laboratory challenge study of Mallards (*Anas platyrhynchos*) and collected from wild birds and environmental samples during avian cholera outbreaks were characterized using amplified fragment length polymorphism (AFLP) analysis, a whole-genome DNA fingerprinting technique. Comparison of the AFLP profiles of 53 isolates from the laboratory challenge demonstrated that *P. multocida* underwent genetic changes during a 3-mo period. Analysis of 120 *P. multocida* serotype 1 isolates collected from wild birds and environmental samples revealed that isolates were distinguishable from one another based on regional and temporal genetic characteristics. Thus, AFLP analysis had the ability to distinguish *P. multocida* isolates of the same serotype by detecting spatiotemporal genetic changes and provides a tool to advance the study of avian cholera epidemiology. Further application of AFLP technology to the examination of wild bird avian cholera outbreaks may facilitate more effective management of this disease by providing the potential to investigate correlations between virulence and *P. multocida* genotypes, to identify affiliations between bird species and bacterial genotypes, and to elucidate the role of specific bird species in disease transmission.

Key words: Amplified fragment length polymorphism, avian cholera, DNA fingerprinting, *Pasteurella multocida*, waterfowl.

INTRODUCTION

Pasteurella multocida serotype 1 is the primary causative agent of avian cholera in North American wild birds, and many species of domestic, feral, and wild birds are susceptible to infection (Samuel et al., 2007). Outbreaks killing thousands of wild waterfowl occur almost annually in some areas of the United States, and the disease has been documented in all major flyways (Botzler, 1991; Friend, 1999).

Pasteurella multocida isolates are classified based on serologic antigen presentation, and there are 16 somatic serotypes designated 1 though 16 (Rhoades and Rimler, 1991). Most pathogenic *P. multocida* isolates cultured from wild waterfowl in the Pacific, Central, and Mississippi flyways of North America are serotype 1 (Botzler, 1991). There have been significant annual and geographic fluctuations in the patterns of avian cholera mortality among wild bird species, and factors influencing the initiation and the course of an avian cholera outbreak are not well understood (Rosen, 1969; Wobeser, 1992; Blanchong et al., 2006; Samuel et al., 2007).

To gain further epidemiological insight into the dynamics of avian cholera transmission and disease spread among wild birds, it is necessary to develop techniques to differentiate *P. multocida* isolates of the same serotype. The use of DNA-based techniques provides this ability (Owen, 1989), but to date studies demonstrating the usefulness of these techniques for understanding the epidemiology of avian cholera in wild birds have been limited (Wilson et al., 1995a, b; Samuel et al., 2003b; Samuel et al., 2007).

A more recently developed genomic fingerprinting technique, fluorescent amplified fragment length polymorphism

(AFLP) analysis, provides a greater capacity than previous techniques to identify polymorphic regions within a genome (Vos et al., 1995) and has been applied to pasteurellae epizootics in domestic birds (Amonsin et al., 2002). Amplified fragment length polymorphism analysis is based on the detection of polymorphic restriction fragments by selective polymerase chain reaction (PCR) and provides the capacity to examine in excess of 1,000 DNA markers per isolate (Vos et al., 1995). To conduct AFLP analysis, genomic DNA is digested with two restriction enzymes, followed by ligation of adapters of defined DNA sequence to the ends of the restriction fragments. Ligation products are reamplified by PCR using primers that include a selective nucleotide at their 3′-end to reduce the complexity of AFLP patterns (Geert et al., 1996). A fluorescent label is incorporated during selective PCR to facilitate analysis of AFLP reaction products using an automated DNA sequencing instrument. Amplified fragment length polymorphism analysis generates reproducible, complex patterns, referred to as genetic fingerprints, that are useful for distinguishing closely related organisms.

The objective of this study was to evaluate the utility of AFLP analysis as an epidemiological tool to distinguish *P. multocida* serotype 1 isolates based on regional and temporal genetic characteristics. Fifty-three serotype 1 isolates cultured during a laboratory challenge of Mallards (*Anas platyrhynchos*; Samuel et al., 2003a) and 120 serotype 1 isolates cultured from wild birds and environmental samples during avian cholera outbreaks were examined. Analysis of the AFLP data revealed that the isolates were distinguishable.

MATERIALS AND METHODS

Bacterial isolates

Amplified fragment length polymorphism analysis was applied to a collection of *P. multocida* serotype 1 isolates from the US Geological Survey–National Wildlife Health Center diagnostic culture collection and from field studies on avian cholera epidemiology (Samuel et al., 2003b). A collection of serotype 1 isolates collected during a 15-wk laboratory challenge study of captive-reared mallard ducks (Samuel et al., 2003a) was also analyzed. The challenge study was conducted as follows (for additional study details, see Samuel et al., 2003a): Six-to-eight-wk-old male Mallards were divided into groups consisting of 30 birds each, and each group was housed in a separate isolation room (designated 4, 5, and 6). Within each room birds were divided into three groups of 10; two groups were challenged with *P. multocida* inocula of different environmental origins, and the third group was unchallenged. Hence, six different *P. multocida* inoculae (designated 1, 2, 3, 4, 7, and 8) were used. The inocula, all originally isolated from environmental samples collected in central California (Samuel et al., 2003a), were utilized as follows: inocula 1 and 2 (room 6), inocula 3 and 4 (room 5), inoculae 7 and 8 (room 4). *Pasteurella multocida* was cultured from tissues and/or swabs collected periodically from live birds during the course of the study, from birds that died, and following euthanasia of birds at the end of the study. During the study some birds inoculated with *P. multocida* cleared the organism, while some uninoculated birds became infected.

AFLP reaction preparation

Genomic DNA was extracted from *P. multocida* isolates using the Puregene DNA purification kit (Gentra Systems Inc., Minneapolis, Minnesota, USA) according to the manufacturer's instructions. Genomic DNA concentration and purity were evaluated by UV spectroscopy and agarose gel electrophoresis. Amplified fragment length polymorphism reactions were prepared as described by Vos et al. (1995). Genomic DNA (500 ng) was digested with the restriction enzymes *EcoRI* and *HpyCH4 IV* for 3 hr and ligated, using T4 ligase (New England Biolabs, Beverly, Massachusetts, USA), to *EcoRI* (5′-CTC GTA GCT GCG TAC C-3′ plus 3′-CAT CTG ACG CAT GGT TAA-5′) and *HpyCH4 IV* (5′-GAC GAT GAG TCC TGA G-3′ plus 3′-TAC TCA GGA CTC GC-5′) double-stranded adapters. The digested and ligated DNA was then diluted fivefold in a 1× solution of *Taq* DNA polymerase reaction buffer without $MgCl_2$ (Promega, Madison, Wisconsin, USA) prior to amplification by preselective PCR using the *EcoRI* and *HpyCH4 IV* primers 5′-

GAC TGC GTA CCA ATT C-3′ and 5′-GAT GAG TCC TGA GCG T-3′, respectively, in a final volume of 50 µl. PCR reactions contained 10 µl diluted ligation mixture, 15 pmol each *EcoRI* and *HpyCH4 IV* primers, 4 µl 40 mM dNTP mix, and 2.5 U *Taq* polymerase in storage buffer B (Promega). Preselective PCR amplification conditions were an initial extension cycle at 72 C for 60 sec, followed by 35 cycles of denaturation at 94 C for 50 sec, annealing at 56 C for 1 min, and extension at 72 C for 2 min. Preselective PCR products were then diluted 10-fold with double-deionized water, and 3 µl of the diluted preselective mixture were used as template for selective amplification. Selective PCR reactions contained 15 pmol fluorescein phosphoramidite (FAM)–labeled *EcoRI*+G selective primer (5′-FAM-GAC TGC GTA CCA ATT CG-3′), 25 pmol unlabeled *HpyCH4 IV*+A selective primer (5′-GAT GAG TCC TGA GCG TA-3′), 3 µl 40 mM dNTP mix, and 1.25 U *Taq* polymerase in storage buffer B (Promega) in a final reaction volume of 25 µl. Selective PCR amplification conditions were 35 cycles of denaturation at 94 C for 50 sec, annealing at 56 C for 1 min, and extension at 72 C for 2 min, with a final extension at 72 C for 10 min. Selective PCR reaction products were diluted fivefold with double-deionized water, and fragment analysis was conducted at the University of Wisconsin–Madison Biotechnology Center using an Applied Biosystems (Foster City, California, USA) 3730 automated capillary DNA sequencing instrument.

