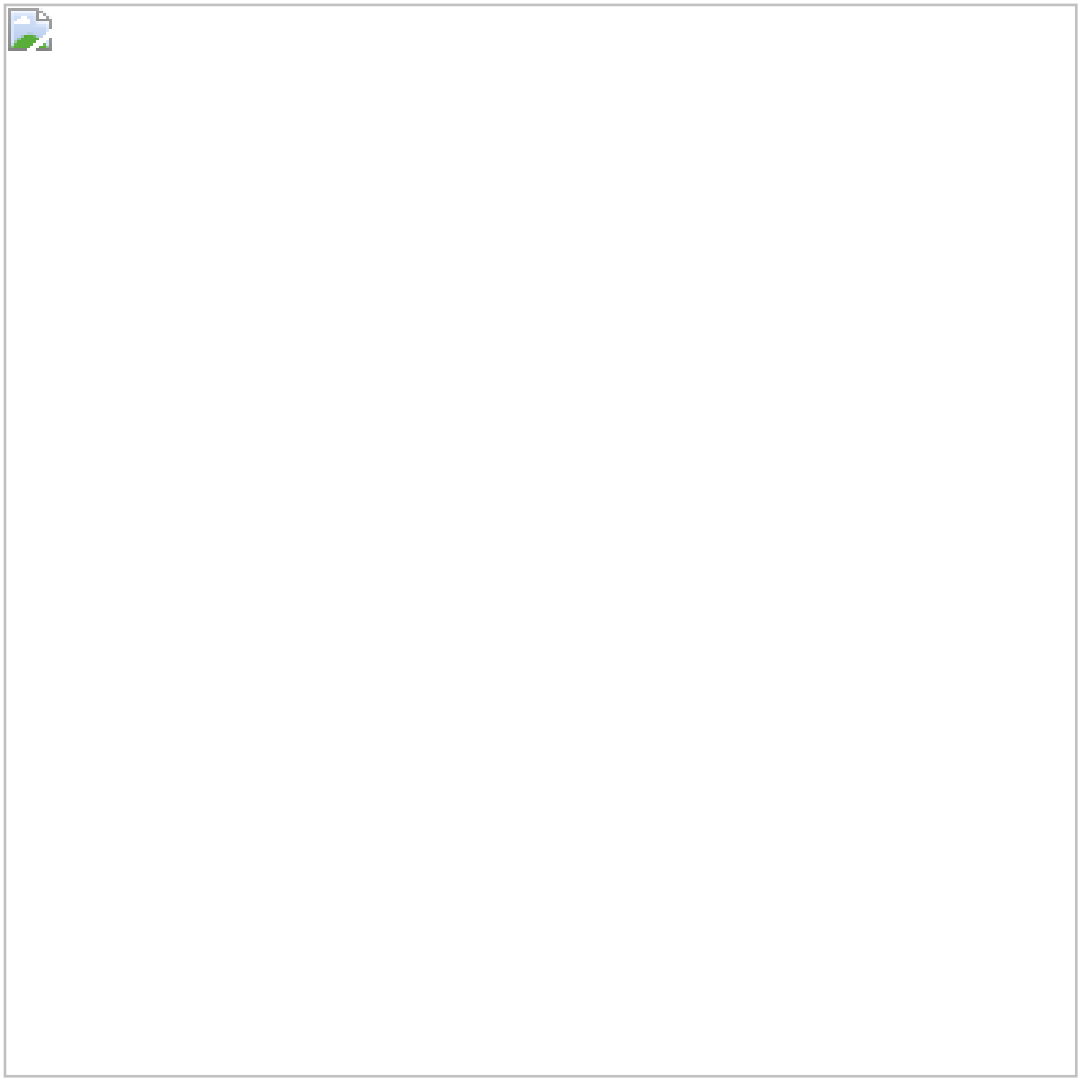

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CHARACTERIZATION OF CANARYPOX-LIKE VIRUSES INFECTING ENDEMIC BIRDS IN THE GALÁPAGOS ISLANDS

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ABSTRACT: The presence of avian pox in endemic birds in the Galápagos Islands has led to concern that the health of these birds may be threatened by avipoxvirus introduction by domestic birds. We describe here a simple polymerase chain reaction–based method for identification and discrimination of avipoxvirus strains similar to the fowlpox or canarypox viruses. This method, in conjunction with DNA sequencing of two polymerase chain reaction–amplified loci totaling about 800 bp, was used to identify two avipoxvirus strains, Gal1 and Gal2, in pox lesions from yellow warblers (*Dendroica petechia*), finches (*Geospiza* spp.), and Galápagos mockingbirds (*Nesomimus parvulus*) from the inhabited islands of Santa Cruz and Isabela. Both strains were found in all three passerine taxa, and sequences from both strains were less than 5% different from each other and from canarypox virus. In contrast, chickens in Galápagos were infected with a virus that appears to be identical in sequence to the characterized fowlpox virus and about 30% different from the canarypox/Galápagos group viruses in the regions sequenced. These results indicate the presence of canarypox-like viruses in endemic passerine birds that are distinct from the fowlpox virus infecting chickens on Galápagos. Alignment of the sequence of a 5.9-kb region of the genome revealed that sequence identities among Gal1, Gal2, and canarypox viruses were clustered in discrete regions. This indicates that recombination between poxvirus strains in combination with mutation led to the canarypox-like viruses that are now prevalent in the Galápagos.

