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# Poxvirus infection in a Steller's sea eagle (*Haliaeetus pelagicus*)

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**ABSTRACT.** A severely emaciated adult Steller's sea eagle *(Haliaeetus pelagicus)* was found dead with electrocution-induced severe wing laceration, and with multiple cutaneous pock nodules at the periocular regions of both sides nearby the medial canthi and rhamphotheca. Histopathological examination of the nodules revealed hyperplasia of the epidermis with vacuolar degeneration and intracytoplasmic inclusion bodies (Bollinger bodies). The proventriculus was severely affected by nematodes and was ulcerated. Nucleotide sequencing of a PCR-amplified product of *Avipoxvirus* 4b core gene revealed 100% identity to the sequence of *Avipoxvirus* derived from other eagle species. This report describes the first detection of *Avipoxvirus* clade A from a Steller's sea eagle.

**KEY WORDS:** 4b gene, *Avipoxvirus*, Japan, Steller's sea eagle

Avian pox is an infectious disease of both domestic and wild birds, caused by *Avipoxvirus*, related to family *Poxviridae* and subfamily *Chordopoxvirinae*. The *Avipoxvirus* is a double-stranded DNA virus, with a large size (mostly measures 270 × 350 nm) and oval shape. The genes located within the central core are involved in the virus replication, whereas the terminal genes encode proteins responsible for host range restriction [20]. The virus is characterized clinically by the development of pock lesions either on the skin of the featherless areas (cutaneous) as well as oral mucosa can be affected by poxvirus [23]. Current studies have reported the transmission of avian poxvirus among wild birds; therefore, infection of this virus is a potential emerging disease [6].

Steller's sea eagle (*Haliaeetus pelagicus*) is one of the predatory bird species related to the family *Accipitridae*, which inhabits the tropical part of Asia. They over-winter primarily in Hokkaido, the southern Kamchatka, and the Kuril Islands, with some birds in northeastern China, the Korean Peninsula, and western Japan [13]. Lead toxicity is a common cause of death among Steller's sea eagles [9, 11, 15] in Hokkaido district, but pox infection in Steller's sea eagles has not been previously reported. In Japan, a few published studies have reported the occurrence of avian pox in wild and captive birds. In wild birds, pox infection was reported in a white-tailed sea eagle (*Haliaeetus albicilla*) [16], field sparrow [21], and carrion (*Corvus corone*) and large-billed crows (*Corvus macrorhynchos*) [6]. In captive birds, pox infection was reported in a Japanese rock ptarmigan (*Lagopus mutus japonicus*) [22] and flamingos (*Phoenicopterus roseus*) [18]. The aim of the present report is to describe, for the first time, avian pox infection in a Steller's sea eagle using pathological findings and phylogenetic classification of the detected avian pox virus 4b gene.

A female Steller's sea eagle was found dead in poor body condition with marked prominence of keel bone, feather excoriation, and a wide skin laceration in the right wing (about 10 cm). These findings indicate that the bird died possibly from electrocution. Multiple cutaneous nodules were seen with and without crust formation on the skin of both sides of the beak mostly on the medial canthi of the right eye (5 mm in diameter) (Fig. 1A) and left eye (30 mm in diameter) (Fig. 1B). The other parts of the beak, mouth commissures, oral cavity, and legs were free of lesions. Postmortem examination revealed large numbers of nematodes in the proventriculus mucosa forming an ulcerative nodule (15 × 15 mm) (Fig. 1C). Skin, liver, heart, spleen, kidney, lungs, trachea, proventriculus, gizzard, duodenum, ileum, caecum, and brain specimens were fixed in 10% neutral buffered formalin. All tissue samples were dehydrated in alcohol, cleared in xylene then embedded in paraffin, sectioned at 5 $\mu$m, and stained with hematoxylin