AFLP data analysis

Amplified fragment length polymorphism profiles were normalized and aligned to one another with respect to an internal sizing standard using BioNumerics 3.5 (Applied Maths Inc., Austin, Texas, USA) software's proprietary algorithm. Continuous AFLP fingerprint data, in the form of densitometry distributions, were then analyzed directly without attempting to identify discrete bands because discretization in a nonarbitrary manner is not possible. Once AFLP densitometry patterns were aligned, a Pearson product moment correlation matrix was computed for all pairwise combinations, exported as a text file, and imported into SAS statistical software (SAS Institute Inc., Cary, North Carolina, USA). The SAS PROC CLUSTER was used to produce the dendrograms, using the average linkage (also known as the unweighted pair group method with arithmetic mean [UPGMA]; Sokal and Michener, 1958) and WARD (Ward, 1963) options for comparison. The UPGMA and WARD dendrogram outputs were virtually the same, and only UPGMA dendrograms are reported. SAS PROC PRINCOMP was used to conduct a principal components analysis (PCA; Johnson and Wichern, 1982) using the Pearson product moment correlation matrix. Principal components analysis uses a different algorithm than dendrogram analysis to evaluate the data, providing a complementary assessment. The second and third principal components were used for the PCA. Although the first principal component explained the majority of the variation in the data, the first component is essentially the mean axis and typically reflects the average signal strength of the fingerprint. Signal strength largely reflects sample preparation attributions and therefore is not usually an informative biologic axis on which to align samples. Fisher's 2-tailed exact test was used to determine whether specific groups of samples fell disproportionately on different branches of the dendrograms. Analysis of variance (ANOVA) followed by pairwise comparisons of the means was used to test whether the principal components scores differed between groups of interest. Values of $P \leq 0.05$ were considered significant.

To understand the resolution AFLP analysis in distinguishing closely related bacteria, a single genomic DNA preparation from a *P. multocida* serotype 1 isolate was used to conduct 10 replicate AFLP analyses. All aspects of AFLP reaction processes were conducted independently. A Pearson product moment correlation matrix comparing the resulting AFLP patterns indicated an average correlation of 0.92 (SD=0.05) among the replicate samples.

RESULTS

Analysis of *P. multocida* serotype 1 isolates from a laboratory infection trial

Amplified fragment length polymorphism analysis was completed for the six *P. multocida* inocula and for 53 isolates cultured from Mallards during the infection trial. Of the 53 isolates examined, 21, 12, and 20 were cultured from birds housed in rooms 4, 5, and 6, respectively. Amplified fragment length polymorphism profiles for each *P. multocida* isolate were used to construct a dendrogram, consisting of four major branches, illustrating genetic relationships among the isolates (Fig. 1). Isolates cultured from birds that

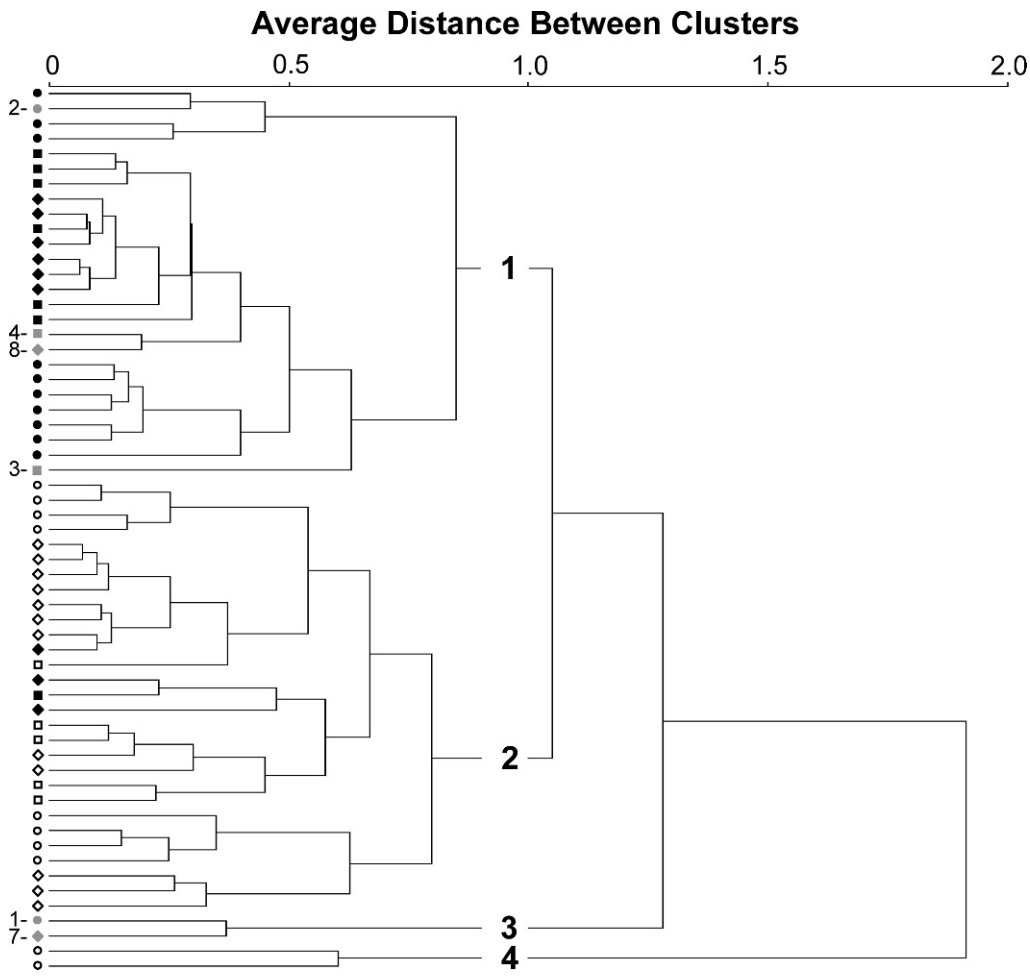

FIGURE 1. Dendrogram analysis of *Pasteurella multocida* serotype 1 isolates cultured from Mallards during a laboratory infection trial. Isolates cultured from birds that died are represented by filled symbols, and isolates cultured from live, apparently healthy birds are represented by open symbols. Dendrogram branches 1–4 are labeled, and inocula 1, 2, 3, 4, 7, and 8 are identified numerically to the left of symbols (gray) indicating in which room they were used: room 4 (diamonds), room 5 (squares), and room 6 (circles).

died (filled symbols) allocated disproportionately to dendrogram branch 1, while isolates obtained from live, apparently healthy birds allocated disproportionately to branches 2 and 4 (open symbols). The distribution of *P. multocida* isolates cultured from dead birds was distinct from those cultured from apparently healthy live birds ($P<0.001$). Analysis of AFLP data using PCA (components 2 and 3; Fig. 2) also supported the division of bacterial isolates into the two major groups observed on the dendrogram, one consisting of isolates from dead birds (filled symbols; quadrant IV), and the other consisting of isolates from live, apparently healthy birds (open symbols; quadrant I; $P<0.001$ for principal component 2). Further, PCA indicated that room 6 bacterial isolates (circles) allocated disproportionately to the right-hand quadrants of the plot (Fig. 2) compared to room 4 and room 5 isolates (diamonds and squares, respectively; $P<0.001$ for principal component 3).

