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Worldwide Phylogenetic Relationship of Avian Poxviruses

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Poxvirus infections have been found in 230 species of wild and domestic birds worldwide in both terrestrial and marine environments. This ubiquity raises the question of how infection has been transmitted and globally dispersed. We present a comprehensive global phylogeny of 111 novel poxvirus isolates in addition to all available sequences from GenBank. Phylogenetic analysis of the *Avipoxvirus* genus has traditionally relied on one gene region (4b core protein). In this study we expanded the analyses to include a second locus (DNA polymerase gene), allowing for a more robust phylogenetic framework, finer genetic resolution within specific groups, and the detection of potential recombination. Our phylogenetic results reveal several major features of avipoxvirus evolution and ecology and propose an updated avipoxvirus taxonomy, including three novel subclades. The characterization of poxviruses from 57 species of birds in this study extends the current knowledge of their host range and provides the first evidence of the phylogenetic effect of genetic recombination of avipoxviruses. The repeated occurrence of avian family or order-specific grouping within certain clades (e.g., starling poxvirus, falcon poxvirus, raptor poxvirus, etc.) indicates a marked role of host adaptation, while the sharing of poxvirus species within prey-predator systems emphasizes the capacity for cross-species infection and limited host adaptation. Our study provides a broad and comprehensive phylogenetic analysis of the *Avipoxvirus* genus, an ecologically and environmentally important viral group, to formulate a genome sequencing strategy that will clarify avipoxvirus taxonomy.

Avian pox is a viral disease affecting more than 230 species in 23 orders of wild and domesticated birds (1). Poxviruses were identified as causative agents of pox lesions almost a century ago (2, 3), but understanding of their phylogenetics and epidemiology remains rudimentary. The genomes of only two well-diverged avian poxviruses (isolated from chicken and canaries) have thus far been sequenced. All avian poxviruses (avipoxviruses) are assigned to the genus *Avipoxvirus* in the subfamily *Chordopoxvirinae* of the *Poxviridae* family. Within the *Avipoxvirus* genus there are currently 10 recognized species (established primarily in the pre-sequence era, with subsequent limited use of restriction fragment length polymorphism analysis): *Fowlpox virus*, *Canarypox virus*, *Juncopox virus*, *Mynahpox virus*, *Psittacinepox virus*, *Sparrowpox virus*, *Starlingpox virus*, *Pigeonpox virus*, *Turkeypox virus*, and *Quailpox virus*, according to the International Committee on Taxonomy of Viruses (www.ictvonline.org). The exact number of existing avipoxvirus species, strains, and variants is unknown, since new isolates continue to be identified from a wide variety of avian species, such as Berthelot's pipit (*Anthus berthelotii*) (4), lesser flamingos (*Phoenicopterus minor*) (5), or crested serpent eagle (*Spilornis cheela*) (6).

Avian pox infections cause significant economic losses in domestic poultry due to decreased egg production, reduced growth, blindness, and increased mortality (7). Effects of avian pox on wild bird species can also be severe. The infection may produce several negative effects including elevated predation among affected birds (8), secondary infections, trauma, reduced male mating success (9) and death (10). The lifestyle of wild birds allows avian poxviruses to reach new hosts through bird migration, species introductions, and habitat change. Avian pox has been identified as an important risk factor in the conservation of small and endangered populations, particularly in island bird species (4). The impact of the introduction of avian pox has been disastrous for the avifauna of various archipelagos (11). Poxvirus infection has been responsible for the population decline of native bird species on Hawaii (12), Galápagos (2, 13), and the Canary Islands (14). Avian pox has also been identified as a risk factor in the reintroduction programs of houbara bustard (*Chlamydotis undulata macqueenii*) in the Middle East, Floreana mockingbirds (*Mimus trifasciatus*) in Galapagos (15, 16), and peregrine falcons (*Falco peregrinus*) in Germany (17). The recent emergence of an epizootic of conspicuous and distinctive avian pox among great tits (*Parus major*) in the United Kingdom (18), and its penetrance of a historically well-studied population near Oxford, allowed detailed study of the epidemiology (19) and population-level impacts (20) of the disease in wild birds.

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The currently available vaccines against fowlpox, canarypox, pigeon pox, and quail pox are each produced using virus strains isolated from the respective avian group. There is an increasing demand for new vaccines against avian poxvirus infections to help protect a wide range of birds, especially endangered species (21).

Fowlpox virus is the type species of the *Avipoxvirus* genus. The complete genomic sequences of *Fowlpox virus* (AF198100) (22) and *Canarypox virus* (AY318871) (23) are available. The two genomes are highly diverged, sharing only ca. 70% sequence identity. The 365-kbp genome of *Canarypox virus* is larger than that of *Fowlpox virus* (288 kbp) and shows significant differences in gene content, particularly in the expansion and diversification of some gene families that are already large in *Fowlpox virus*, notably the ankyrin repeat proteins (19). The phylogenetic relationships among avipoxviruses are only partially characterized. Comparative analysis of genomic sequences is the most informative and reliable method for comparing closely related viral genomes, so a definite phylogeny will have to await additional genome sequencing. The relationships of avian poxviruses isolated from free-ranging birds have been analyzed using DNA sequences of the 4b core protein coding genomic region (21, 24–27). Until recently, the significant divergence among avipoxviruses impeded the efforts to identify other pan-genus PCR primers. Jarmin et al. (25) and Manarolla et al. (21) sequenced the fpv140 locus (FPV140 gene; virion envelope protein, p35) of some avian poxvirus strains, while Thiel et al. (13) sequenced the intergenic region between CA.X (CNPV114 gene; HT motif protein), and TK (CNPV113 gene; thymidine kinase) genes. Unfortunately, these markers appeared to fail to identify some clades or subclades that were identified by the 4b core protein-based PCR system. These phylogenetic studies have concluded that the vast majority of avian poxvirus isolates clustered into three major clades, represented by the *Fowlpox virus* (clade A), the *Canarypox virus* (clade B), and the *Psittacinepox virus* (clade C). However, other pan-genus markers, similar to the 4b core protein coding genomic region, are needed in order to achieve a more robust phylogenetic classification of avian poxviruses.

This study was aimed at identifying another such pan-genus marker from the wider set of genomic core genes (the DNA polymerase gene) and combining it with sequences from the 4b region to provide a robust and global phylogenetic framework for the study and classification of avian poxviruses. Our analysis included partial 4b core protein and DNA polymerase gene sequences of virus strains isolated from natural pox infection cases occurring in 111 wild and captive birds from 57 different species sampled in North and South America, Europe, Asia, Antarctica, and the Pacific Ocean.

MATERIALS AND METHODS

Sample collection and preparation. Samples were collected by biopsy or during postmortem examinations from a wide range of clinically ill or dead birds in the United States, Ecuador (Galapagos Islands), Argentina, Chile, Hungary, Spain, Netherlands, Belgium, United Kingdom, South Korea, and Antarctica (Table 1). Tissue samples were frozen at −20 or −80°C or fixed in 10% neutral buffered formalin and embedded in paraffin blocks.

Virus isolation on muscovy duck embryo fibroblasts (MSDEF) (28, 29) or the chorioallantoic membrane (CAM) of embryonated chicken eggs (28, 29) was carried out in several cases (Table 1). A lesion (ca. 1g) was homogenized for 2 min using a tissue grinder in 10 ml of Hanks' balanced salt solution (Gibco-Invitrogen, Carlsbad, CA) supplemented with 5% glycerin (Sigma-Aldrich, St. Louis, MO) and 5% gelatin (Difco-BD, Franklin Lakes, NJ). The tissue suspension was centrifuged at 800 × *g* at 4°C for 30 min. About 0.2 ml of supernatant was inoculated onto the CAM of 13-day-old embryonated chicken eggs after filtration through a 0.45-μm-pore-size filter. The eggs were incubated for 5 days at 37°C before harvesting. The CAM was excised under microscope and observed for generalized thickening or lesions. MSDEF cell culture was prepared and handled by the method of Docherty and Slota (28, 29). About 0.5 ml of supernatant, after filtration through a 0.45-μm-pore-size filter, was inoculated into a 7-day-old confluent T-75 flask of MSDEF. The flask was incubated at 37°C and 5% CO_2 in a humidified air incubator and read on days 3 to 7 after inoculation to observe for cytopathic effect (CPE). The flask was freeze-thawed for blind passage 7 days after the original inoculation if no CPE was seen (28, 29).

DNA was extracted from frozen tissue samples, CAM homogenates, tissue cultures, and paraffin-embedded samples with a QIAamp DNA minikit (Qiagen, Inc., Valencia, CA) according to the manufacturer's recommendations.

Primers, PCR, and sequencing. In order to amplify a fragment of the avian poxviruses DNA polymerase gene, a PCR system was designed based on the known *Fowlpox virus* DNA polymerase gene sequence (30) utilizing the primer pair: PoPr1, 5′-CGCCGCATCATCTACTTATC-3′; and PoPr2, 5′-CCACACAGCGCCATTCATTA-3′. Since this method was not able to detect all poxvirus strains, a pair of universal primers (PPolF [5′-GGCYAGTACKCTTATYAAAGG-3′] and PPolR [5′-CGTCTCTACGT GTTTCGCT-3′]) was designed from the consensus sequence of the aligned DNA polymerase gene sequences of *Fowlpox* and *Canarypox virus*. Alignments were generated with the web-based Multalin software (31), while PRIMER2 (Scientific and Educational Software, Cary, NC) and PrimerSelect from the Lasergene software package (DNASTAR, Inc., Madison, WI) were used for primer design. The PCR amplifying a sequence of the 4b core protein gene was used as described by Lee and Lee (32).

All PCRs were performed in a 25-μl total volume containing 10 to 100 ng of target DNA diluted in, 5 μl of 5× Green GoTaq Flexi Buffer (Promega, Inc., Madison, WI), 2 μl of $MgCl_2$ (25 mM), 0.75 μl of deoxynucleoside triphosphates (10 mM; Qiagen), 2 μl of each primer (10 pmol/μl), and 0.2 μl of GoTaq DNA polymerase (5 U/μl; Promega). The PCR was performed in DNA Engine Thermal Cyclers PTC-0200 (Bio-Rad Laboratories Inc., Hercules, CA).