Key words: Avian pox, avipoxvirus, canary pox, fowlpox, Galápagos.

INTRODUCTION

The Galápagos Islands are volcanic in origin (Christie et al., 1992; White et al., 1993) and located on the equator almost 1,000 km west of mainland Ecuador in South America. Their isolation and relative desolation delayed permanent colonization by humans, and biodiversity remains mostly intact, with only about 5% of species having been lost (Gibbs et al., 1999); this includes none of the 28 breeding land bird species, 26 of which are endemic. Although 90% of the archipelago was set aside as a national park in 1959, the resident human population, along with tourism, has grown rapidly, and exotics are continually being introduced despite increasing efforts to exclude them. Because pathogens can have especially severe effects when introduced to previously isolated avian populations, as well documented in Hawaii (Wikelski et al., 2004), there is increasing concern about the introduction of avian diseases that could result in extinctions of Galápagos avifauna. The appearance of avian pox–like lesions in both domestic chickens and endemic birds on Galápagos has heightened these concerns.

Avian pox is a mild to severe disease of birds that has been reported worldwide in approximately 60 avian species representing 20 families. Avian pox is caused by DNA viruses of the family *Poxviridae,* genus *Avipoxvirus,* and transmission can occur through introduction into a break in the skin or, more commonly, when vectored by a biting insect. Disease is most commonly characterized by cutaneous proliferative lesions that harden to thick scabs, but a diptheritic or wet form with mucosal lesions within the digestive and upper-respiratory tracts can occur (Ger-

lach, 1999). The cutaneous form is most commonly observed in passerine birds (Gerlach, 1999). In Galápagos, the order Passeriformes is represented by eight families with 28 species (Castro and Phillips, 1996). Several of these species are severely threatened including the mangrove finch (*Cactospiza heliobates*; total population approximately 100 individuals), the Floreana mockingbird (*Nesomimus trifasciatus*; approximately 200 individuals), the Española mockingbird (*Nesomimus macdonaldi*; approximately 2,500 individuals), the medium tree finch (*Camarhynchus pauper*), and the large tree finch (*Camarhynchus psittacula*). The diptheritic form of the disease is observed most frequently in Psittaciformes, Phasianiformes, and several Columbiformes (Gerlach, 1999). Columbiformes present on Galápagos include a single endemic species, the Galápagos dove (*Zenaida galapagoensis*), and the introduced rock pigeon (*Columba livia*).

Thirteen strains of avipoxvirus have been identified worldwide, and strains vary in virulence and host specificity. Avipoxviruses from endemic forest birds in Hawaii (Apapane [*Himatione sanguinea*] and Hawaiian crow [*Corvus hawaiiensis*]) include two strains that differ significantly from fowlpox virus by restriction fragment length polymorphism genetic analysis (Tripathy et al., 2000); their pathogenicity was mild in chickens. Protective immunity also is strain related, and in vaccine trials, birds are often unprotected if challenged with different strains (Winterfield and Reed, 1985; Sarma and Sharma, 1988; Saini et al., 1990a, b). This indicates that significant antigenic differentiation may exist among strains, and in part, this may result from the rapid evolution of avipoxviruses by recombination between strains. Replication in avipoxviruses and other viruses in *Poxviridae* involve genomic intermediates comprising many tandem repeats of the entire genome (Moyer and Graves, 1981). Recombination, which occurs at extraordinarily high frequencies in these viruses, is an essential part of replication involving a single strain of virus (Ball, 1987); however, recombination between coinfecting viruses may also contribute to the wide diversity observed between avipoxviruses.

The dynamics of multihost pathogens in natural populations is important to understanding general patterns of rapid evolution of viruses and their effect on natural populations (Woolhouse et al., 2001; Cleaveland et al., 2002). In Galápagos, pox-like symptoms have been described in several species of endemic birds, including Galápagos mockingbirds (*N. parvulus parvulus*), Galápagos doves, yellow warblers (*D. petechia*), and some Galápagos finches (*Geospiza* spp.) (Jimenez, 2003). Most data on the effects of avian pox are from the mockingbirds.

During the 1982–83 El Niño events, 56% of mockingbirds displaying pox-like lesions died on Genovesa, compared with 39% of asymptomatic individuals (Curry and Grant, 1989). In that study, significantly more adults were affected than juveniles, partly because the epidemic peaked before peak hatching. Prevalence in Galápagos mockingbirds was higher in nestlings and juveniles than in adults on the island of Santa Cruz; a higher resighting rate for young birds without symptoms than those with lesions indicated higher mortality for infected birds (Vargas, 1987). Pox-like lesions were also observed among mockingbirds on Champion (an islet off Floreana) during the 1982–83 El Niño (Grant et al., 2000).