Amplified fragment length polymor-

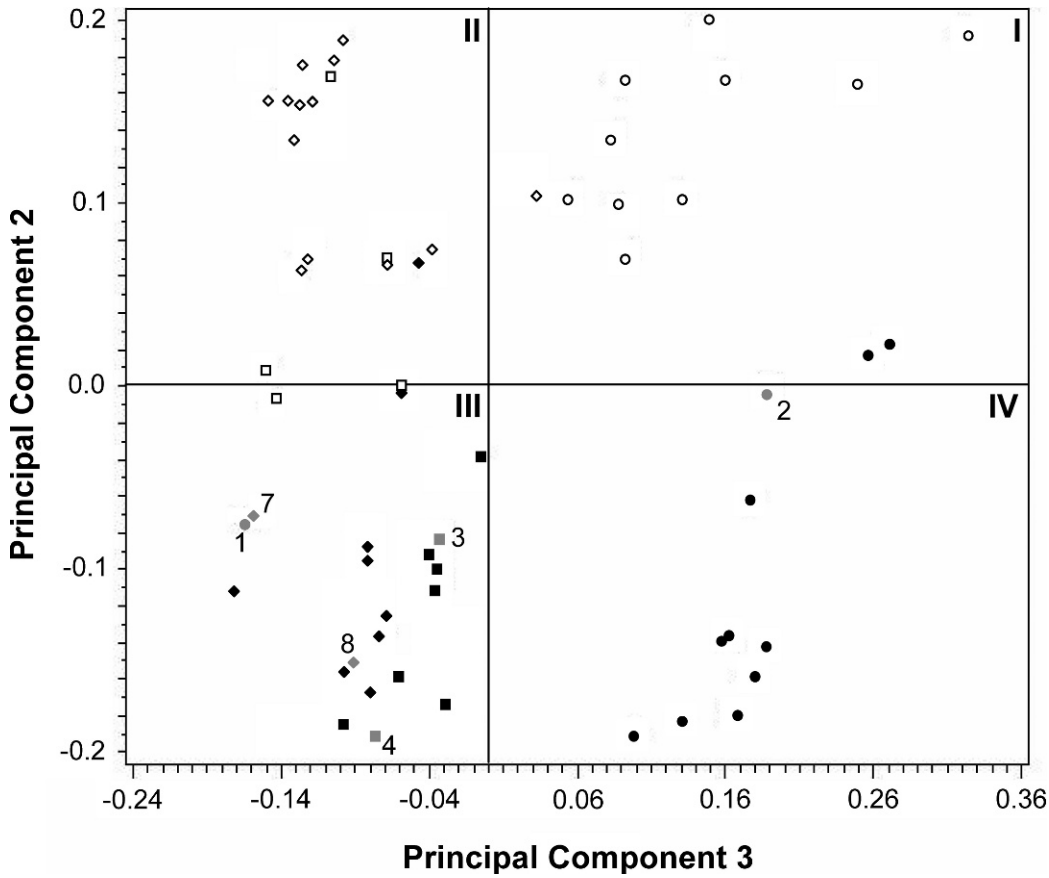

FIGURE 2. Principal components analysis of *Pasteurella multocida* serotype 1 isolates cultured from Mallards during a laboratory infection trial. Isolates from birds that died are represented by filled symbols, and isolates from live, apparently healthy birds are represented by open symbols. Quadrants I–IV are labeled, and inocula 1, 2, 3, 4, 7, and 8 are identified numerically and with symbols (gray) indicating in which room they were used: room 4 (diamonds), room 5 (squares), and room 6 (circles).

phism profiles were also used to infer the genetic correlation of parental inocula to the descendant isolates cultured during each infection trial. For this analysis relatedness was determined by comparing the correlation (determined from the Pearson product moment correlation matrix) of the AFLP profile generated from a single *P. multocida* isolate from each bird to the AFLP profiles of both parental inocula used within the infection trial isolation room. The inoculum with the most closely correlated AFLP profile to a descendant isolate was identified as the parent. For room 4, five isolates were likely descendants of inoculum 7, and 15 isolates were likely descendants of inoculum 8; for room 5, four isolates were likely descendants of inoculum 3, and seven isolates were likely descendants of inoculum 4; for room 6, no likely descendants of inoculum 1 were isolated, but 19 isolates were likely descendants of inoculum 2. For each infection trial one isolate was equally correlated to both inocula used, and these isolates were excluded from this analysis. A plot (not shown) of the correlation of each bacterial isolate to the most closely related inoculum within each infection trial yielded a line described by the following equation (correlation to parent $= -0.005 \times$ week of isolation $+ 0.89$).

This analysis indicated that over time the correlation of parental inocula to descendant isolates decreased (slope = −0.005; P=0.046). Serial *P. multocida* isolations from individual birds were insufficient to evaluate genetic changes in the bacteria at greater resolution than the population level.

Analysis of *P. multocida* serotype 1 isolates from wild birds and environmental samples

One-hundred-twenty serotype 1 isolates from a variety of wild bird species (72 total; Table 1) and environmental samples (48 total; Table 2) were examined using the AFLP technique. Isolate collection locations included California (89 isolates), Hawaii (one isolate), Iowa (two isolates), Missouri (one isolate), Nebraska (14 isolates), Utah (12 isolates), and Wisconsin (one isolate). The predominant bird species from which *P. multocida* was cultured included Eared Grebes (*Podiceps nigricollis*; 20 isolates), Snow Geese (*Chen caerulescens*; 13 isolates), and Ruddy Ducks (*Oxyura jamaicensis*; 12 isolates). To facilitate the comparison of bacterial isolates by collection date, the following categories were established: January 1981 to March 1997 (20 isolates), November 1997 to April 1998 (62 isolates), October 1998 to March 1999 (20 isolates), and January 2000 to October 2004 (18 isolates). These categories were determined based on the availability of isolates surrounding significant avian cholera mortality events from 1997 to 1999. The largest group of bird and environmental samples was collected in central California (61 isolates) during the winter of 1997/1998 (54 of the 61 isolates).

As indicated on the dendrogram (Fig. 3a), the isolates segregated into four major clusters (labeled 1–4) consisting of 12 branches (labeled A–L). Cluster 1 (branches A and B) contained six isolates (three bird and three environmental) from California (three isolates) and Nebraska (three isolates; Fig. 3a). Cluster 2 (branches C–F) contained 47 isolates (38 bird and nine environmental) from California (27 isolates), Iowa (one isolate), Missouri (one isolate), Nebraska (nine isolates), Utah (eight isolates), and Wisconsin (one isolate; Figs. 3a and 3b). Cluster 3 (branches G–K) contained 66 serotype 1 isolates (31 bird and 35 environmental) from California (59 isolates), Iowa (one isolate), Nebraska (two isolates), and Utah (four isolates; Figs. 3a and 3c). A serotype 3 type strain was located on divergent branch K within cluster 3. A single serotype 1 isolate cultured from a Hawaiian Coot (*Fulica americana alai*) was the only member of cluster 4 (branch L).