For the PCR amplifying the DNA polymerase gene segment with the PoPr1/2 primers the reaction consisted of initial denaturation for 5 min at 95°C, followed by 35 amplification cycles consisting of denaturation for 30 s at 95°C, primer annealing at 53°C for 30 s, and extension at 72°C for 1 min. The final extension step was performed for 5 min at 72°C. For the PPolF and PPolR primers, the annealing temperature was set to 50°C, with the rest of the protocol unaltered. In the PCR amplifying the 4b core protein sequence the amplification was extended to 45 cycles and consisted of 1 min of denaturation at 95°C, 1 min of annealing at 60°C, and 1 min of extension at 72°C.

After amplification, 5 μl of each reaction mixture was subjected to electrophoresis in 1% agarose gel, and the amplified gene products were visualized under UV light after ethidium bromide staining. PCR products were isolated from agarose gel (QIAquick gel extraction kit; Qiagen), and direct cycle sequencing was performed with the primers used for amplification on an ABI 373A or an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA).

Phylogenetic methods. Nucleic acid databases were searched using BLASTN (33). Multiple alignments of the obtained DNA sequences were performed with CLUSTAL W in the DAMBE software package (34) using the translated amino acid sequence alignment as a template for the precise alignment of the DNA sequences. Alignments were edited and shaded with BioEdit software (35). The concatenated alignment containing the sections of both 4b core protein and DNA polymerase gene sequences was also produced in DAMBE.

TABLE 1 List of samples with their information and GenBank accession numbers of derived sequences used in the study

Subclade (clade)[a]	Sample code	GenBank ID		English name	Latin name	Order	Family	Origin[b]	Yr	Pox lesion category	Source for DNA extraction
		4b core protein gene sequence	DNA polymerase gene sequence								
A1	P1	KC017960	KC017850	Domestic fowl	*Gallus domesticus*	Galliformes	Phasianidae	Hungary†	2003	Cutaneous	Skin lesion
A1	P2	KC017961	KC017866	Domestic turkey	*Meleagris gallopavo*	Galliformes	Phasianidae	Nevada (USA)	2005	Cutaneous-oral mucosa	Tissue culture
A1	P3	KC017962	KC017867	Domestic fowl	*Gallus domesticus*	Galliformes	Phasianidae	Hawaii† (USA)	1996	Cutaneous	Tissue culture
A1	P4	KC017963	KC017883	Superb parrot	*Polytelis swainsonii*	Psittaciformes	Psittacidae	Chile†	2004	Cutaneous	CAM
A1	P5	KC017964	KC017851	Blue-eared pheasant	*Crossoptilon auritum*	Galliformes	Phasianidae	Hungary†	2005	Cutaneous	Skin lesion
A2	P6	KC017965	KC017868	Rock dove	*Columba livia*	Columbiformes	Columbidae	Hawaii (USA)	1994	Cutaneous	Skin lesion
A2	P7	KC017966	KC017885	Rock dove	*Columba livia*	Columbiformes	Columbidae	Georgia (USA)	1995	Cutaneous	Tissue culture
A2	P8	KC017967	KC017852	Eastern imperial eagle	*Aquila heliaca*	Accipitriformes	Accipitridae	Hungary	2000	Cutaneous	Skin lesion
A2	P9	KC017968	KC017853	Rock dove	*Columba livia*	Columbiformes	Columbidae	Hungary	2003	Cutaneous	Skin lesion
A2	P10	KC017969	KC017854	Rock dove	*Columba livia*	Columbiformes	Columbidae	Hungary	Unknown	Unknown	Unknown
A2	P11	KC017970	KC017855	Great bustard	*Otis tarda*	Gruiformes	Otidae	Hungary	2003	Cutaneous	Skin lesion
A2	P12	KC017971	KC017856	Rock dove	*Columba livia*	Columbiformes	Columbidae	Hungary	2005	Cutaneous	Skin lesion
A2	P13	KC017972	KC017886	Oriental turtle-dove	*Streptopelia orientalis*	Columbiformes	Columbidae	South Korea	Unknown	Cutaneous	Skin lesion
A2	P14	KC017973	KC017887	Oriental turtle-dove	*Streptopelia orientalis*	Columbiformes	Columbidae	South Korea	Unknown	Cutaneous	Skin lesion
A2	P15	KC017974	KC017890	Great bustard	*Otis tarda*	Gruiformes	Otidae	Spain	2003	Cutaneous	Skin lesion
A2	P16	KC017975	KC017857	Indian peafowl	*Pavo cristatus*	Galliformes	Phasianidae	Hungary†	2003	Cutaneous-oral mucosa	Skin lesion
A2	P17	KC017976	KC017891	Booted eagle	*Hieraaetus pennatus*	Accipitriformes	Accipitridae	Spain	2000	Cutaneous	CAM
A2	P18	KC017977	KC017892	Red-legged partridge	*Alectoris rufa*	Galliformes	Phasianidae	Spain	2000	Cutaneous	Skin lesion
A2	P19	KC017978	KC017893	Red kite	*Milvus milvus*	Accipitriformes	Accipitridae	Spain	2003	Cutaneous	CAM
A2	P20	KC017979	KC017894	Booted eagle	*Hieraaetus pennatus*	Accipitriformes	Accipitridae	Spain	2003	Cutaneous	CAM
A2	P21	KC017980	KC017895	Red-legged partridge	*Alectoris rufa*	Galliformes	Phasianidae	Spain	2002	Cutaneous	CAM
A3	P22	KC017981	KC017898	Southern giant petrel	*Macronectes giganteus*	Procellariiformes	Procellariidae	Antarctica	2004	Cutaneous-oral mucosa	CAM
A3	P23	KC017982	KC017899	Pelagic cormorant	*Phalacrocorax pelagicus*	Suliformes	Phalacrocoracidae	Alaska (USA)	1989	Cutaneous	CAM
A3	P24	KC017983	KC017888	Eurasian eagle owl	*Bubo bubo*	Strigiformes	Strigidae	South Korea	Unknown	Cutaneous	Skin lesion
A3	P25	KC017984	KC017889	Eurasian eagle owl	*Bubo bubo*	Strigiformes	Strigidae	South Korea	Unknown	Cutaneous	Skin lesion
A3	P26	KC017985	KC017902	Common murre	*Uria aalge*	Charadriiformes	Alcidae	Washington (USA)	1991	Cutaneous-oral mucosa	Tissue culture
A3	P27	KC017986	KC017904	Laysan albatross	*Phoebastria immutabilis*	Procellariiformes	Diomedeidae	Midway Islands (USA)	1983	Cutaneous	CAM
A3	P28	KC017987	KC017905	Magellanic penguin	*Spheniscus magellanicus*	Sphenisciformes	Spheniscidae	Argentina	2007	Cutaneous	Skin lesion
A4	P29	KC017988	KC017858	Peregrine falcon	*Falco peregrinus*	Falconiformes	Falconidae	Hungary	2005	Cutaneous	Skin lesion
A4	P30	KC017989	KC017859	Red-footed falcon	*Falco vespertinus*	Falconiformes	Falconidae	Hungary	2007	Cutaneous	Skin lesion
A5	P31	KC017990	KC017906	Trumpeter swan	*Cygnus buccinator*	Anseriformes	Anatidae	Wisconsin (USA)	1991	Cutaneous	Tissue culture
A5	P32	KC017991	KC017920	Mottled duck	*Anas fulvigula*	Anseriformes	Anatidae	Texas (USA)	2005	Cutaneous	Skin lesion
A5	P33	KC017992	KC017907	Blue-winged teal	*Anas discors*	Anseriformes	Anatidae	Wisconsin (USA)	1991	Cutaneous	Tissue culture
A5	P34	KC017993	KC017908	Redhead duck	*Aythya americana*	Anseriformes	Anatidae	Wisconsin (USA)	1991	Cutaneous	Tissue culture
A5	P35	KC017994	KC017924	Mallard duck	*Anas platyrhynchos*	Anseriformes	Anatidae	New York (USA)	1994	Cutaneous	Skin lesion
A5	P36	KC017995	KC017909	Trumpeter swan	*Cygnus buccinator*	Anseriformes	Anatidae	Wisconsin (USA)	1989	Unknown	CAM
A5	P37	KC017996	KC017910	Wood duck	*Aix sponsa*	Anseriformes	Anatidae	Wisconsin (USA)	1991	Cutaneous	Skin lesion
A6	P38	KC017997	KC017926	Mourning dove	*Zenaida macroura*	Columbiformes	Columbidae	Illinois (USA)	1993	Cutaneous	CAM
A6	P39	KC017998	KC017928	Mourning dove	*Zenaida macroura*	Columbiformes	Columbidae	California (USA)	1993	Oral mucosa	Tissue culture
A6	P40	KC017999	KC017911	Mourning dove	*Zenaida macroura*	Columbiformes	Columbidae	Wisconsin (USA)	1994	Cutaneous	Skin lesion
A6	P41	KC018000	KC017912	Mourning dove	*Zenaida macroura*	Columbiformes	Columbidae	Wisconsin (USA)	1987	Cutaneous	Tissue culture
A6	P42	KC018001	KC017929	Rock dove	*Columba livia*	Columbiformes	Columbidae	California (USA)	1980	Cutaneous	Skin lesion
A6	P43	KC018002	KC017913	Canada goose	*Branta canadensis*	Anseriformes	Anatidae	Wisconsin (USA)	1992	Cutaneous	Tissue culture
A7	P44	KC018003	KC017932	Bald eagle	*Haliaeetus leucocephalus*	Accipitriformes	Accipitridae	Florida (USA)	1993	Cutaneous	Tissue culture
A7	P45	KC018004	KC017933	Bald eagle	*Haliaeetus leucocephalus*	Accipitriformes	Accipitridae	Florida (USA)	1992	Cutaneous	Tissue culture
A7	P46	KC018005	KC017935	Bald eagle	*Haliaeetus leucocephalus*	Accipitriformes	Accipitridae	Minnesota (USA)	1993	Cutaneous	Skin lesion
A7	P47	KC018006	KC017914	Red-tailed hawk	*Buteo jamaicensis*	Accipitriformes	Accipitridae	Wisconsin (USA)	1985	Cutaneous	Skin lesion
A7	P48	KC018007	KC017934	Bald eagle	*Haliaeetus leucocephalus*	Accipitriformes	Accipitridae	Florida (USA)	1989	Cutaneous	Tissue culture
A7	P49	KC018008	KC017860	Northern goshawk	*Accipiter gentilis*	Accipitriformes	Accipitridae	Hungary	2003	Cutaneous	Skin lesion
A7	P50	KC018009	KC017861	Common buzzard	*Buteo buteo*	Accipitriformes	Accipitridae	Hungary	2000	Cutaneous	Skin lesion
A7	P51	KC018010	KC017896	Red kite	*Milvus milvus*	Accipitriformes	Accipitridae	Spain	2000	Cutaneous	Skin lesion
A7	P52	KC018011	KC017900	Bald eagle	*Haliaeetus leucocephalus*	Accipitriformes	Accipitridae	Alaska (USA)	1981	Cutaneous	CAM
A7	P53	KC018012	KC017901	Bald eagle	*Haliaeetus leucocephalus*	Accipitriformes	Accipitridae	Alaska (USA)	1991	Oral mucosa	Tissue culture
A7	P54	KC018013	KC017915	Mallard duck	*Anas platyrhynchos*	Anseriformes	Anatidae	Wisconsin (USA)	1991	Cutaneous	Skin lesion
B1	P55	KC018014	KC017869	Canary	*Serinus canaria*	Passeriformes	Fringillidae	Hawaii† (USA)	1996	Cutaneous	Tissue culture
B1	P56	KC018015	KC017870	Canary	*Serinus canaria*	Passeriformes	Fringillidae	Hawaii† (USA)	1996	Cutaneous	Tissue culture
B1	P57	KC018016	KC017871	Canary	*Serinus canaria*	Passeriformes	Fringillidae	Hawaii† (USA)	1996	Cutaneous	Tissue culture
B1	P58	KC018017	KC017872	Canary	*Serinus canaria*	Passeriformes	Fringillidae	Hawaii† (USA)	1996	Cutaneous	Tissue culture
B1	P59	KC018018	KC017873	Apapane	*Himatione sanguinea*	Passeriformes	Fringillidae	Hawaii (USA)	1996	Cutaneous	Tissue culture