The first objective of this study was to develop a simple, specific diagnostic test for avian pox that could be adapted for use in the Galápagos, where traditional virus propagation techniques are not economically feasible. The second objective was to characterize the avipoxviruses that are infecting native birds. The availability of large published regions of sequence for the fowlpox and canarypox viruses provides the foundation for development of rapid polymerase chain reaction–based methods for detection of these viruses.

METHODS

Field methods

Between May and July of 2002 and 2003, wild birds were captured with mistnets near the Charles Darwin Research Station on the island of Santa Cruz, Galapagos, Ecuador. In January 2003 and July 2003, birds were captured on the island of Isabela, Galapagos Ecuador. Samples of cutaneous lesions were removed using sterile scalpels; these were transferred to a plastic vial and frozen in liquid nitrogen for transport. In some instances, samples were suspended in 95% ethanol and frozen. Any bleeding related to sample collection was stopped by applying mild pressure with sterile cotton.

DNA extraction

Samples were frozen in liquid nitrogen, pulverized to powder, and incubated at 65 C for at least 6 hr in 250 μl Longmire's lysis buffer (0.1 M Tris-HCl, pH 8.0, 0.1 M EDTA, 10 mM NaCl, 0.5% SDS) with Proteinase-K (final concentration, 1.0 mg/ml^{-1}). Samples were extracted with phenol/$CHCl_3$/isoamyl alcohol (25:24:1), and total DNA was precipitated with ethanol and resuspended in 100 μl sterile TE (10 mM Tris-HCl, pH 8.0; 10 mM EDTA).

Primer design

Only one sequence was available for canarypox virus at the time this project began (6181 nucleotides; GenBank D86731), and this was aligned with the homologous region from fowlpox virus (complete genome; AF198100) using ClustalW (Thompson et al., 1994). Overall, the sequence identity between the two avipoxvirus species in this region approximates 70%. The alignment was visually inspected for regions of divergence, particularly insertions or deletions (indels) that would allow rapid differentiation of PCR products on the basis of size. The intergenic region between the canarypox virus genes CA.X and TK (thymidine kinase), designated CAX, was chosen because it provides an indel of 52 bp in a region of 426 bp. Primers were based on highly conserved sequences within the coding regions of CA.X and TK and were designed to amplify DNA from both fowlpox and canarypox viruses (CAX′F AGATATAGTAGAATTTAGTG; CAX′R TTCTGCAAGATTTAATATC). The second locus, designated CA3-2, is a region spanning the CA.2 and the CA.3 genes of canarypox viruses. A number of indels in this region led to a predicted difference of 18 nucleotides between PCR products from the fowlpox and canarypox viruses. Highly conserved regions within CA.2 and CA.3 provided the sequence for primers that amplified DNA from both viruses (CA3-2F CTAATAGATACTAACGGAGAAG; CA3-2R TTAAATAAAGAAATGTAAAGAC).

PCR amplification and sequencing

PCR amplification with primer set CA3-2 or CAX was performed in 50-μl volumes of 67 mM Tris-HCl (pH 8.8), 16 mM $(NH_4)_2SO_4$, 0.01% Tween0, 1.5 mM $MgCl_2$, 2.0 mM each dNTP, 0.01 mg bovine serum albumen, 0.6 μM for each primer, 1 U *Taq* polymerase (Bioline, Randolph, Massachusetts, USA), and 2 μl extracted DNA (concentration unknown). DNA isolated from fowlpox virus from a chicken in the United States (kindly provided by D.N. Tripathy) was used as a positive control. A touchdown PCR program was used, beginning with an annealing temperature of 50 C and decreasing 0.5 C every cycle for 14 cycles to a final annealing temperature of 43 C for an additional 25 cycles. Denaturation was at 94 C, and extension was 72 C, with a 45-sec hold at each temperature in the cycle. Amplicon size was determined after electrophoresis in a 1.5% agarose gel by comparison with markers of known size. Amplification with the other primers (see previous section) was performed as described for CA3-2, except that the initial annealing temperature varied with the T_m of each primer pair. For sequencing the 6-kb region of DNA from lesions of samples 502F (designated Gal1) and 100W (designated Gal2) (Table 1), 20 sets of primers were designed that produced 20 amplicons of 350–400 bp, with approximately 50 bp of overlap for adjacent amplicons, covering the 6-kb region. Amplicons were sequenced in both directions with the same primers used for amplification with the ABI Big Dye protocol on an ABI 377 sequencer. Sequencing of several amplicons of CA3-2 revealed the presence of *Spe*I or *Age*I restriction sites in amplicons from some strains, but not from others; therefore, amplicons of CA3-2 were digested with *Spe*I or *Age*I in the buffer supplied with the enzyme. The accession number for the sequenced region of Gal1 is AY631870, and for Gal2 it is AY631871

Phylogenetic analysis

Using ClustalW (Thompson et al., 1994), the sequenced regions of Gal1 and Gal2 were aligned with each other and with the sequence of fowlpox virus (Afonso et al., 2000; GenBank AF19810), and sequences from two canarypox viruses described in Tulman et al. (2004; ATCC VR-111; GenBank AY318871) and Amano et al. (1999; GenBank D86731). The sequence of the culture-adapted fowlpox virus, FP9 (Laidlaw and Skinner, 2004; GenBank AJ581527), was

TABLE 1. Avipoxviruses from birds in the Galápagos Islands.