Amplified fragment length polymorphism results were analyzed to determine whether dendrogram branching patterns were related to collection locations or collection dates (Fig. 3a). Collection location categories analyzed included central California, the Salton Sea in southern California, the Midwest (Iowa, Missouri, Nebraska, and Wisconsin, collectively), and the Great Salt Lake in Utah. Central California isolates (51/61) allocated disproportionately to cluster 3 (Figs. 3a and 3c; P<0.001). Salton Sea (19/25) and Midwestern isolates (12/18) allocated disproportionately to cluster 2 (Figs. 3a and 3b; P<0.001). The majority of Great Salt Lake isolates (8/12) allocated to cluster 2, but this result was not determined to be significant (P=0.22). Analysis of isolates based on collection dates indicated that isolates collected between January 1981 and March 1997 (14/20) allocated disproportionately to cluster 2 (Figs. 3a and 3b; P<0.001). Isolates collected between November 1997 and April 1998 (50/62) allocated disproportionately to cluster 3 (Figs. 3a and 3c; P<0.001). Although the majority of isolates collected between October 1998 and March 1999 (12/20) and between January 2000 and October 2004 (11/18) allocated within cluster 2, these groupings were not determined to be significant (P=0.16 and 0.21, respectively).

Principal components analysis of the

TABLE 1. Summary of *Pasteurella multocida* serotype 1 isolates cultured from wild birds.

Branch[a]	Collection					Dendrogram ID no.[c]
	Date	State	County	Location[b]	Bird species	
A	6 Feb 1992	NE	Clay	RWB WMD	Snow Goose (*Chen caerulescens*)	—
B	13 Dec 1994	CA	Sutter	Sutter NWR	Snow Goose	—
B	24 Mar 1999	CA	Imperial	Salton Sea NWR	Ruddy Duck (*Oxyura jamaicensis*)	—
C	20 Jan 1981	IA	Fremont	Riverton Wildlife Area	Mallard (*Anas platyrhynchos*)	23
C	5 Dec 1984	NE	Not known	RWB WMD	Mallard	25
C	24 Feb 1989	CA	Imperial	Salton Sea NWR	Ring-billed Gull (*Larus delawarensis*)	22
C	3 Feb 1992	NE	Clay	RWB WMD	Snow Goose	39
C	22 Feb 1992	NE	Clay	RWB WMD	White-fronted Goose (*Anser albifrons*)	19
C	8 Nov 1994	CA	Imperial	Salton Sea NWR	Snow Goose	43
C	14 Nov 1994	UT	Davis	Great Salt Lake	Eared Grebe (*Podiceps nigricollis*)	8
C	14 Nov 1994	UT	Davis	Great Salt Lake	Eared Grebe	17
C	3 Jan 1995	CA	Sutter	Sutter NWR	Snow Goose	10
C	5 Mar 1998	NE	Clay	RWB WMD	Snow Goose	20
C	16 Oct 1998	UT	Davis	Great Salt Lake	Eared Grebe	7
C	18 Dec 1998	CA	Imperial	Salton Sea NWR	Ruddy Duck	45
C	4 Dec 2003	MO	Buchanan	Sugar Lake	Snow Goose	26
C	20 Feb 2004	CA	Riverside	Salton Sea NWR	Eared Grebe	21
C	19 Mar 2004	NE	Clay	RWB WMD	Snow Goose	9
C	30 Mar 2004	CA	Imperial	Salton Sea NWR	Ring-billed Gull	11
C	25 Oct 2004	UT	Tooele	Great Salt Lake	Eared Grebe	42
C	25 Oct 2004	UT	Tooele	Great Salt Lake	Eared Grebe	44
C	25 Oct 2004	UT	Tooele	Great Salt Lake	Eared Grebe	24
D	15 Mar 1989	NE	Kearney	RWB WMD, Harvard WPA	Snow Goose	30
D	6 Feb 1992	NE	Clay	RWB WMD	Snow Goose	47
D	10 Sept 1992	WI	Dodge	Not specified	Northern yellow-shafted Flicker (*Colaptes auratus*)	32
D	11 Jan 2000	CA	Riverside	Salton Sea NWR	Eared Grebe	46
D	7 Mar 2000	CA	Riverside	Salton Sea NWR	Eared Grebe	31
D	11 Jan 2000	CA	Riverside	Salton Sea NWR	Eared Grebe	33
E	8 Nov 1994	CA	Imperial	Salton Sea NWR	Snow Goose	49
E	3 Jan 1995	CA	Sutter	Sutter NWR	Snow Goose	48
E	16 Oct 1998	UT	Davis	Great Salt Lake	Eared Grebe	52
E	16 Oct 1998	UT	Davis	Great Salt Lake	California Gull (*Larus californicus*)	56
E	18 Dec 1998	CA	Imperial	Salton Sea NWR	Ruddy Duck	51

TABLE 1. Continued.

Branch[a]	Collection					Dendrogram ID no.[c]
	Date	State	County	Location[b]	Bird species	
E	18 Dec 1998	CA	Imperial	Salton Sea NWR	Ruddy Duck	54
E	18 Dec 1998	CA	Imperial	Salton Sea NWR	Ruddy Duck	55
E	11 Jan 1999	CA	Imperial	Salton Sea NWR	Ruddy Duck	57
E	1 Mar 1999	CA	Imperial	Salton Sea NWR	Ruddy Duck	50
E	22 Mar 1999	CA	Riverside	Salton Sea NWR	Ruddy Duck	58
E	24 Mar 1999	CA	Imperial	Salton Sea NWR	Ruddy Duck	53
E	24 Mar 1999	CA	Imperial	Salton Sea NWR	Ruddy Duck	59
F	7 Jan 2000	CA	Riverside	Salton Sea NWR	Eared Grebe	34
G	8 Jan 1998	CA	Glenn	Sacramento NWR	Northern Pintail (*Anas acuta*)	94
G	1 Mar 1999	CA	Imperial	Salton Sea NWR	Ring-billed Gull	93
G	7 Jan 2000	CA	Riverside	Salton Sea NWR	Eared Grebe	29
G	11 Jan 2000	CA	Riverside	Salton Sea NWR	Eared Grebe	28
G	25 Oct 2004	UT	Tooele	Great Salt Lake	Eared Grebe	91
H	2 Jan 1998	CA	Butte	Sacramento River NWR, Llano Seco	Northern Pintail	78
H	22 Jan 1998	CA	Sutter	Sutter NWR	White-faced Ibis (*Plegadis chihi*)	65
H	22 Jan 1998	CA	Sutter	Sutter NWR	Northern Shoveler (*Anas clypeata*)	66
H	22 Jan 1998	CA	Sutter	Sutter NWR	Black-crowned Night heron (*Nycticorax nycticorax*)	73
H	28 Jan 1998	CA	Merced	Merced MWR, Arena Plains Unit	American Widgeon (*Anas americana*)	76
H	2 Feb 1998	CA	Merced	Merced NWR, Mud Slough Unit 5	American Coot (*Fulica americana*)	74
H	16 Oct 1998	UT	Davis	Great Salt Lake	Eared Grebe	86
H	16 Oct 1998	UT	Davis	Great Salt Lake	Eared Grebe	95
H	16 Dec 1998	CA	Stanislaus	San Joaquin River NWR, Page Lake	Aleutian Canada Goose (*Branta canadensis leucopareia*)	75, 77, 79
H	33 Mar 1999	CA	Riverside	Salton Sea NWR	Ruddy Duck	85
H	14 Jan 2000	CA	Riverside	Salton Sea NWR	Eared Greb	96
H	3 Dec 2003	IA	Fremont	Riverton Wildlife Area	Snow Goose	97
H	17 Dec 2003	CA	Sutter	Butte Sink NWR	Mallard	90
H	25 Oct 2004	UT	Tooele	Great Salt Lake	Eared Grebe	89
I	16 Dec 1997	CA	Stanislaus	San Joaquin River NWR	Mallard	108
I	14 Jan 1998	CA	Merced	Los Banos WMA	American Coot	100

TABLE 1. Continued.