B1	P60	KC018019	KC017874	Canary	*Serinus canaria*	Passeriformes	Fringillidae	Hawaii† (USA)	1996	Cutaneous	Tissue culture
B1	P61	KC018020	KC017875	Apapane	*Himatione sanguinea*	Passeriformes	Fringillidae	Hawaii (USA)	1996	Cutaneous	Tissue culture
B1	P62	KC018021	KC017876	Canary	*Serinus canaria*	Passeriformes	Fringillidae	Hawaii† (USA)	1996	Cutaneous	Tissue culture
B1	P63	KC018022	KC017936	Dark-eyed junco	*Junco hyemalis hyemalis*	Passeriformes	Emberizidae	Utah (USA)	1986	Cutaneous	CAM
B1	P64	KC018023	KC017938	House finch	*Carpodacus mexicanus*	Passeriformes	Fringillidae	Arizona (USA)	1991	Cutaneous	Tissue culture
B1	P65	KC018024	KC017942	House finch	*Carpodacus mexicanus*	Passeriformes	Fringillidae	Oregon (USA)	1995	Cutaneous	Tissue culture
B1	P66	KC018025	KC017939	House finch	*Carpodacus mexicanus*	Passeriformes	Fringillidae	Arizona (USA)	1996	Cutaneous	Tissue culture
B1	P67	KC018026	KC017943	House finch	*Carpodacus mexicanus*	Passeriformes	Fringillidae	Oregon (USA)	1998	Cutaneous	Tissue culture
B1	P68	KC018027	KC017944	House finch	*Carpodacus mexicanus*	Passeriformes	Fringillidae	Oregon (USA)	1998	Cutaneous	Tissue culture
B1	P69	KC018028	KC017937	House finch	*Carpodacus mexicanus*	Passeriformes	Fringillidae	Utah (USA)	2001	Cutaneous	Tissue culture
B1	P70	KC018029	KC017940	House finch	*Carpodacus mexicanus*	Passeriformes	Fringillidae	Arizona (USA)	2001	Cutaneous	Tissue culture
B1	P71	KC018030	KC017903	House finch	*Carpodacus mexicanus*	Passeriformes	Fringillidae	Washington (USA)	1988	Cutaneous	Tissue culture
B1	P72	KC018031	KC017945	American crow	*Corvus brachyrhynchos,*	Passeriformes	Corvidae	Washington, DC (USA)	1999	Cutaneous	Tissue culture
B1	P73	KC018032	KC017877	House finch	*Carpodacus mexicanus*	Passeriformes	Fringillidae	Hawaii (USA)	1987	Cutaneous	Tissue culture
B1	P74	KC018033	KC017946	Medium ground finch	*Geospiza fortis*	Passeriformes	Emberizidae	Galapagos Islands (Ecuador)	2008	Cutaneous	Skin lesion
B1	P75	KC018034	KC017947	Galapagos mockingbird	*Mimus parvulus*	Passeriformes	Mimidae	Galapagos Islands (Ecuador)	2008	Cutaneous	Skin lesion
B1	P76	KC018035	KC017941	Northern (masked) bobwhite	*Colinus virginianus ridgwayi*	Galliformes	Odontophoridae	Arizona (USA)	1993	Conjunctiva, infraorbital sinus	Tissue culture
B1	P77	KC018036	KC017950	American crow	*Corvus brachyrhynchos*	Passeriformes	Corvidae	Massachusetts (USA)	2008	spleen	Spleen
B1	P78	KC018037	KC017951	Black-billed magpie	*Pica hudsonia*	Passeriformes	Corvidae	Colorado (USA)	1997	Lung	Lung
B1	P79	KC018038	KC017952	Black-hooded siskin	*Carduelis atrata*	Passeriformes	Fringillidae	The Netherlands†	2003	Cutaneous	Paraffin-embedded tissue
B1	P80	KC018039	KC017930	Common raven	*Corvus corax*	Passeriformes	Corvidae	California (USA)	2004	Cutaneous	Tissue culture
B1	P81	KC018040	KC017953	American crow	*Corvus brachyrhynchos*	Passeriformes	Corvidae	Maryland (USA)	2005	Cutaneous	Skin lesion
B1	P82	KC018041	KC017931	Common murre	*Uria aalge*	Charadriiformes	Alcidae	California (USA)	1980	Cutaneous	Skin lesion
B1	P83	KC018042	KC017948	Medium ground finch	*Geospiza fortis*	Passeriformes	Emberizidae	Galapagos Islands (Ecuador)	2008	Cutaneous	Skin lesion
B1	P84	KC018043	KC017949	Woodpecker finch	*Camarhynchus pallidus*	Passeriformes	Thraupidae	Galapagos Islands (Ecuador)	2008	Cutaneous	Skin lesion
B1	P85	KC018044	KC017956	American crow	*Corvus brachyrhynchos*	Passeriformes	Corvidae	Pennsylvania (USA)	1999	Cutaneous	Tissue culture
B1	P86	KC018045	KC017897	Northern (hen) harrier	*Circus cyaneus*	Accipitriformes	Accipitridae	Spain	2000	Cutaneous	Skin lesion
B1	P87	KC018046	KC017957	Common bullfinch	*Pyrrhula pyrrhula*	Passeriformes	Fringillidae	Belgium†	2008	Cutaneous	Skin lesion
B1	P88	KC018047	KC017862	Great tit	*Parus major*	Passeriformes	Paridae	Hungary	2007	Cutaneous	Skin lesion
B1	P89	KC018048	KC017958	Mississippi sandhill crane	*Grus canadensis*	Gruiformes	Gruidae	Mississippi (USA)	1992	Cutaneous	Tissue culture
B1	P90	KC018049	KC017916	Swainson's thrush	*Catharus ustulatus*	Passeriformes	Turdidae	Wisconsin (USA)	1994	Cutaneous	CAM
B1	P91	KC018050	KC017959	Gray-crowned rosy finch	*Leucosticte tephrocotis*	Passeriformes	Fringillidae	Montana (USA)	1985	Cutaneous	Skin lesion
B1	P92	KC018051	KC017917	Humboldt penguin	*Spheniscus humboldti*	Sphenisciformes	Spheniscidae	Wisconsin (USA)†	2008	Cutaneous	Tissue culture
B1	P93	KC018052	KC017878	Hawai'i amakihi	*Hemignathus virens*	Passeriformes	Fringillidae	Hawaii (USA)	1987	Cutaneous-oral mucosa	Tissue culture
B1	P94	KC018053	KC017927	Dark-eyed junco	*Junco hyemalis hyemalis*	Passeriformes	Emberizidae	Illinois (USA)	1986	Cutaneous	Tissue culture
B1	P95	KC018054	KC017918	Canada goose	*Branta canadensis*	Anseriformes	Anatidae	Wisconsin (USA)	1989	Cutaneous	CAM
B1	P96	KC018055	KC017879	Elepaio	*Chasiempis sandwichensis*	Passeriformes	Monarchidae	Hawaii (USA)	1996	Cutaneous	Tissue culture
B1	P97	KC018056	KC017880	Apapane	*Himatione sanguinea*	Passeriformes	Fringillidae	Hawaii (USA)	1996	Cutaneous	Tissue culture
B1	P98	KC018057	KC017881	Apapane	*Himatione sanguinea*	Passeriformes	Fringillidae	Hawaii (USA)	1996	Cutaneous	Tissue culture
B1	P99	KC018058	KC017863	Golden eagle	*Aquila chrysaetos*	Accipitriformes	Accipitridae	Spain	2000	Cutaneous	CAM
B1	P100	KC018059	KC017882	Apapane	*Himatione sanguinea*	Passeriformes	Fringillidae	Hawaii (USA)	1996	Cutaneous	Tissue culture
B1	P101	KC018060	KC017884	Canary	*Serinus canaria*	Passeriformes	Fringillidae	Chile†	2008	Cutaneous	CAM
B1	P102	KC018061	KC017921	Common grackle	*Quiscalus quiscula*	Passeriformes	Icteridae	Texas (USA)	1993	Cutaneous	Tissue culture
B1	P103	KC018062	KC017922	Boat-tailed grackle	*Quiscalus major*	Passeriformes	Icteridae	Texas (USA)	1989	Cutaneous	Skin lesion
B2	P104	KC018063	KC017954	European starling	*Sturnus vulgaris*	Passeriformes	Sturnidae	Maryland (USA)	1984	Cutaneous	Tissue culture
B2	P105	KC018064	KC017919	European starling	*Sturnus vulgaris*	Passeriformes	Sturnidae	Wisconsin (USA)	1985	Cutaneous	Skin lesion
B2	P106	KC018065	KC017955	European starling	*Sturnus vulgaris*	Passeriformes	Sturnidae	Maryland (USA)	1985	Cutaneous	CAM
B2	P107	KC018066	KC017864	Great bustard	*Otis tarda*	Gruiformes	Otidae	Hungary	2005	Cutaneous	Skin lesion
B2	P108	KC018067	KC017865	Common hill myna	*Gracula religiosa*	Passeriformes	Sturnidae	Hungary†	Unknown	Cutaneous	Skin lesion
B3	P109	KC018068	KC017923	American robin	*Turdus migratorius*	Passeriformes	Turdidae	Texas (USA)	2005	Cutaneous	Tissue culture
C	P110	KC018069	KC017925	Yellow-crowned amazon	*Amazona ochrocephala*	Psittaciformes	Psittacidae	New York† (USA)	1980	Cutaneous	Tissue culture
C	P111	AM050383	KC017849	Parrot	Undescribed	Psittaciformes	Psittacidae	UK†	Unknown	unknown	Tissue culture