Strain	Species	Location	Pox type
300C	Chicken (*Gallus gallus*)	Santa Cruz	Fowlpox
301C	Chicken (*Gallus gallus*)	Santa Cruz	Fowlpox
302C	Chicken (*Gallus gallus*)	Santa Cruz	Fowlpox
303C	Chicken (*Gallus gallus*)	Santa Cruz	Fowlpox
304C	Chicken (*Gallus gallus*)	Santa Cruz	Fowlpox
305C	Chicken (*Gallus gallus*)	Santa Cruz	Fowlpox
306C	Chicken (*Gallus gallus*)	Santa Cruz	Fowlpox
307C	Chicken (*Gallus gallus*)	Santa Cruz	Fowlpox
308C	Chicken (*Gallus gallus*)	Santa Cruz	Fowlpox
309C	Chicken (*Gallus gallus*)	Santa Cruz	Fowlpox
100W	Warbler (*Dendroica petechia*)	Santa Cruz	Gal2
101W	Warbler (*Dendroica petechia*)	Santa Cruz	Gal1
102W	Warbler (*Dendroica petechia*)	Santa Cruz	Gal2
500F	Small ground finch (*Geospiza fuliginosa*)	Santa Cruz	Gal2
501F	Small ground finch (*Geospiza fuliginosa*)	Santa Cruz	Gal1
502F	Medium ground finch (*Geospiza fortis*)	Santa Cruz	Gal1
503F	Medium ground finch (*Geospiza fortis*)	Santa Cruz	Gal1
504F	Medium ground finch (*Geospiza fortis*)	Santa Cruz	Gal1
505F	Finch (*Geospiza* sp.)	Santa Cruz	Gal1
508F	Small ground finch (*Geospiza fuliginosa*)	Isabela	Gal1
509F	Small ground finch (*Geospiza fuliginosa*)	Isabela	Gal1
510F	Medium ground finch (*Geospiza fortis*)	Isabela	Gal1
512F	Finch (*Geospiza* sp.)	Unknown	Gal1
513F	Cactus finch (*Geospiza scandens*)	Isabela	Gal1
514F	Small ground finch (*Geospiza fuliginosa*)	Isabela	Gal2
515F	Cactus finch (*Geospiza scandens*)	Santa Cruz	Gal2
516F	Finch (*Geospiza* sp.)	Santa Cruz	Gal1
518F	Finch (*Geospiza* sp.)	Santa Cruz	Gal1
519F	Finch (*Geospiza* sp.)	Santa Cruz	Gal1
702M	Mockingbird (*Nesomimus parvulus*)	Santa Cruz	Gal2
703M	Mockingbird (*Nesomimus parvulus*)	Santa Cruz	Gal1

identical to the other fowlpox virus sequence (Afonso et al., 2000; GenBank AF19810) throughout this region; therefore, it was not included in the analysis.

Given the limited nature of our sampling, we sought a simple genetic distance-based analysis to portray the gross phylogenetic relationships among the five avipoxvirus sequences. To this end, the 5.9-kb alignment was converted to a distance matrix and analyzed via the neighbor-joining method in PAUP*4.0 (Swofford, 2002). However, the branch leading to fowlpox virus, which was much longer relative to canarypox virus and canarypox-like Gal1 and Gal2 virus sequences, yielded a tree that was biologically untenable (fowlpox virus was a joined sister to the Gal strains). Thus, we used a maximum-likelihood approach to minimize the problem associated with long branches in phylogenetic analysis (Felsenstein, 1978). A bootstrapped (10,000 replications) maximum-likelihood tree (with TBR branch swapping) was produced for these aligned sequences using PAUP*4.0 (Swofford, 2002), rooted with fowlpox virus. Maximum-likelihood evaluates trees using explicit evolutionary models. MODELTEST 3.06 (Posada and Crandall, 1998) selected the TrN+I+G evolutionary model as the most likely of the 56 possible evolutionary models. The log-likelihood score of the best tree was 14,404.83620.