Branch[a]	Collection				Bird species	Dendrogram ID no.[c]
	Date	State	County	Location[b]		
I	14 Jan 1998	CA	Merced	Los Banos WMA	Eared Grebe	110
I	14 Jan 1998	CA	Merced	Los Banos WMA	American Coot	114
I	14 Jan 1998	CA	Merced	Los Banos WMA	American Coot	117
I	27 Jan 1998	CA	Merced	Merced MWR, Arena Plains Unit	Ross's Goose (*Chen rossii*)	107
J	28 Jan 1998	CA	Merced	Merced MWR, Arena Plains Unit	American Coot	109
K	10 Dec 1997	CA	Lassen	Eagle Lake	Bufflehead (*Bucephala albeola*)	35
K	10 Dec 1997	CA	Lassen	Eagle Lake	Ruddy Duck	38
L	29 Oct 1986	HI	Not applicable	Hawaiian Pacific Islands NWR	Hawaiian Coot (*Fulica americana alai*)	—

[a] Designations refer to dendrogram branches defined in Figure 3a.

[b] NWR = national wildlife refuge; RWB WMD = Rain Water Basin wetlands management district; WMA = wildlife management area; WPA = waterfowl production area.

[c] Numbers refer to isolate identifiers in Figures 3b and 3c.

wild bird and environmental isolate AFLP data using components 2 and 3 supported the allocation of isolates into the same branch-based clusters observed on the dendrogram (data not shown). When examined based on collection location, PCA supported the existence of a visually distinct cluster of isolates from central California (Fig. 4, open circles; $P<0.001$ for component 2). This same cluster, consisting primarily of isolates cultured from samples collected between November 1997 and April 1998, was also apparent by PCA when samples were identified based on collection date ($P<0.001$ for component 2; data not shown). Statistical comparison of the contributions of regional ($P<0.001$ for component 2) and temporal ($P=0.026$ for component 2) genetic characteristics of the isolates to the observed clusters indicated that both exerted significant contributions.

A more detailed analysis of the *P. multocida* isolates collected in central California established that the majority of these isolates (48/61) allocated to branches H (30 isolates) and I (18 isolates) of the dendrogram (Figs. 3a and 3c). Examination of the central California isolates based on specific collection locations (16 sites distributed among the Sacramento and San Joaquin Valley Refuge Complexes) indicated that although branch H isolates were cultured from samples collected at both the Sacramento and San Joaquin Valley Refuge Complex sites, branch I isolates were only cultured from samples collected at San Joaquin Valley Refuge Complex sites (Fig. 5). Furthermore, branch I isolates were exclusively cultured during the 1997/1998 season (Tables 1 and 2). In contrast, consistent with their broader local distribution within central California, branch H isolates were also identified at collection sites outside of central California, including the Salton Sea, the Great Salt Lake, and the Midwestern United States (Tables 1 and 2 and

TABLE 2. Summary of *P. multocida* serotype 1 isolates cultured from environmental samples.

Branch[a]	Collection						Dendrogram ID no.[c]
	Date	State	County	Location[b]	Latitude	Longitude	
A	17 Mar 1996	NE	Clay	RWB WMD, Sandpiper	40°38′N	97°58′W	—
A	8 Jan 1998	CA	Colusa	Colusa NWR, T15A	39°09′N	122°03′W	—
A	3 Apr 1998	NE	Phelps	RWB WMD, Johnson	40°34′N	99°20′W	—
C	7 Jan 1998	CA	Merced	Los Banos WMA, Gadwall Unit 2	37°03′N	120°47′W	12
C	7 Jan 1998	CA	Merced	Los Banos WMA, Gadwall Unit 2	37°03′N	120°47′W	14
C	8 Jan 1998	CA	Colusa	Colusa NWR, T15A	39°09′N	122°03′W	18
C	14 Jan 1998	CA	Imperial	Salton Sea NWR	33°11′N	115°37′W	41
C	15 Jan 1998	CA	Colusa	Delevan NWR, T6	39°20′N	122°07′W	16
C	15 Jan 1998	CA	Colusa	Delevan NWR, T6	39°20′N	122°07′W	15
C	2 Feb 1998	CA	Merced	Merced NWR, Mud Slough Unit 5	37°04′N	120°46′W	13
C	2 Apr 1998	NE	Phelps	RWB WMD, Funk	40°31′N	99°13′W	87
E	3 Apr 1998	NE	Phelps	RWB WMD, Johnson	40°34′N	99°20′W	40
G	23 Jan 1998	CA	Colusa	Delevan NWR, T16	39°19′N	122°07′W	92
H	17 Mar 1996	NE	Fillmore	RWB WMD, Mallard Haven	40°27′N	97°45′W	99
H	20 Mar 1997	NE	Clay	RWB WMD, Sandpiper	40°38′N	97°58′W	88
H	25 Nov 1997	CA	Colusa	Colusa NWR, T15A	39°09′N	122°03′W	67
H	7 Jan 1998	CA	Merced	Los Banos WMA, Gadwall Unit 2	37°03′N	120°47′W	70
H	7 Jan 1998	CA	Merced	Los Banos WMA, Gadwall Unit 2	37°03′N	120°47′W	71
H	7 Jan 1998	CA	Merced	Los Banos WMA, Gadwall Unit 3	37°02′N	120°46′W	82
H	8 Jan 1998	CA	Colusa	Colusa NWR, T13A	39°09′N	122°03′W	60
H	15 Jan 1998	CA	Stanislaus	San Joaquin River NWR, East Page Lake	37°40′N	121°12′W	62
H	15 Jan 1998	CA	Stanislaus	San Joaquin River NWR, East Page Lake	37°40′N	121°12′W	63
H	15 Jan 1998	CA	Stanislaus	San Joaquin River NWR, East Page Lake	37°40′N	121°12′W	64
H	15 Jan 1998	CA	Stanislaus	San Joaquin River NWR, East Page Lake	37°40′N	121°12′W	68
H	15 Jan 1998	CA	Stanislaus	San Joaquin River NWR, East Page Lake	37°40′N	121°12′W	69
H	15 Jan 1998	CA	Stanislaus	San Joaquin River NWR, East Page Lake	37°40′N	121°12′W	72
H	15 Jan 1998	CA	Colusa	Delevan NWR, T6	39°20′N	122°07′W	81
H	15 Jan 1998	CA	Colusa	Delevan NWR, T6	39°20′N	122°07′W	83
H	15 Jan 1998	CA	Stanislaus	San Joaquin River NWR, East Page Lake	37°40′N	121°12′W	84
H	15 Jan 1998	CA	Stanislaus	San Joaquin River NWR, Page Lake	37°40′N	121°12′W	104
H	23 Jan 1998	CA	Colusa	Delevan NWR, T16	39°19′N	122°07′W	98
H	30 Jan 1998	CA	Colusa	Delevan NWR, T15A	39°19′N	122°05′W	61
H	3 Feb 1998	CA	Merced	Los Banos WMA, Gadwall Unit 1	37°03′N	120°47′W	80
H	3 Feb 1998	CA	Merced	Los Banos WMA, Gadwall Unit 1	37°03′N	120°47′W	106
H	4 Feb 1998	CA	Merced	Merced NWR, Arena Plains Unit	37°17′N	120°44′W	105
I	7 Jan 1998	CA	Merced	Los Banos WMA, Gadwall Unit 2	37°03′N	120°47′W	112
I	7 Jan 1998	CA	Merced	Los Banos WMA, Gadwall Unit 2	37°03′N	120°47′W	115
I	7 Jan 1998	CA	Merced	Los Banos WMA, Gadwall Unit 2	37°03′N	120°47′W	116
I	15 Jan 1998	CA	Stanislaus	San Joaquin River NWR, Page Lake	37°40′N	121°12′W	102
I	15 Jan 1998	CA	Stanislaus	San Joaquin River NWR, Page Lake	37°40′N	121°12′W	111
I	15 Jan 1998	CA	Stanislaus	San Joaquin River NWR, East Page Lake	37°40′N	121°12′W	113
I	2 Feb 1998	CA	Merced	Merced NWR, Mud Slough Unit 5	37°04′N	120°46′W	101
I	2 Feb 1998	CA	Merced	Merced NWR, Mud Slough Unit 5	37°04′N	120°46′W	120
I	3 Feb 1998	CA	Merced	Los Banos WMA, Gadwall Unit 1	37°03′N	120°47′W	118
I	3 Feb 1998	CA	Merced	Los Banos WMA, Gadwall Unit 1	37°03′N	120°47′W	121
I	4 Feb 1998	CA	Merced	Merced NWR, Arena Plains Unit	37°17′N	120°44′W	103