[a] That is, subclades of fowlpox and canarypox clades and the *Psittacinepox virus* clade in Bayesian analysis of concatenated, 4b, and DNA polymerase gene sequences.

[b] †, Samples collected from captive birds (aviaries, zoos, etc.).

Phylogenies were generated separately for the 4b gene and DNA polymerase gene sequences and for the concatenated sequences of these two genes. Trees were constructed using three methods: neighbor joining (NJ), maximum likelihood (ML), and a Bayesian approach. To determine the most likely model of evolution, jModelTest (36, 37) was performed. Based on Akaike's information criterion, the most likely model for the DNA polymerase gene and the concatenated sequences was a general time reversible model with a gamma distribution (GTR+G), while for the 4b gene, it was the transitional model TIM1+G. The gamma rates for the three gene sequences were as follows: concatenated = 0.2590, 4b = 0.3260, and polymerase = 0.2670. The model and parameter estimates for the closest matching model (see below) was entered using NJ in MEGA 5.0 (38, 39), ML analyses in PAUP* 4.0b (40), and Bayesian analysis in MrBayes 3.1 (41, 42). The LogDet model (43) with the estimated gamma rate was used for NJ analysis bootstrapped for 1,000 replicates. ML analyses utilized the PAUP block from jModelTest for each gene region in a heuristic search with tree bisection and reconnection (TBR) branch swapping, bootstrapped for 100 replicates. Bayesian analyses were run for 1 to 2.5 million generations, with sampling at every 100th generation, until model convergence was achieved. Four chains and a 25% burn-in that was then discarded for all analyses were used. A 50% majority rule consensus tree was built from the resulting trees. Initial phylogenies were generated with *Molluscum contagiosum* (NC001731) as the outgroup, according to the method of Jarmin et al. (25). Tree topologies within the avian poxviruses were unchanged when the following outgroups were used (*Deerpox virus* AY689437, *Tanapox virus* EF420157, and *Yaba-like disease virus* AJ293568) (44). Subsequent trees excluded these outgroup taxa, and the isolates clustered in the most basal group were used as an outgroup. The use of orthopoxviruses for outgroup(s) did not affect the tree topologies (data not shown).

The average evolutionary divergence between sequences was estimated with the MEGA 5.0 software (39) both between and within Avipoxvirus clades, subclades and Orthopoxvirus clades. Analyses were conducted using the Tamura-Nei model with standard error estimated through 1,000 bootstrap replicates. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1) using all codon positions. Between group and within group analyses were performed on the partial (555 bp) alignment of avipoxvirus DNA polymerase sequences complemented with Orthopoxvirus type sequences available from GenBank (Old World clade: X94355, *Coxpox virus*; M35027, *Vaccinia virus*; L22579, *Variola virus*; AY009089, *Camelpox virus*; DQ437594, *Taterapox virus*; DQ792504, *Horsepox virus*; AY484669, *Rabbitpox virus*; HM172544, *Monkeypox virus*; and AF012825, *Ectromelia virus*; North American clade: FJ807738, *Volepox virus*; DQ066529, *Skunkpox virus*; DQ066531, *Raccoonpox virus*) ($n = 121$), while additional within group analyses were also conducted on concatenated (981 bp) avipoxvirus DNA polymerase and 4b core protein sequences ($n = 109$). Potential recombinant sequences were excluded from the analysis.

A recombination analysis was performed on the concatenated sequence alignment using the RDP 3 software (45) in order to detect potential recombination events resulting in incongruent topology of the two single gene trees. The analysis focused on identifying events involving large sequence segments, or indeed the whole of the partial 4b core and DNA polymerase sequences (426 and 555 bp, respectively). The default selection of detection methods (RDP, GeneConv, and MaxChi) and general settings were used to perform the analyses but sequences were treated as linear, the power of detection was set to 0.01, the number of permutations to 100 with the shuffle column option.

The relationship between the phylogeny of avian hosts and avipoxvirus isolates were analyzed based on the most basic fowlpox virus (clade A) and canarypox virus (clade B) groupings. First, an alignment of cytochrome *b* sequences was generated for all available avipoxvirus hosts from GenBank; this gene contained the largest number of comparable and phylogenetically informative sequences across these species. When a sequence was not available for the specific host, the taxonomically closest available species was chosen. The taxa in the analyses were pared down to a single representative for each host species to avoid bias due to highly sampled taxa with the same poxvirus genotype. The final data set contained 61 sequences; 29 from canarypox virus hosts and 32 from fowlpox virus hosts (Table 2). Sequences were trimmed to 589 bp shared among all of the taxa in Sequencher 4.10 (Gene Codes, Ann Arbor, MI). A maximum-likelihood phylogeny of the hosts was generated to visualize the distribution of different poxvirus groupings. Evolutionary divergence was estimated between and within the canarypox virus and fowlpox virus group. In order to estimate the evolutionary divergence between sequences, pairwise genetic distances, a measure of the genetic similarity between two groups based on shared nucleotides, were calculated with the MEGA 5.0 software (39). Both analyses estimated differences over sequence pairs using a maximum composite likelihood model with standard error estimated through 100 bootstrap replicates.

RESULTS

Molecular phylogeny of the avipoxvirus sequences. The primers PPolF and PPolR for DNA polymerase gene were successfully used to amplify sequences from all tested isolates which encompassed all previously known clades. These primers yielded products of ~900 bp. However, only a 555-bp length part was included in the phylogenetic analysis since older samples were examined only with the PoPr1/2 primers, which produced a smaller PCR product. A 426-bp long sequence of the 4b core protein gene was used to prepare an additional alignment. Thus, the concatenated sequences of both genes were 981 bp long.

Partial sequences of both DNA polymerase and 4b core protein genes were amplified successfully from 111 avian pox lesion samples and virus isolates. The topologies of the phylogenetic trees created with different methods (neighbor joining [NJ], maximum likelihood [ML], and Bayesian) from the concatenated (Fig. 1), 4b core protein gene (Fig. 2), and DNA polymerase gene (Fig. 3) sequence alignments were very similar. Based on the posterior probability values and most consistent tree topology, the Bayesian trees were considered the most reliable, followed by the NJ analysis, while the ML trees had the lowest bootstrap values and poorest resolution. Based on these results we primarily used the topology of the concatenated Bayesian tree through our analysis. Avipoxviruses form two major clades (A and B) with strong support (Fig. 1), while the placement of the third major clade (C) is less certain.

Clade A represents seven subclades (A1 to A7). Subclade A1 comprises viruses isolated from birds of the order Galliformes (domestic fowl, blue-eared pheasant) with a wide geographic distribution. A poxvirus isolated from a superb parrot originating from Chile also clustered in subclade A1. Subclade A2 consists of viruses originating from birds of the order Columbiformes (rock doves, oriental turtle doves) from North America, Europe, and the Republic of Korea, with additional samples from a peacock, raptors, red-legged partridges, and great bustards from Europe. Subclade A3 formerly consisted of only an albatross virus and a falcon virus, but it has been expanded by isolates from other seabirds (southern giant petrel, pelagic cormorant, common murre, Laysan albatross, Magellanic penguin) from the coasts of the Pacific and Atlantic Ocean and Eurasian eagle-owls from Korea. Subclade A4 still forms an outlier and contains viruses from peregrine falcon and red-footed falcon from Hungary and a United Arab Emirates falcon isolate. A new subclade, A5, sharing a common ancestor with subclade A1, comprises isolates from Anseriformes (trumpeter swans, mottled duck, blue-winged teal, redhead duck, wood duck, mallard duck) originating from the United States.