Recombination analysis

In *Poxviridae,* recombination plays an important role in replication, and thus, the family's evolutionary history is not strictly one of association by descent. This has clear conservation implications should a bird become infected with two viral strains simultaneously. Thus, we conducted a preliminary recombination analysis. Specifically, we used statistical analysis to detect the extent of historical recombination among ancestors of the five *Poxviridae* lineages. This was implemented using the GENECONV version 1.81 program (an extension

of Sawyer, 1989, 2004). This program is a substitution-based approach that determines whether segments of DNA between two taxa in the alignment are more similar to each other than would be expected given their overall level of similarity. After detecting recombination events, GENECONV then ranks them according to statistical significance and reports where the recombinatory segment begins and ends within the sequence and its total length. This widely used approach (Millman et al., 2001; Drouin, 2002), is more powerful than other recombination-detection methods in computer simulations and does not overestimate recombination events (Posada and Crandall, 2001). In our study, pairs of segments of sequences within the alignment that showed significant recombination are reported as global inner *P*-values. Significant recombination between a segment of a sequence from within the alignment and an unknown taxon outside the alignment, or a taxon within the alignment obscured by other evolutionary processes, are reported here as global outer *P*-values. Both are based on 10,000 permutations, and are corrected for multiple comparisons.

RESULTS

Identification of avipoxviruses

Lesions were collected from 21 endemic passerine birds on the inhabited islands of Santa Cruz and Isabela in the Galápagos Islands and from domestic chickens on Santa Cruz (Table 1). The CAX primers amplified a 374-bp fragment of DNA from lesions from Galápagos chickens (the size predicted from the genome of fowlpox virus) compared to a 426-bp amplicon from lesions from Galápagos finches, yellow warblers, and mockingbirds. The CA3-2 primers amplified a 374-bp fragment of DNA from lesions from Galápagos chickens (the size predicted from the genome of fowlpox virus) compared to 392 or 381 bp (depending on the avipoxvirus strain) from lesions from Galápagos finches, warblers, and mockingbirds. Each set of primers produced an amplicon of the expected size for fowlpox virus from DNA extracted from a control fowlpox virus from a U.S. chicken. The sizes of both the CAX and CA3-2 amplicons from the lesions from the Galápagos finches, warblers, and mockingbirds were comparable to those predicted for canarypox virus and larger than the amplicons produced from the pox lesions from chickens.

The sequences of the CAX and CA3-2 amplicons from five viruses from chickens from the Galápagos and one from the United States were identical to the published sequence for fowlpox virus at both loci. Thus, chickens in Galápagos were infected with an avipoxvirus that is very similar, if not identical, to the strain that infects poultry in the United States. In contrast, the sequences of several CA3-2 amplicons from the passerine birds indicated distinct viruses, but both were very similar to canarypox virus. One of these amplicons, Gal1 (sequenced for 10 strains), contained restriction sites for *Spe*I and *Age*I, and the other, Gal2 (sequenced for five strains), did not. The Gal1 strain was more similar to canarypox virus than was Gal2 and was also the more prevalent of the two strains, particularly in the finches (Table 1). Evidence of avipoxvirus infection was restricted to chickens on Santa Cruz that were infected with fowlpox virus and the passerine birds that were infected with two variants of canarypox virus. Because the primers used in this study were designed using the sequences of fowlpox and canarypox virus, it is likely that they would not amplify DNA from all avipoxvirus strains. Hence, other avipoxviruses may be present in birds in the Galápagos.

Similarity of avipoxviruses from Galápagos

To determine the similarities among the avipoxviruses, we sequenced a 5.9-kb region of a Gal1 (strain 502F) and a Gal2 (strain 100W) representative strain corresponding to most of a sequenced region of the canarypox virus genome that contains TK gene (Amano et al., 1999; GenBank D86731). DNA from these two strains produced single amplicons with all primer pairs tested, and the sequences of each amplicon were consistent with the presence of a single virus strain in each specimen. Recently the complete genome sequence of a slightly different canarypox vi-