TABLE 2. Continued.

Branch[a]	Collection						Dendrogram ID no.[c]
	Date	State	County	Location[b]	Latitude	Longitude	
I	4 Feb 1998	CA	Merced	Merced NWR, Arena Plains Unit	37°17′N	120°44′W	119
K	24 Nov 1997	CA	Siskiyou	Tule Lake NWR	41°55′N	121°39′W	36

[a] Designations refer to dendrogram branches defined in Figure 3a.

[b] NWR = national wildlife refuge; RWB WMD = Rain Water Basin wetlands management district; WMA = wildlife management area.

[c] Numbers refer to isolate identifiers in Figures 3b and 3c.

Fig. 3c). Collection dates for all branch H isolates ranged from 1996 to 2004.

DISCUSSION

This work demonstrated that *P. multocida* serotype 1 isolates are genetically heterogeneous and that AFLP analysis is a useful technique for distinguishing them. Amplified fragment length polymorphism analysis of serotype 1 isolates cultured during a 3-mo laboratory infection trial of Mallards (Samuel et al., 2003a) indicated that the bacteria underwent detectable genetic changes during the trial. Temporal genetic change among the isolates was demonstrated by graphic analysis (data not shown), which indicated a decrease in the correlation of each isolate cultured during the infection trial to its most closely related parent. The *y*-intercept (0.89) indicated that the isolates at the beginning of the trial were similar to the parental inocula within the resolution of the technique, 0.92 (SD=0.05). By the end of the trial, however, descendent isolates were 0.83 correlated to the parental inocula. The decrease in the correlation of descendant to parental AFLP patterns over the course of the infection trial is consistent with the finding that as a result of single nucleotide changes and genetic recombination, bacterial genomes are in a continuous state of flux (Levin and Bergstrom, 2000). Analyses to determine whether the observed genetic changes among the *P. multocida* isolates examined affected functional properties (e.g., virulence) of the bacteria were beyond the scope of this project. However, genome plasticity may enhance a bacterium's ability to adapt to diverse natural environments (Earl et al., 2007), and genetic change leading to fluctuations in *P. multocida* virulence levels provides a possible mechanism to explain observed temporal variations in North American *P. multocida* restriction enzyme fingerprints (Wilson et al., 1995b; Samuel et al., 2003b) and avian cholera mortality patterns (Lehr et al., 2005; Blanchong et al., 2006). Unlike previously employed techniques for differentiating *P. multocida* serotype 1 isolates, including restriction enzyme (Wilson et al., 1995a, b; Samuel et al., 2003b) and serotyping (Rhoades and Rimler, 1991) analyses, AFLP analysis does not currently allow the ready assignment of isolates to predefined groupings. It does, however, allow comparison of the relationships among collections of *P. multocida* isolates, and provides a context for genetic changes that accumulate over the short term (during a disease outbreak) in contrast to geographic and temporal genetic differences among *P. multocida* from one outbreak season to the next.

A pattern revealed by both dendrogram (Fig. 1) and principal components (Fig. 2) analyses of the infection trial AFLP data was the presence of a cluster of *P. multocida* isolates cultured from live birds distinct from those cultured from dead birds. We hypothesize that this pattern resulted from genetic drift leading to divergence of the *P. multocida* isolates cultured from live birds later in the trial compared to isolates cultured from dead

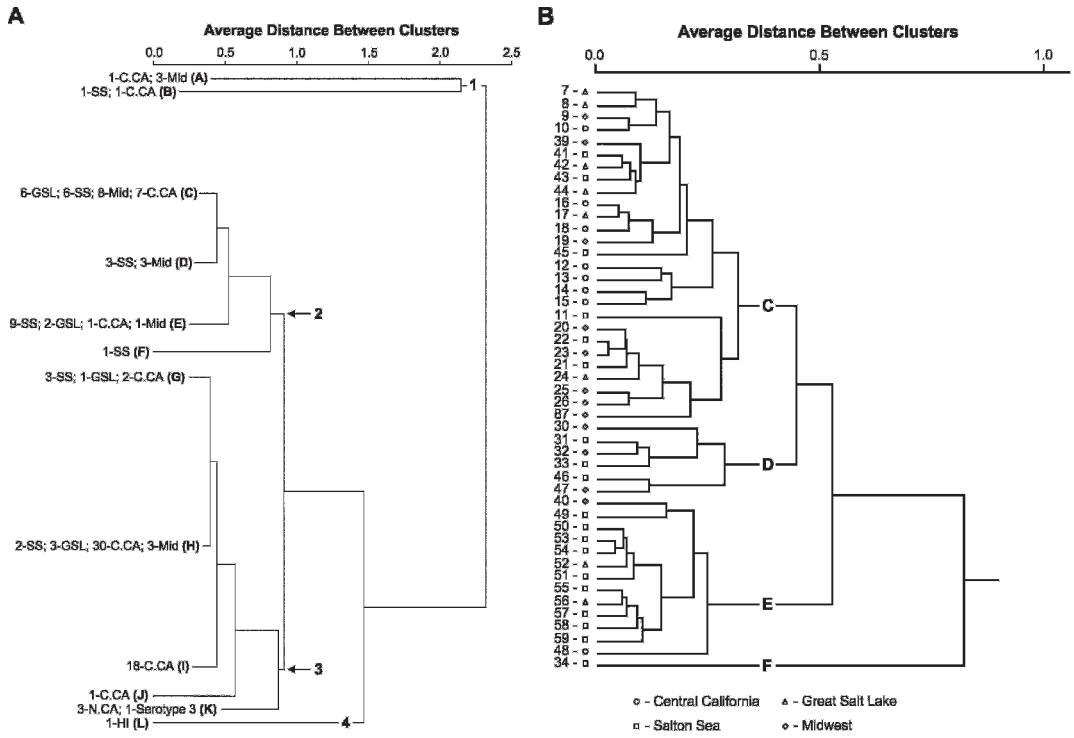

FIGURE 3. Dendrogram analyses of *Pasteurella multocida* serotype 1 isolates cultured from wild birds and environmental samples. (a) Dendrogram in which each branch was truncated at its root representative of all isolates included in this study. Four major clusters (1–4) and 12 branches (A–L) are labeled. Location categories (C.CA = central California, GSL = Great Salt Lake, HI = Hawaii, Mid = Midwestern states, N.CA = northern California, and SS = Salton Sea) for each group of isolates are labeled. The number of isolates from each location category precedes the location designation. (b) and (c) Details of all isolates within clusters 2 and 3, respectively. Symbols indicate the source of each isolate, and dendrogram identification numbers are defined in Tables 1 and 2.