TABLE 2 List of cytochrome *b* sequences for avipoxvirus hosts from GenBank

Pox type	Host (English name)	Host (Latin name)	Alternate host sequence	GenBank no.
Canarypox	Yellow-crowned amazon	*Amazona ochrocephala*		AY194411.1
	Golden eagle	*Aquila chrysaetos*		EU345512.1
	Canada goose	*Branta canadensis*		NC_007011.1
	Woodpecker finch	*Cactospiza pallida*		AF108793.1
	Black-hooded siskin	*Carduelis atrata*		L76385.1
	House finch	*Carpodacus mexicanus*		AF447364.1
	Swainson's thrush	*Catharus ustulatus*		EU619788.1
	Elepaio	*Chasiempis sandwichensis*	Eiao monarch *Pomarea iphis fluxa*	AY262704.1
	Northern (hen) harrier	*Circus cyaneus*	Western marsh-harrier *Circus aeruginosus*	AY987305.1
	Northern (masked) bobwhite	*Colinus virginianus*		EU372675.1
	American crow	*Corvus brachyrhynchos*		AY509619.1
	Common raven	*Corvus corax*		AY527266.1
	Medium Ground finch	*Geospiza fortis*		AF108773.1
	Common hill myna	*Gracula religiosa*	Common myna *Sturnus tristis*	NC_015195.1
	Mississippi sandhill crane	*Grus canadensis*		FJ769855.1
	Hawai'i amakihi	*Hemignathus virens*		AF015755.1
	Apapane	*Himatione sanguinea*		AF015754.1
	Dark-eyed junco	*Junco hyemalis hyemalis*		AF290161.1
	Gray-crowned rosy finch	*Leucosticte tephrocotis*		AY156380.1
	Galapagos mockingbird	*Mimus parvulus*	Le Conte's thrasher *Toxostoma lecontei*	AY329478.1
	Great tit	*Parus major*		EU167009.1
	Black-billed magpie	*Pica hudsonia*		AY030114.1
	Common bullfinch	*Pyrrhula pyrrhula*		HQ284613.1
	Boat-tailed grackle	*Quiscalus major*		AF089055.2
	Common grackle	*Quiscalus quiscula*		AF089058.2
	Canary	*Serinus canaria*		AY914127.1
	Humboldt penguin	*Spheniscus humboldti*		DQ137220.1
	European starling	*Sturnus vulgaris*		AF285790.1
	American robin	*Turdus migratorius*		EU619827.1
Fowlpox	Northern goshawk	*Accipiter gentilis*		NC_011818.1
	Wood duck	*Aix sponsa*		EU585605.1
	Red-legged partridge	*Alectoris rufa*		AM850840.1
	Blue-winged teal	*Anas discors*		EU914146.1
	Mottled duck	*Anas fulvigula*	Mallard duck *Anas platyrhynchos*, alt. haplotype	EU755252.1
	Mallard duck	*Anas platyrhynchos*		EU755253.1
	Eastern imperial eagle	*Aquila heliaca*		Z73465.1
	Redhead duck	*Aythya americana*		NC_000877.1
	Canada goose	*Branta canadensis*		NC_007011.1
	Eurasian eagle owl	*Bubo bubo*		AJ003961.1
	Common buzzard	*Buteo buteo*		NC_003128.3
	Red tailed hawk	*Buteo jamaicensis*		GQ264785.1
	Rock dove	*Columba livia*		NC_013978.1
	Blue-eared pheasant	*Crossoptilon auritum*		AF534552.1
	Trumpeter swan	*Cygnus buccinator*	Tundra swan *Cygnus columbianus*	DQ083161.1
	Peregrine falcon	*Falco peregrinus*		EU233100.1
	Red-footed falcon	*Falco vespertinus*		EU233132.1
	Domestic fowl	*Gallus domesticus*	Red junglefowl *Gallus gallus*	NC_007236.1
	Bald eagle	*Haliaeetus leucocephalus*		GQ264818.1
	Booted eagle	*Hieraaetus pennatus*		Y15760.1
	Southern giant petrel	*Macronectes giganteus*		AF076060.1
	Domestic turkey	*Meleagris gallopavo*		NC_010195.2
	Red kite	*Milvus milvus*		AY987312.1
	Great bustard	*Otis tarda*		NC_014046.1
	Indian peafowl	*Pavo cristatus*		DQ010648.1
	Pelagic cormorant	*Phalacrocorax pelagicus*		EU167011.1
	Laysan albatross	*Phoebastria immutabilis*		AB276050.1
	Superb parrot	*Polytelis swainsonii*	Red-winged parrot *Aprosmictus erythropterus*	AB177959.1
	Magellanic penguin	*Spheniscus magellanicus*		DQ137218.1
	Oriental turtle-dove	*Streptopelia orientalis*	Spotted dove *Streptopelia chinensis*	AF483341.1
	Common murre	*Uria aalge*		DQ485892.1
	Mourning dove	*Zenaida macroura*	Eared dove *Zenaida auriculata*	NC_015203.1

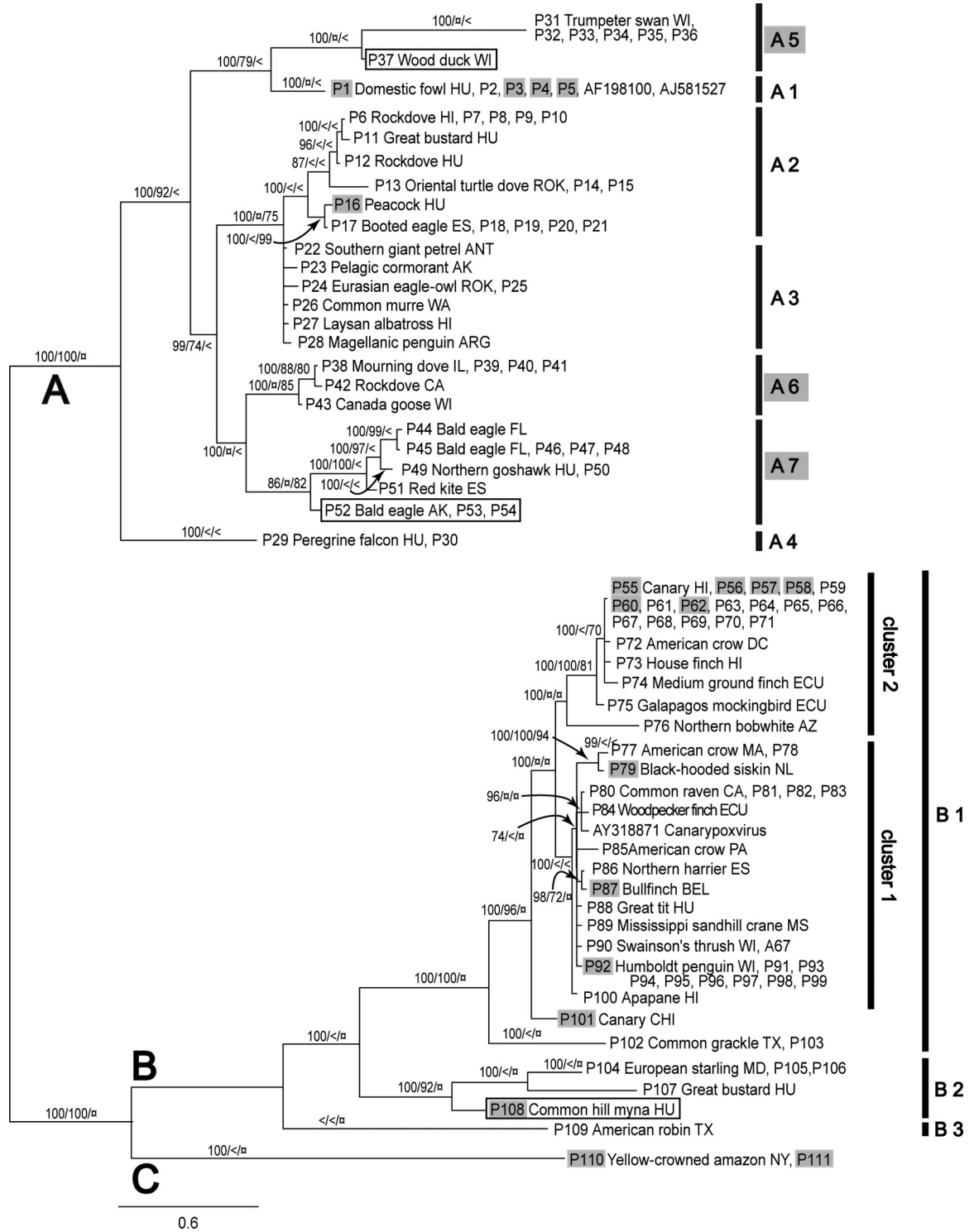

FIG 1 Bayesian phylogeny of concatenated DNA sequences from genes encoding 4b core and DNA polymerase proteins of avipoxviruses. Posterior probability values of the Bayesian trees (1,000 replicates) and neighbor-joining and maximum likelihood bootstrap values (1,000 replicates) of >70 are indicated (MB/NJ/ML). Symbols: <, lower than 70; ¤, branch does not exist with that method. Avipoxvirus clades A to C, subclades, and clusters are labeled according to the nomenclature of Jarmin et al. (25) and Jarvi et al. (46). Novel subgroups described in the present study are highlighted by gray. Isolate origins are given either as U.S. state abbreviations or using the following location codes: Antarctica (ANT), Argentina (ARG), Belgium (BEL), Chile (CHI), Ecuador (ECU), Germany (GER), Hungary (HU), Italy (ITA), Netherlands (NL), Norway (NOR), Portugal (POR), Spain (ES), South Korea (ROK), United Arab Emirates (UAE), and United Kingdom (UK). Avian poxviruses which were isolated from captive birds (aviaries, zoos, etc.) are highlighted by gray, isolates containing potential recombinations are set in a box. The scale represents the number of substitutions per site.