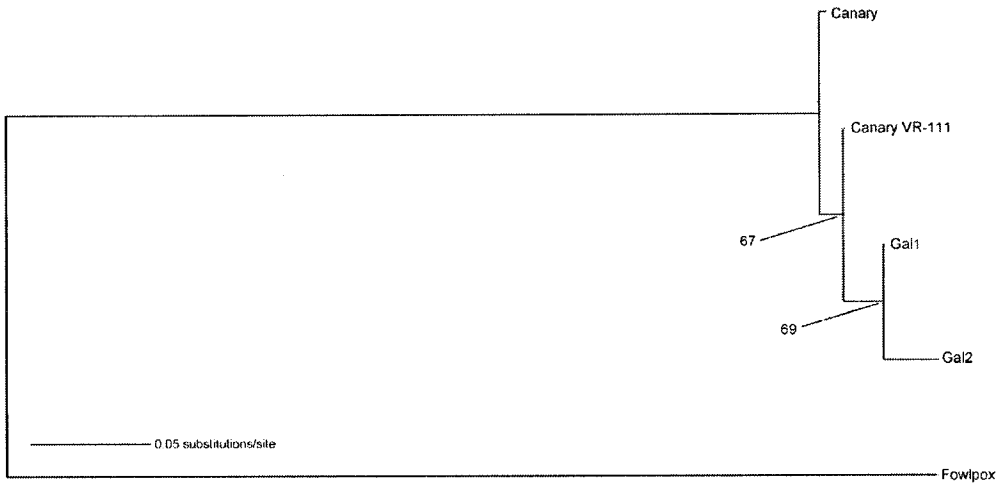

FIGURE 1. A maximum-likelihood phylogenetic tree (with TBR branch-swapping) of an alignment of a 5.9-kb region of DNA sequence from five avipoxviruses rooted with fowlpox virus and bootstrapped (10,000 replications); support values are below the branches. Taxon labels are as follows: CanaryVR-111 (Genbank AY31887), Canary (GenBank D86731), Fowlpox (AF198100).

rus strain has been published (Tulman et al., 2004; GenBank AY318871), providing another strain for comparison to Gal1 and Gal2. The 5.9-kb sequenced region spans from within gene CNPV117 to within gene CNPV109 (using the nomenclature of the genes for the complete canarypox virus genome [Tulman et al., 2004]). Alignment of the Gal1 and Gal2 sequences with the two canarypox virus sequences and with fowlpox virus confirmed that Gal1 and Gal2 were most similar to both canarypox viruses. Within this region, sequences for Gal1 and Gal2 were 97.6% identical, the two published canarypox virus strains were 98.7% identical, Gal1 was 97–98% identical to the two canarypox virus strains, and Gal2 was 95–96% identical to the two canarypox virus strains. In contrast, sequence from fowlpox virus was approximately 70% identical to these other strains. The aligned sequences were analyzed using maximum likelihood to produce a phylogenetic tree (Fig. 1). These results were fairly congruent with the pairwise comparisons. The canarypox virus, Gal1, and Gal2 strains clustered together and were separated by a long branch leading to fowlpox virus.

The quantitative analysis of recombination using the GENECONV program indicated 19 significant recombination events (global inner fragments) between ancestors of four of the five taxa included in the analysis (Table 2). Notably, identical sequence segments or overlapping segments showing evidence of recombination occurred between more than one pair of sequences in many cases. More generally, there was statistical evidence of recombination at most nucleotide sites along the 5.9-kb alignment between at least two lineages (Table 2).

A qualitative analysis of the alignment of the 5.9-kb region revealed that the identities among Gal1, Gal2, and canarypox virus were clustered in discrete regions, further supporting recombination between strains. In the first 500 nucleotides, Gal1 matched canarypox perfectly, whereas Gal2 differed by about 5%. This was followed by a region of over 1,500 nucleotides in which Gal1 and Gal2 matched perfectly, and the sequence from canarypox virus matched them in some regions but not in others. The next 350 nucleotides showed more variability, followed by a similar size region of identity among all three stains. For the remainder of the sequence, Gal1 matched canarypox virus,

TABLE 2. Putative recombination events between ancestors of avipoxvirus strains[a].

	Fragment	Sequence 1	Sequence 2	P[b]	Begin[c]	End[d]	Length[e]
1	GI	Canary	CanaryVR-111	0.0206	3605	4402	798
2	GI	Canary	Gal1	0.0007	464	897	434
3	GI	Canary	Gal1	0.0128	2591	2973	383
4	GI	Canary	Gal1	0.0000	3605	4402	798
5	GI	Canary	Gal2	0.0000	535	897	363
6	GI	Canary	Gal2	0.0047	2591	2838	248
7	GI	Canary	Gal2	0.0005	3326	3603	278
8	GI	Canary	Gal2	0.0002	3605	3940	336
9	GI	CanaryVR-111	Gal1	0.0001	1	927	927
10	GI	CanaryVR-111	Gal1	0.0000	2476	4431	1956
11	GI	CanaryVR-111	Gal1	0.0000	4433	5966	1534
12	GI	CanaryVR-111	Gal2	0.0001	535	927	393
13	GI	CanaryVR-111	Gal2	0.0126	2521	2838	318
14	GI	CanaryVR-111	Gal2	0.0000	3209	3940	732
15	GI	CanaryVR-111	Gal2	0.0000	4552	5258	707
16	GI	CanaryVR-111	Gal2	0.0020	5343	5684	342
17	GI	Gal1	Gal2	0.0000	535	2193	1659
18	GI	Gal1	Gal2	0.0001	3209	3940	732
19	GI	Gal1	Gal2	0.0013	4552	5258	707
20	GO	Fowlpox	N/A	0.0000	535	905	371
21	GO	Fowlpox	N/A	0.0160	2591	2838	248
22	GO	Fowlpox	N/A	0.0000	3209	3940	732
23	GO	Canary	N/A	0.0294	2152	2160	9