birds during the first 2 wk. Additionally, segregation of all trial 6 isolates along with inoculum 2 from the other inocula and isolates was consistent with the determination that 19 of 20 trial 6 isolates likely descended from inoculum 2. Thus, the ability to distinguish identified descendants of inoculum 2 throughout the infection trial demonstrated the utility of AFLP analysis to track bacteria with distinct genetic characteristics.

The capacity of AFLP analysis to distinguish *P. multocida* serotype 1 isolates during epidemiologic investigations of avian cholera outbreaks was demonstrated by using this technique to characterize 120 isolates cultured from wild birds (Table 1) and environmental samples (Table 2). The most distinct epidemiological cluster of *P. multocida* isolates examined originated from samples collected in central California (Figs. 4 and 5), and the majority of isolates from this region, regardless of whether they were collected from birds or from environmental samples, allocated to branches H and I of the dendrogram (Figs. 3a and 3c). The inability to distinguish *P. multocida* isolates originating from birds or the environment by AFLP analysis is consistent with the transmission of bacterial isolates between birds and their environment within an outbreak area (Samuel et al., 2004). Within central California, branch H isolates exhibited a wider distribution pattern than branch I isolates (Fig. 5). The wider spatial and

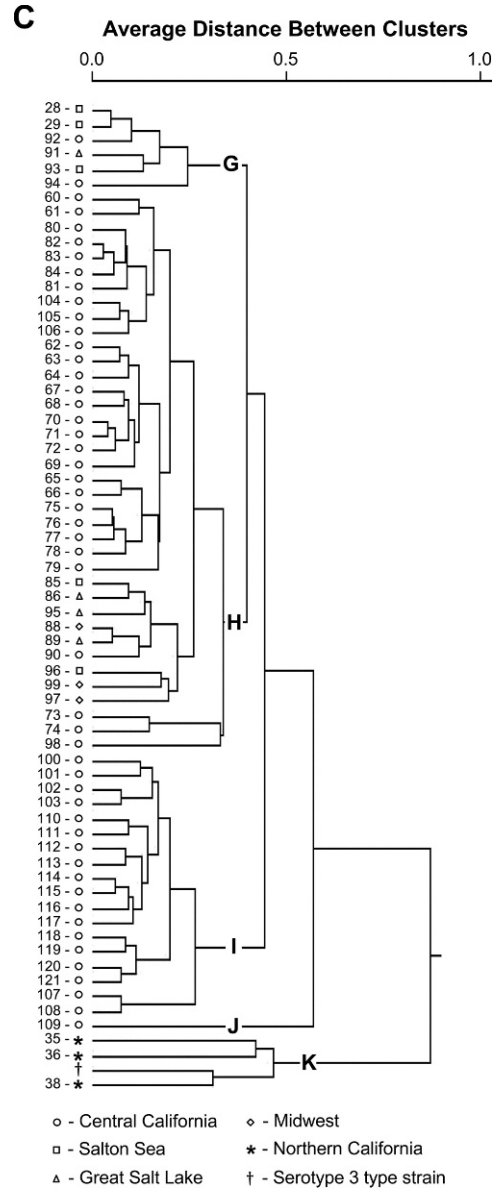

FIGURE 3. Continued.

temporal distribution of branch H isolates in central California was also consistent with a more diverse national distribution pattern for branch H isolates. Samuel et al. (2005) demonstrated that healthy wild waterfowl have the potential to transmit *P. multocida* to other birds and locations. Accordingly, the wider spatial and temporal distribution of branch H isolates, both locally and nationally, may have resulted from the movements of carrier birds. Identification of a group of bacterial isolates primarily associated with a distinct geographic region and a single season indicates that AFLP analysis provides a potentially useful tool to further elucidate the transmission of *P. multocida* between bird species, across the landscape, and over time.

Both dendrogram (Figs. 3a and 3b) and principal components (Fig. 4) analyses revealed that Great Salt Lake and Salton Sea *P. multocida* isolates clustered together. The majority of Great Salt Lake isolates analyzed for this study were cultured from Eared Grebes, while Salton Sea isolates were primarily cultured from Eared Grebes and Ruddy Ducks (Table 1). Large numbers of Eared Grebes move between the Great Salt Lake and the Salton Sea each year (Jehl, 1993; Jehl et al., 1999). Thus, interactions between Great Salt Lake and Salton Sea Eared Grebe populations may facilitate the transmission of disease agents between these two sites resulting in genetic homogenization, as measured by AFLP analysis, of the *P. multocida* isolates cultured from waterfowl of this geographic region. Application of AFLP analysis to *P. multocida* isolates from additional sites along waterfowl migratory routes may provide information on the transmission of avian cholera useful to wildlife resource managers for predicting where future disease outbreaks might occur.

The previously perceived homogeneity of *P. multocida* serotype 1 has been a limiting factor in understanding the epidemiology of avian cholera infections in wild bird populations (Samuel et al., 2007). Using AFLP analysis, we have demonstrated that the *P. multocida* genome is subject to genetic drift and that there is sufficient diversity among *P. multocida* serotype 1 isolates to distinguish regional and temporal epidemiologic patterns. The utility of this technique for tracing avian cholera outbreaks among wild birds was most clearly exemplified