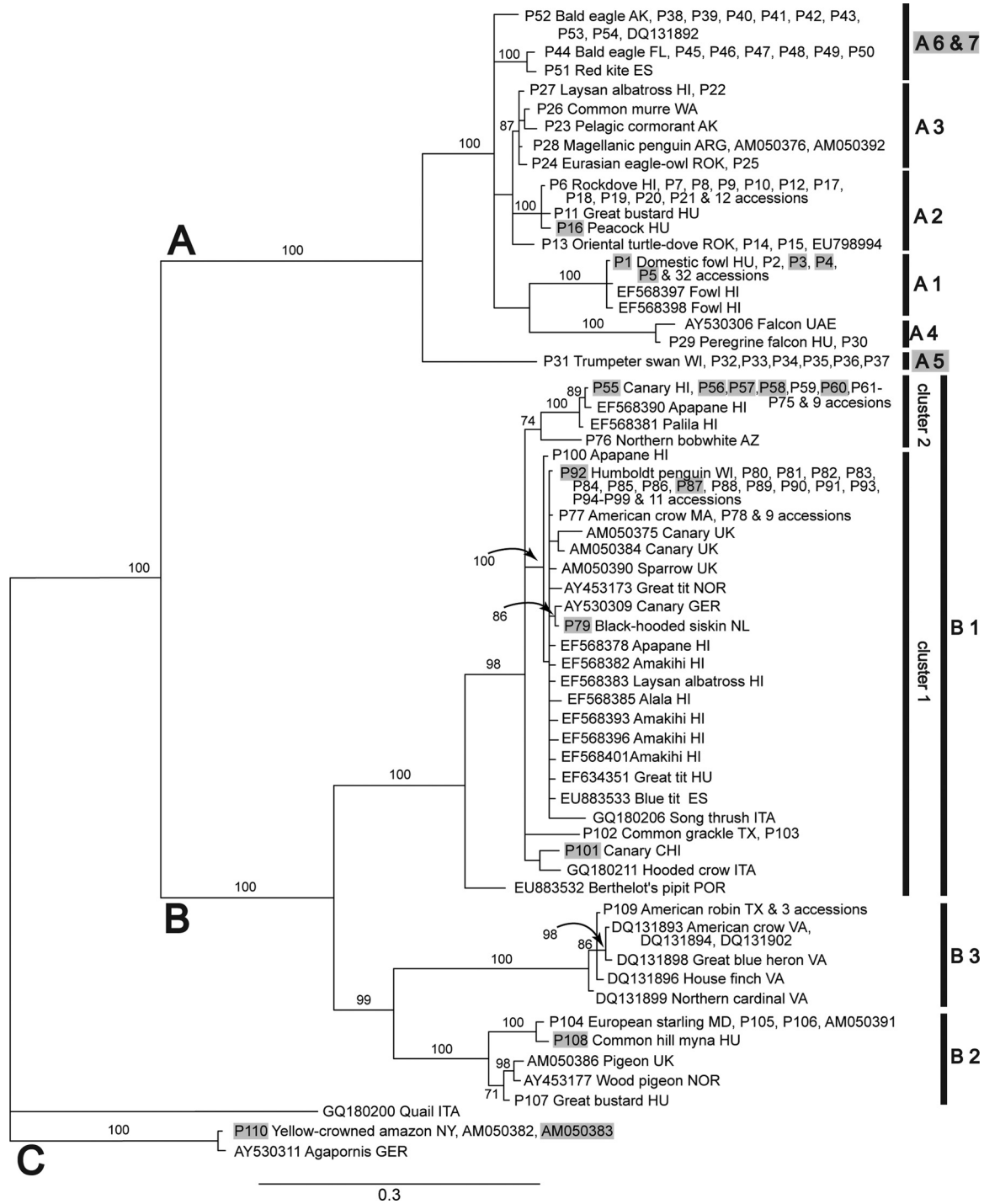

FIG 2 Bayesian phylogram of DNA sequences from genes encoding 4b core proteins of avipoxviruses. Posterior probability values of >70 are shown. Avipoxvirus clades A to C, subclades, and clusters are labeled according to the nomenclature of Jarmin et al. (25) and Jarvi et al. (46). Novel subgroups described in the present study are highlighted by gray. Isolate origins are given either as U.S. state abbreviations or using the following location codes: Antarctica (ANT), Argentina (ARG), Belgium (BEL), Chile (CHI), Ecuador (ECU), Germany (GER), Hungary (HU), Italy (ITA), Netherlands (NL), Norway (NOR), Portugal (POR), Spain (ES), South Korea (ROK), United Arab Emirates (UAE), and United Kingdom (UK). Avian poxviruses that were isolated from captive birds (aviaries, zoos, etc.) are highlighted by gray. The scale represents the number of substitutions per site. Due to the large number of avian poxvirus isolates in the 4b gene analyses ($n = 226$), we abbreviated the names for isolates with identical sequences from GenBank accessions as follows: (i) P1 genotype, AB292647, AF198100, AJ005164, AJ581527, AM050377, AM050378, AM050379, AM050380, AY453171, AY453172, AY530302, AY530304, AY530307, DQ873808, EF568377, EF634347, EF634348, M25781, GU108500, GU108501, GU108502, GU108503, GU108504, GU108505, GU108506, GU108507, GQ221269, GQ180212, GQ180207, GQ180201, GU108509, and GU108508; (ii) P6 genotype, AM050385, AM050387, AM050388, AY530303, AY530305, DQ873809, DQ873810, DQ873811, EF016108, GQ180210, GQ180208, and GQ180204; (iii) P55 genotype, EF568379, EF568384, EF568386, EF568387, EF568388, EF568389, EF568391, EF568399, and EF568400; (iv) P77 genotype, AM050381, AM050389, AY530308, GQ487567, GU108510, GQ180202, GQ180203, GQ180205, and GQ180209; (v) P92 genotype, AY530310, AY318871, AY453174, AY453175, EF568380, EF568392, EF568394, EF568395, EF634349, EF634350, and GQ180213; and (vi) P109 genotype, DQ131895, DQ131897, DQ131900, and DQ131901.

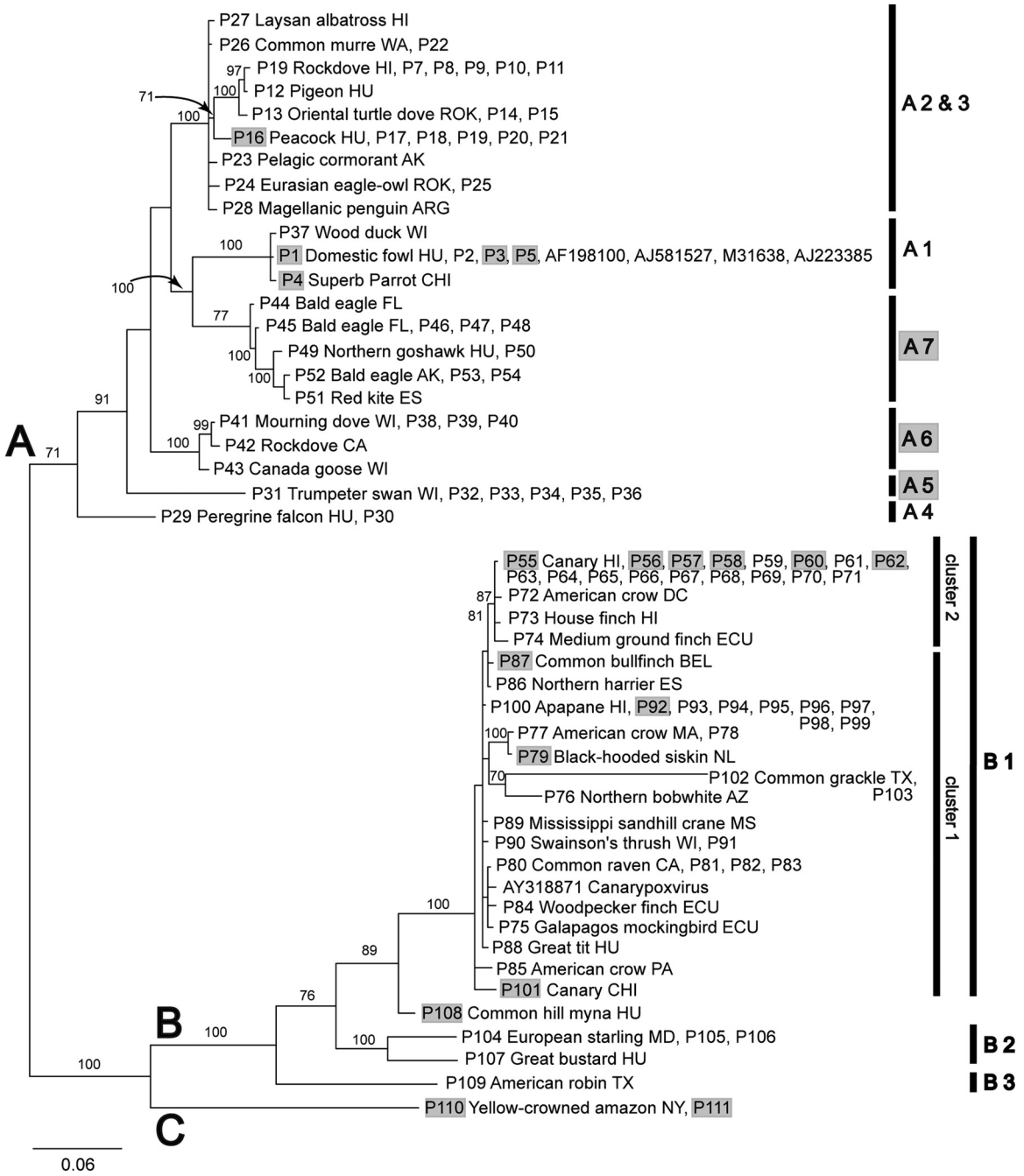

FIG 3 Bayesian phylogeny of DNA sequences from gene encoding DNA polymerase protein of avipoxviruses. Posterior probability values of >70 are shown. Avipoxvirus clades A to C, subclades, and clusters are labeled according to the nomenclature of Jarmin et al. (25) and Jarvi et al. (46). Novel subgroups described in the present study are highlighted by gray. Isolate origins are given either as U.S. state abbreviations or using the following location codes: Antarctica (ANT), Argentina (ARG), Belgium (BEL), Chile (CHI), Ecuador (ECU), Germany (GER), Hungary (HU), Italy (ITA), Netherlands (NL), Norway (NOR), Portugal (POR), Spain (ES), South Korea (ROK), United Arab Emirates (UAE), and United Kingdom (UK). Avian poxviruses which were isolated from captive birds (aviaries, zoos, etc.) are highlighted by gray. The scale represents the number of substitutions per site.