[a] Putative recombination events detected using GENECONV version 1.81 (Sawyer, 1989). Nineteen significant ($P < 0.05$) recombination events were detected between members of the five-taxon sequence alignment (GI = Global inner fragments), and four significant recombination events were inferred between one taxon within and one unknown taxon outside the alignment or taxa within the alignment obscured by other evolutionary processes (GO = Global outer fragments).
[b] Based on global P value obtained by simulation via 10,000 permutations, corrected for muliple comparisons.
[c] Corresponds to the first nucleotide base of the recombinatory region.
[d] Corresponds to the last nucleotide base of the recombinatory region.
[e] Corresponds to the entire length of the recombinatory region.

and Gal2 matched in some regions but not in others. A region that showed many differences among all the strains was in the 5′ end of the gene encoding TK (CNPV113) (Fig. 2). Many of these differences would result in changes in amino acids in the amino-terminal region of the deduced proteins. Even the genes of the two canarypox virus strains showed differences in the amino acids encoded in this region. The mosaic pattern of sequence identity combined with the many substitutions in the TK gene indicates that recombination between avipoxvirus strains, combined with mutation, led to the variants of canarypox-like viruses that are now prevalent on Santa Cruz and Isabela.

DISCUSSION

The introduction of domestic animal diseases to archipelagos poses a threat to endemic species that have not evolved with or have been previously exposed to such pathogens. The losses of endemic birds in Hawaii have been attributed to the introduction of diseases of domestic birds and exotic wild birds, including avian pox and malaria (Warner, 1968; van Riper et al., 1986, 2002; Tripathy et al., 2000). To date there has been no report of *Plasmodium* in Galápagos; however, avian pox has been prevalent on the islands for decades, infecting both domestic chickens and wild birds. Viruses in *Poxviridae,* including avipoxviruses often cross species barriers (Boosinger et al., 1982; Reed et al., 2004). The presence of chickens on the inhabited islands of Galápagos, therefore, has led to local concern that an avipoxvirus of domestic birds could spread to the endemic wild bird populations.

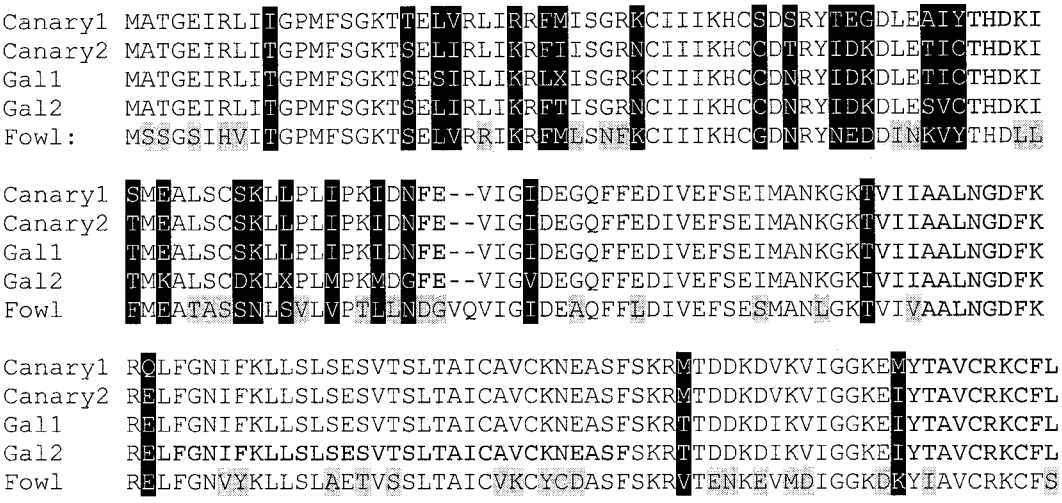

FIGURE 2. Alignment of thymidine kinase. The deduced amino acids for the putative thymidine kinase genes of five avipoxviruses were aligned using ClustalW. Black highlights indicate residues that show some variability among all five strains. Grey highlights indicate residues that differ only in fowlpox virus. Canary1 from GenBank D86731; Canary2 from Genbank AY31887; Fowl from Genbank AF198100.

Restriction fragment length polymorphisms for the CA3-2 amplicon, as well as sequencing of a 5.9-kb region of the genome, indicate that endemic passerine birds, including finches, warblers, and mockingbirds, were infected with one of two closely related variants of canarypox virus, but that none were infected with fowlpox virus. In contrast, chickens from Santa Cruz were infected with fowlpox virus but the canarypox-like virus strains were not detected. These results indicated that on Santa Cruz and Isabela, there was no evidence of avipoxvirus transmission between endemic and domestic birds. However, the similarity of the Gal1 and Gal2 strains to canarypox virus indicated that these viruses originated from either wild or domestic passerine birds, through human involvement or natural migrations. It is unknown whether the canarypox-like strains detected in this study represent divergence from a single ancestor by a combination of mutation and recombination, or whether they are the result of multiple introductions. It will be interesting to characterize avipoxvirus strains from birds on the other islands of the Galápagos, particularly the uninhabited islands where avipoxviruses may have been introduced by migrant birds from other islands. Because these viruses are mechanically vectored, it can be transferred to a new host by any number of biting insects, as well as by introduction of virions through any break in the skin. Vector communities differ between uninhabited islands and the human-inhabited islands where biting insects that may require fresh water are found (e.g., the mosquito *Culex quinquefasciatus* and the blackfly *Simulium bipunctatum*) (Peck et al., 1998). On islands without fresh water, the mosquito *Ochlerotatus taeniorhynchus* and a number of hippoboscid parasitic flies and ceratopogonid biting midges are more common.