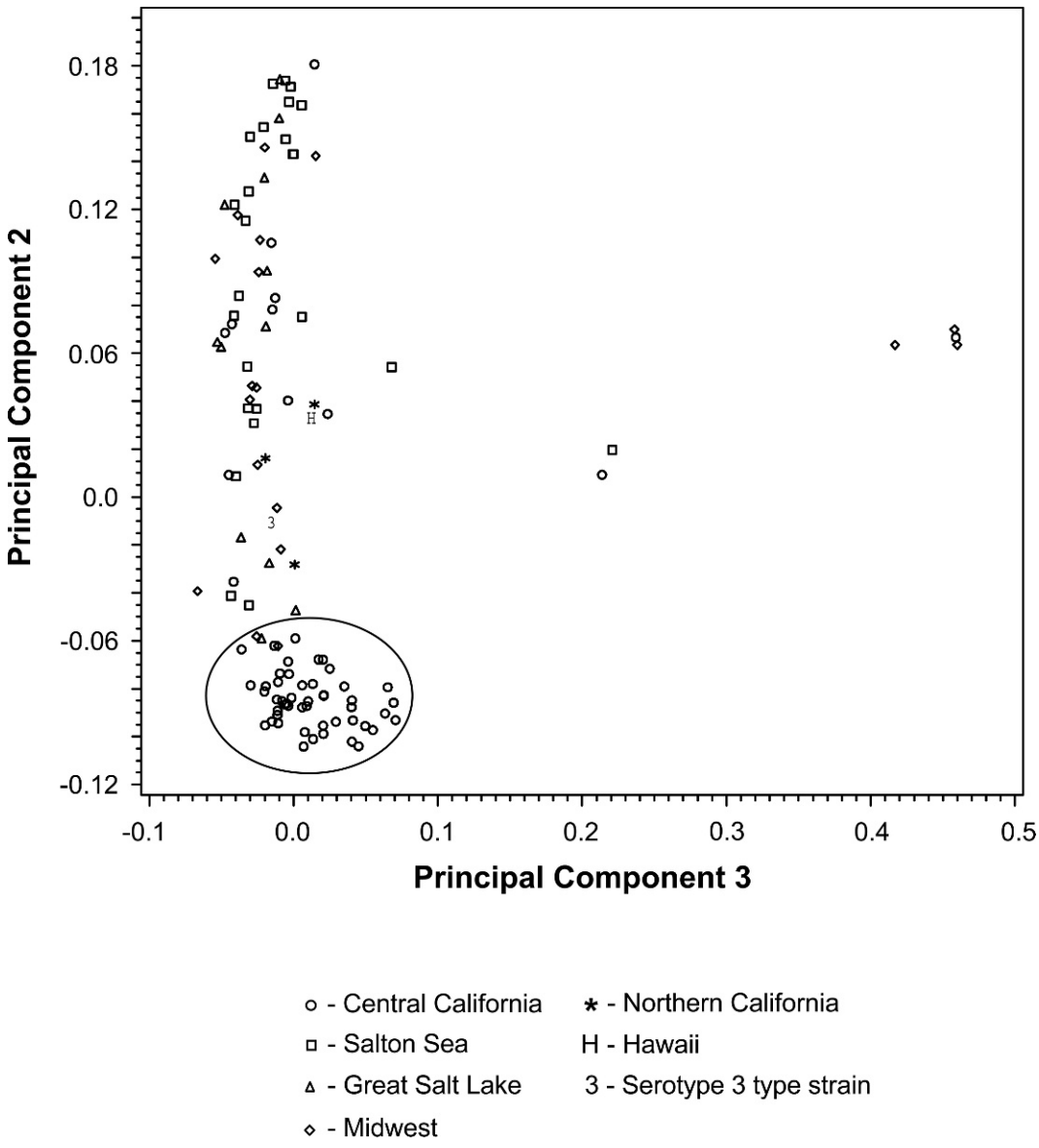

FIGURE 4. Principal components analysis of *Pasteurella multocida* serotype 1 isolates cultured from wild birds and environmental samples. Each isolate is designated according to its collection location; the cluster encompassing 49 of the 61 central California *P. multocida* isolates is circled.

by the group of genetically distinct *P. multocida* isolates originating from central California bird and environmental samples. Further application of AFLP technology to the study of avian cholera epidemiology has the potential ability to link bacterial isolates from the environment to those from infected hosts, to determine spatiotemporal patterns of isolate types, to identify specific affiliations between *P. multocida* genotypes and bird species, to correlate virulence with bacterial genotypes, and to understand the role of specific bird species in transmitting or spreading this disease. Accomplishment of these goals will provide wildlife resource managers with the means to predict local bird population impacts during ongoing

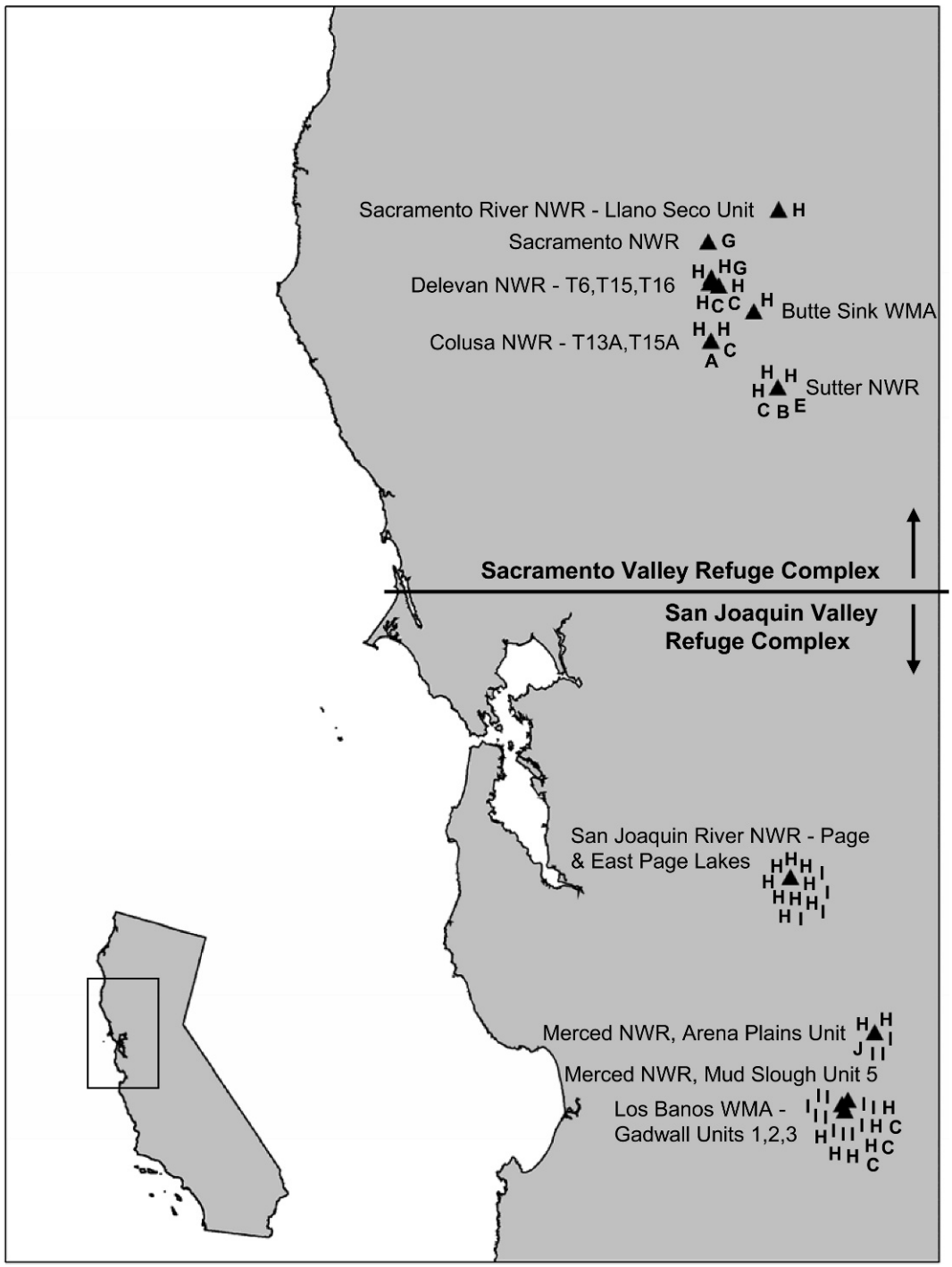

FIGURE 5. Distribution of *Pasteurella multocida* serotype 1 isolates cultured from wild birds and environmental samples collected in central California. Isolates are represented on the map according to their collection location using dendrogram branch designations from Figure 3a. Collection sites are indicated with triangles.

avian cholera die-offs, to identify bird species that may be at greatest risk for infection during a mortality event, and to correlate bird movement patterns with the potential for disease spread. Amplified fragment length polymorphism analysis promises to be a valuable tool to both enhance understanding of the epidemiology of avian cholera infection and more effectively manage this disease among wild birds.

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