New subclades A6 and A7 share a ancestor with subclades A2 and A3. Subclade A6 comprises viruses from Columbiformes (mourning doves, rock doves) and a Canada goose from North America. Isolates from Accipitriformes (bald eagles, red tailed hawk, common buzzard, northern goshawk, red kite) from the United States and Europe and a mallard duck group under subclade A7.

Clade B is comprised of three subclades (B1 to B3). Previously reported subclade B1 comprises viruses isolated from a wide range of passerine species (Passeriformes) of worldwide distribution, although several nonpasserine hosts (e.g., northern harrier, Mississippi sandhill crane, Humboldt penguin, etc.) are represented as well. This subclade further diversifies into three outliers and a main branch consisting of two clusters. Nine house finch isolates from our study and further two from a previous work (46) with a

TABLE 3 Estimates of average evolutionary divergence of sequence pairs between and within avipoxvirus subclades and orthopox virus clades[a]

	Avg evolutionary divergence (SE)														
	Avipox											Orthopox		Distance (SE)	
Clade	A1	A2	A3	A4	A5	A6	A7	B1	B2	B3	C	OW	NA	1	2
Avipox A1		(0.016)	(0.016)	(0.024)	(0.021)	(0.019)	(0.016)	(0.043)	(0.046)	(0.048)	(0.050)	(0.097)	(0.102)	0.001 (0.000)	0.000 (0.000)
Avipox A2	0.107		(0.004)	(0.024)	(0.021)	(0.016)	(0.013)	(0.042)	(0.049)	(0.049)	(0.047)	(0.104)	(0.111)	0.016 (0.004)	0.014 (0.003)
Avipox A3	0.098	0.020		(0.024)	(0.020)	(0.014)	(0.013)	(0.042)	(0.050)	(0.048)	(0.046)	(0.105)	(0.110)	0.005 (0.002)	0.006 (0.001)
Avipox A4	0.169	0.165	0.158		(0.027)	(0.022)	(0.024)	(0.044)	(0.043)	(0.051)	(0.054)	(0.099)	(0.104)	0.000 (0.000)	0.000 (0.000)
Avipox A5	0.151	0.142	0.132	0.190		(0.022)	(0.024)	(0.051)	(0.054)	(0.050)	(0.060)	(0.087)	(0.094)	0.000 (0.000)	0.000 (0.003)
Avipox A6	0.120	0.099	0.078	0.149	0.140		(0.016)	(0.039)	(0.049)	(0.044)	(0.052)	(0.088)	(0.089)	0.005 (0.002)	0.003 (0.001)
Avipox A7	0.104	0.086	0.079	0.161	0.171	0.103		(0.038)	(0.052)	(0.051)	(0.043)	(0.105)	(0.110)	0.010 (0.003)	0.007 (0.003)
Avipox B1	0.354	0.356	0.359	0.345	0.386	0.312	0.327		(0.026)	(0.034)	(0.048)	(0.120)	(0.124)	0.017 (0.003)	0.024 (0.003)
Avipox B2	0.391	0.392	0.402	0.335	0.419	0.390	0.436	0.206		(0.033)	(0.054)	(0.115)	(0.131)	0.050 (0.008)	0.036 (0.006)
Avipox B3	0.385	0.401	0.400	0.378	0.391	0.369	0.426	0.262	0.226		(0.059)	(0.114)	(0.116)	n/c	n/c
Avipox C	0.421	0.406	0.401	0.435	0.477	0.423	0.366	0.409	0.45	0.481		(0.107)	(0.119)	0.000 (0.000)	0.000 (0.000)
Orthopox OW	0.778	0.827	0.83	0.775	0.751	0.745	0.819	0.942	0.917	0.907	0.871		(0.019)	0.013 (0.003)	0.016 (0.003)
Orthopox NA	0.804	0.864	0.854	0.823	0.789	0.758	0.849	0.975	0.973	0.882	0.933	0.148		0.076 (0.011)	

[a] Estimates of average evolutionary divergence of sequence pairs between and within avipoxvirus subclades (A1 to A7, B1 to B3, and C) and orthopoxvirus clades (Old World [OW] and North American [NA]). The number of base substitutions per site from averaging over all sequence pairs between (matrix) and within (columns) groups is shown. Standard error estimates are shown in parentheses. The results of within-group analyses are presented in the last two columns: the within-group analysis for column 1 was performed on a partial DNA polymerase sequence (555 bp) alignment ($n = 121$), while the within-group analysis for column 2 was conducted on concatenated (981-bp) DNA polymerase and 4b core protein sequences ($n = 109$). Potential recombinants were excluded from the analysis. Evolutionary analyses were conducted in MEGA5 (39). n/c, not calculated.

diverse range of isolation dates and geographic origins were analyzed and found to group within cluster 2 of subclade B1. The three outliers were formed by two strains from grackles, a virus from a Chilean canary and a strain described from Berthelot's pipit (Fig. 2). Previously reported subclade B2 consisted of isolates from starlings and mynahs. It was found that starlings in Europe and North America host the same virus strain. Viruses isolated from a great bustard in Hungary and a rock and wood pigeon from Europe also clustered into subclade B2. Isolates from a wide range of different bird species presented to a wildlife center in Virginia in 2003 and 2004 form a new subclade, B3 (Fig. 2). From our samples, only a 2003 American robin isolate from Texas clustered into this subclade. Clade C consists exclusively of isolates from psittacine species. The location of this clade is ambiguous. It formed either a separate clade, or it was a weakly supported member of clade B.

The within-group mean genetic distances of the concatenated avian poxvirus sequences were 0.087 ± 0.007 standard error (SE) in clade A and 0.059 ± 0.005 SE in clade B, while the sequences were identical (genetic distance = 0.000) in clade C. Mean genetic distances of the concatenated sequences within avipoxvirus subclades ranged from 0.000 to 0.035 (Table 3). The results of the partial DNA polymerase sequence analysis allowing a comparison with orthopoxviruses (Old World and North American clade) are summarized in Table 3.

Possible recombination between 4b and DNA polymerase loci. Apparent recombination breakpoints were located and confirmed with multiple analysis methods available in the RDP 3 software in five of the concatenated sequences at the junction of the 4b core protein and DNA polymerase (nucleotide [nt] 426). Isolates P52, P53, and P54 (from two bald eagles and a mallard) were identified by the RDP method ($P = 8.719 \times 10^{-07}$) as apparent interlocus recombinants of isolate P41 (mourning dove) as minor parent (4b core protein sequence) and P51 (red kite) as major parent (DNA polymerase sequence). It should be noted that in the concatenated sequence tree (Fig. 1), the apparent recombinants (P52, P53, and P54) are basal to the apparent parents P51 and P41. It is therefore possible that the apparent recombinant carries the ancestral sequences, whereas the apparent parents carry recombined loci. However, the topology of the three trees (shown in Fig. 1, 2, and 3) is ambiguous for these isolates, so it would be premature to speculate on the actual nature of the event.

Isolate P37 (from a wood duck) was also identified ($P = 1.613 \times 10^{-13}$) as an apparent interlocus recombinant, with P32 (mottled duck) as minor parent (4b core protein sequence) and P2 (domestic turkey) as major parent (DNA polymerase sequence).

A fifth apparent recombination event, in this case intralocus, affecting only a part of the DNA polymerase gene, was detected in a common hill mynah isolate P108 ($P = 9.469 \times 10^{-13}$) with one breakpoint identified at the sequence junction (nt 426) and an additional ending breakpoint at nt 763 of the alignment. The minor parent was identified as the dark-eyed junco isolate P94 (DNA polymerase gene sequence), while the major parent was a European starling isolate P104. All of the above apparent recombination events were confirmed with similarly significant *P* values by the GeneConv, BootScan, MaxChi, Chimaera, SiScan, and 3Seq methods.

Concordance of host and virus phylogeny. When assessing genetic diversity between the avian hosts, the mean between-group genetic distance for the hosts of canarypox viruses (clade B) and fowlpox viruses (clade A) was 0.209 ± 0.011 SE. The mean within-group genetic distances were 0.175 ± 0.009 SE for hosts of clade B viruses and 0.186 ± 0.107 SE for hosts of clade A viruses. Within-group distances were not significantly different based on the overlap of the 95% confidence intervals with the means. Overall, there was significantly greater between- than within-group genetic diversity, indicating two distinct groups of hosts. Nonetheless, although the phylogenetic distribution of hosts shows overall grouping congruent with the clade of virus, it has been possible to isolate clade A and B viruses from some closely related hosts (Fig. 4).