The genomes of all characterized poxviruses comprise a single chromosome of linear double-stranded DNA that has telomeric ends with covalently closed terminal hairpin loops. The virus encodes all the proteins required for DNA replication, which occurs in the cytoplasm of the host cell. Replication of the genome occurs through intermediates called concatamers, comprising many tandem repeats of the entire genome (Moyer and Graves, 1981). Recombination, which occurs at extraor-

dinarily high frequency, is an essential part of concatamer formation and replication (Ball, 1987). The virus-encoded DNA polymerase of vaccinia virus mediates recombination; mutants lacking this polymerase do not recombine DNA (Merchlinsky, 1989; Willer et al., 1999, 2000). Consistent with the role for recombination in the replication of avipoxviruses, the recombination pathway in vaccinia virus can very efficiently recombine pairs of linear molecules and requires only 12–20 bp of homology (Willer et al., 2000; Yao and Evans, 2001).

Recombination is thought to serve a number of possible functions in viruses. Because there is no known primase, recombination may play a role in the priming of DNA replication. It can function to repair double-stranded breaks in DNA, and it may provide a mechanism for the acquisition of new genes from a coinfecting virus or from the host cell (Yao and Evans, 2001). The similarity of many viral genes with mammalian genes provides strong evidence for the acquisition of host genes by poxviruses, although the mechanism is not understood (Yao and Evans, 2001). Intermolecular recombination between the genomes of different viruses has been implicated in the formation of new recombinant poxvirus strains. Malignant rabbit fibroma virus, a lethal tumorigenic poxvirus of rabbits, resulted from recombination between Shope fibroma virus, which induces benign tumors in rabbits, and myxoma virus, which causes myxomatosis (Block et al., 1985; Upton et al., 1988). In another instance, the genome structures of one capripoxvirus isolate indicated that the progenitor of this strains resulted from recombination between the genomes of two other capripoxvirus strains (Gershon and Black, 1988; Gershon et al., 1989). Thus, recombination between different avipoxvirus strains may provide a powerful mechanism for rapid evolution of poxviruses in wild animals.

The gross phylogenic relationships among fowlpox virus, two canarypox virus strains, Gal1, and Gal2 portrayed here indicates that the two avipoxvirus strains identified from endemic, wild, Galápagos passerines are close relatives within the canarypox lineage. However, because no strains from native, wild, mainland South American birds were included in this analysis, it is impossible to determine the nature and number of avipoxvirus introductions into the Galápagos Archipelago. Nevertheless, the Gal1 and Gal2 strains are much more similar to other passerine avipoxviruses (e.g., canarypox virus) than they are to fowlpox virus. This phylogenetic reconstruction should be accepted with caution because of its narrow scope and because recombination can cause considerable error in tree estimation. There is clear evidence of recombination in the sequences of the 5.9-kb region. On Galápagos, the sympatry of fowlpox virus in the introduced chickens and the canary poxvirus variants we describe here in endemic birds presents an opportunity for further recombinants of unknown effect.

The TK gene showed the greatest divergence of any gene in the 5.9-kb region, even in the canarypox-like viruses reported in this study. This viral enzyme is part of the salvage pathway that allows the poxviruses to phosphorylate nucleotides for DNA synthesis. Although the gene is not essential, it is present in the genomes of all poxviruses sequenced to date except *Molluscum contagiosum* (Gubser et al., 2004). The difference in the amino acid sequences of the enzymes from very closely related strains indicates that it may evolve rapidly, and hence it may serve as a marker for identification of different avipoxvirus strains and for measuring the rate of viral evolution on different islands in the Galápagos.

Avipoxvirus infection can significantly increase mortality of Galapagos mockingbirds (Curry and Grant, 1989; Vargas, 1987). Presumably, it can have similar consequences for the other susceptible Galapagos endemics in which it occurs, although these have not been measured.

Management plans for small populations further threatened by pathogens requires characterizing the pathogens and an understanding of how such pathogens are introduced. This work indicates that the two lineages of avipoxvirus described in endemic passerines did not arrive through introduced chickens. The work also indicates, however, that significant recombination continues to occur among the strains in the endemic birds, which could continue to generate new forms with possible differences in pathogenicity and host range. Efforts to understand the pathogenicity of the two extant strains, to understand predominant routes of transmission, especially with regard to vectors, and to continue to characterize avipoxviruses from both endemic and exotic avian species are recommended.

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