DISCUSSION

The phylogenetics and epidemiology of avian poxviruses is only partially understood. This study contributed to our understanding of this group of viruses by studying a broad range of isolates

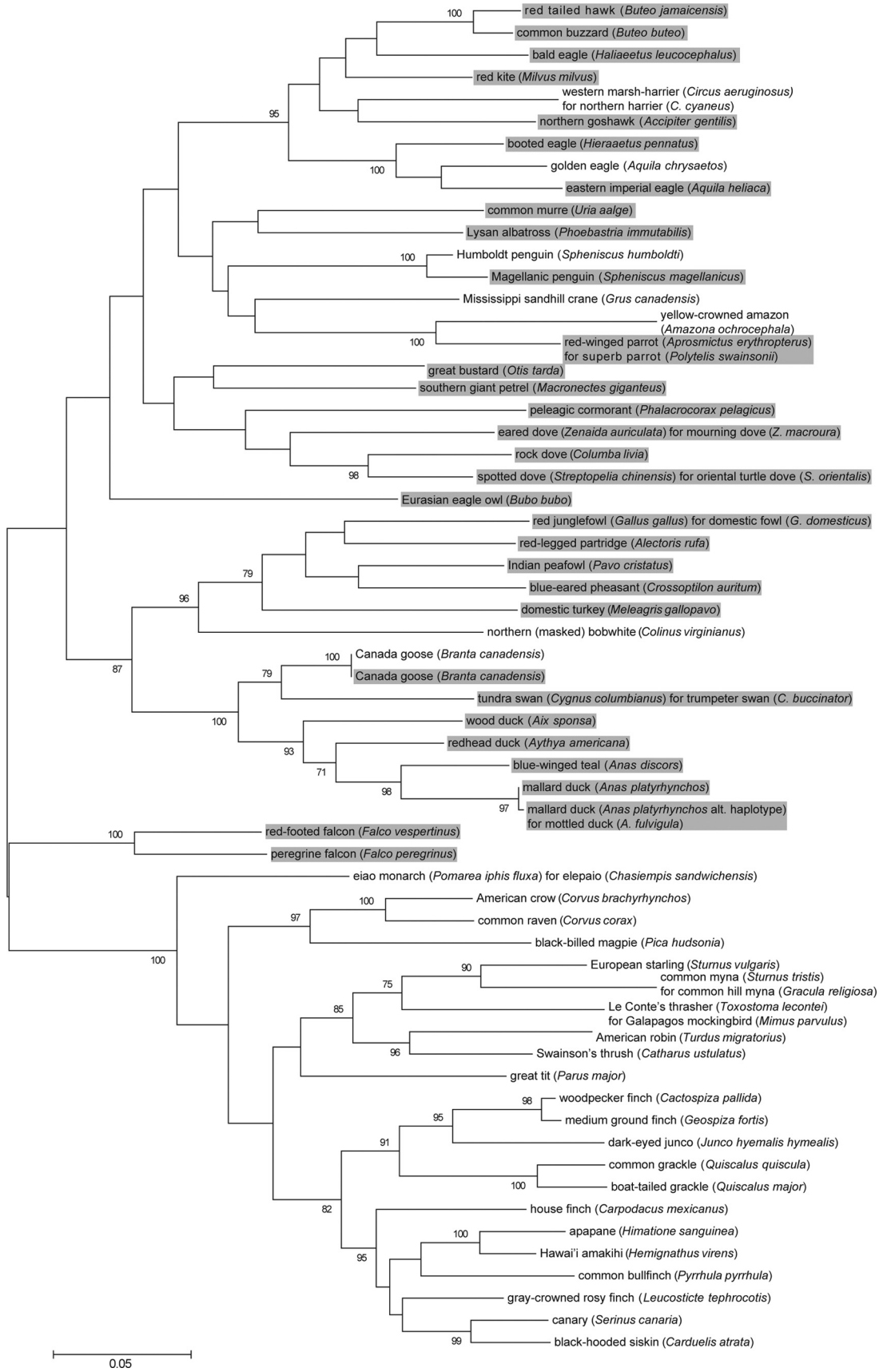

FIG 4 Maximum-likelihood phylogeny of the hosts generated from the cytochrome *b* sequences from GenBank. Hosts of fowlpox viruses are highlighted by gray, and canarypox viruses are without highlight. Bootstrap values of >70 are shown. The scale represents the number of substitutions per site.

collected from around the world. Until now, the highly conserved 4b core protein gene was used as the sole pan-genus marker both in diagnostics and phylogeography (21, 24–27, 47). We show that the DNA polymerase gene is useful as another pan-genus marker, and the results of phylogenetic analyses are comparable to those based on the 4b core protein gene while the use of this additional gene provided the first opportunity to study the potential role of recombination in the evolution of avipoxviruses.

The updated classification of avian poxviruses, based on our concatenated Bayesian phylogeny and described below, primarily follows the nomenclature of Jarmin et al. (25). Three main clades (A to C) are differentiated within avipoxviruses (Fig. 1). Clade A appears to be the fowlpox clade, clade B the canarypox clade, and clade C the *Psittacinepox virus* clade. Clade A further differentiates into seven subclades. Subclades A1 to A4 were previously described (25). Subclade A1 is formed by *Fowlpox virus* in the narrowest sense. Subclade A2 was identified as *Turkeypox virus*, but it now appears to be more representative of a subset of pigeonpox viruses, as a large number of geographically diverse viruses isolated from the order Columbiformes are grouped here. When initially described by Jarmin et al. (25), subclades A3 and A4 contained only two and one sequences, respectively. Our study contributed a large number of novel sequences to these groups. It is now apparent that subclade A3 represents poxviruses of marine birds and subclade A4 those of falcons. Novel subclades A5 to A7 were identified in the present study. Subclade A5 appears to represent poxviruses of waterfowl, subclade A6 as a second, distinct group of pigeonpox viruses, and subclade A7 as poxviruses of raptors. Clade B was found to have three subclades as described earlier (25). Subclade B1 comprises the strict canarypox viruses. *Mynahpox* and *Starlingpox viruses* grouped together in subclade B2 and thus the use of the term "Sturnidaepox virus" is proposed. Considering that the isolates of subclade B3 originate from a narrow temporal and geographic range, we suggest it should be known as "Virginian epidemic avipoxvirus." The finding of Jarvi et al. (46) establishing that subclade B1 has two main clusters was confirmed by our study. The outlier containing the Berthelot's pipit isolate from Macronesia was described earlier (4), but two further outliers, including one from grackles, were identified here.

The mean genetic distance within clades A and B of avian poxviruses appears to be similar to that of the North American clade of orthopoxviruses, but it is about four to five times the mean distance between Old World orthopoxviruses. However, since the average divergence values within Avipoxvirus subclades are generally quite similar to those calculated for orthopoxvirus clades, we may equally consider the option that the current subclades could eventually be viewed as equivalent taxonomical units. This relatively large genetic divergence among avian poxviruses, as well as the topology of the phylogenetic trees, indicates that the *Avipoxvirus* genus is one of the more widely diverged genera of the *Chordopoxvirinae* subfamily.

There is some evidence in our data for recombination events in the evolutionary history of the studied avipoxviruses. Although the limited number of loci ($n = 2$) examined, their length, and their genomic separation (103 kbp in fowlpox virus AF198100) constrains the possible conclusions, it seems likely that the detected events occurred in relatively well defined ecological and phylogenetic frameworks. These events primarily involved viruses (within subclades A5, A6, or A7) circulating in closely interacting hosts, providing a natural interface for potential virus exchange and coinfection (e.g., between and within Accipitriformes, Columbiformes, and Anseriformes), while the case involving Sturnidae (subclade B2) additionally highlights the potential of virus diversification and adaptation linked to extensive, primarily anthropogenic changes in the geographic distribution and concomitant "unnatural" contacts between species (in zoo collections or between alien, invasive, and native resident species in the wild). The confirmation of the nature of these events and elucidation of their role in the evolution and function (e.g., pathogenicity, adaptation, etc.) of avian poxviruses would, however, require the study of complete genomic sequences.

The range of hosts infected by fowlpox viruses (clade A), as estimated by within-group genetic diversity, was not significantly greater than that for those infected by canarypox viruses (clade B), indicating that each clade infects a similarly diverse range of bird hosts. One caveat is that the effect of sampling bias on the phylogenetic results is unknown. Sampling for avipoxviruses is not systematic across hosts and some taxa, e.g., poultry and songbirds are more intensively sampled than other groups. Several isolates originated from quarantine facilities, aviaries, or zoos where unusual transmissions may occur (particularly between already stressed or diseased birds), resulting in lesions but probably representing "dead-end" events that would rarely occur in the wild and would not lead to sustained epornitics. Such phenomena could have occurred, for example, in the cases of the fowlpox virus-infected superb parrot in subclade A1, the canarypox virus-infected Humboldt penguin in cluster 1 of subclade B1, or the isolates of subclade B3, which were isolated from a wide range of different bird species within a short time range during hospitalization in a wildlife center in Virginia.

In general, avian poxviruses tend to be host family or order specific, but ecological niche, habitat, and geography may modulate this pattern. A clear example of host family/order specificity is the European starling, which harbors the same virus strain both in Europe and North America and is a close relative of mynahs, infected with a closely related virus. The viruses isolated from and largely specific to falcons and raptors are other good examples.

The circulation of certain poxviruses within a prey-predator system can be recognized in several subclades (e.g., subclades A2 and B1). For example, we hypothesize that eastern imperial eagles may acquire pox infection from their dove prey (subclade A2) and northern harriers from a passerine species (subclade B1).

Another example of the role of the ecological niche and/or habitat lies with the poxviruses of marine birds (subclade A3), where evolutionarily distinct avian species with similar lifestyles harbor related viruses. In this case, although the isolates showed wide spatial separation, the effect of geography could not be excluded completely since these hosts migrate widely and share breeding sites where poxvirus infections could be transmitted and sustained. Except for this situation, geography seems to have only a minor effect on the avipoxvirus phylogeny, but it should not be dismissed, as in the case of the "Virginian epidemic avipoxvirus," where the hospitalized birds infected each other.

An interesting phenomenon can be observed in cluster 2 of subclade B1. Viruses of this cluster infect different passerines, including all of the analyzed house finch isolates, with diverse retrieval dates and geographic origins. The timeline of sample collection indicates that the ancestor of cluster 2 might have been a house finch virus (see samples P64 to P71 and P73 in Fig. 1 to 3),

the variants of which were subsequently dispersed around the Western Hemisphere and infected other bird species.

The data presented here provide novel insights into the complex relationship between avian poxviruses and their hosts. Generation of a significant number of whole-genome sequences of viruses from key points in the tree presented here would help to solve emerging problems in the conservation of endemic bird species and decrease pox-related economic losses in the poultry industry.

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