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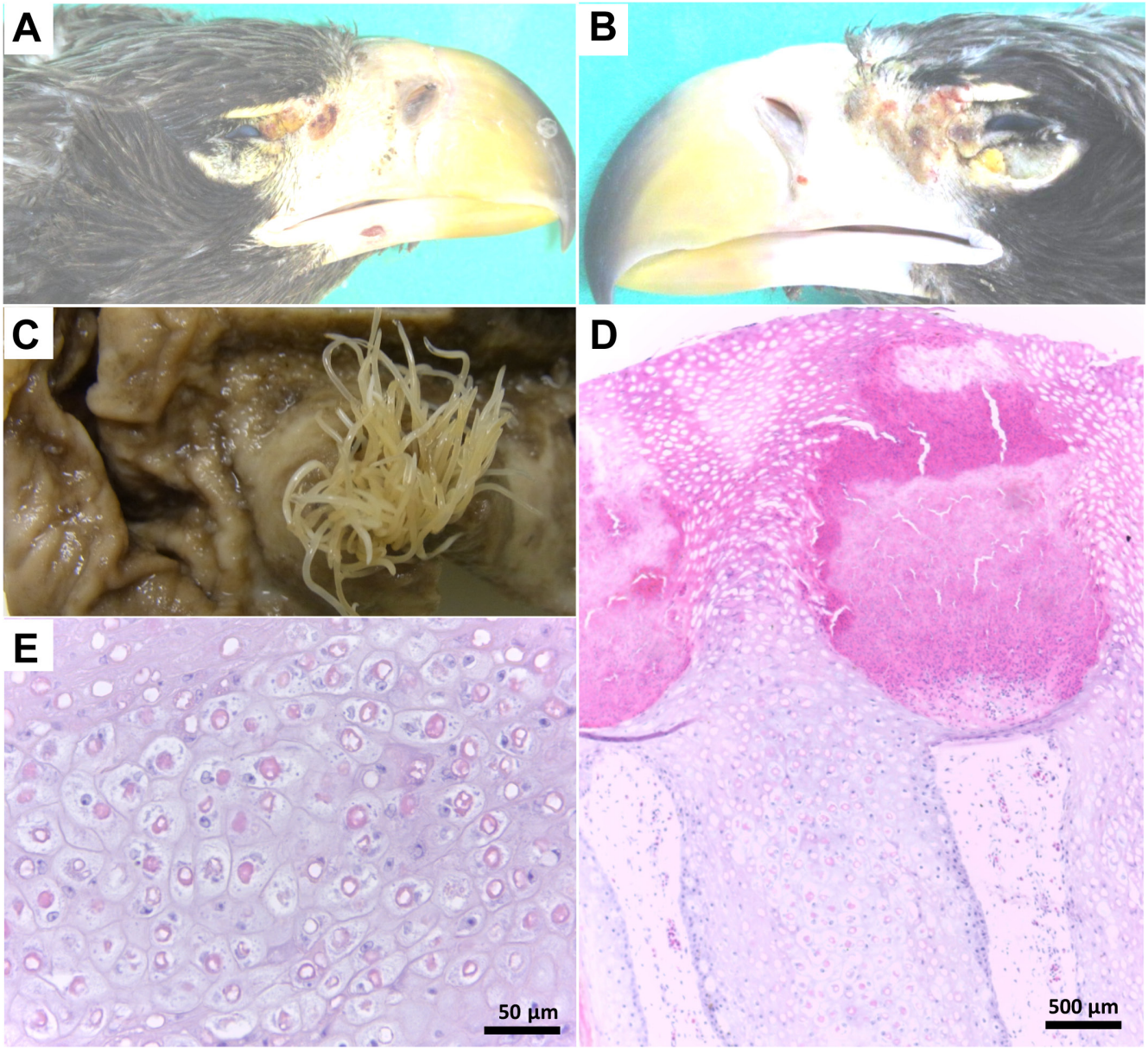


**Fig. 1.** Figure 1A and 1B showed multiple crusts on the skin of both sides of the beak, mostly on the medial canthi of the right (A) and left eyes (B). (C) Ulcerative parasitic nodule of the proventriculus mucosa with large numbers of nematodes. (D) Cutaneous lesion in a Steller's sea eagle. Necrosis and proliferation were observed in the stratum spinosum. H&E, bar=500 $\mu$m. (E) Large eosinophilic inclusion bodies (Bollinger inclusions) were seen in the cytoplasm of epidermal cells. H&E, bar=50 $\mu$m.

and eosin (H&E).

The histopathological findings of the skin nodules of both sides of the eagle's beak revealed hyperplasia of the epidermis with vacuolar degeneration (Fig. 1D) and intracytoplasmic inclusion bodies (Bollinger bodies) (Fig. 1E). Most of keratinocytes appeared swollen (ballooning degeneration) with necrosis of the overlying layer, which was admixed with bacterial colonization. The dermis showed congestion and severe heterophilic and eosinophilic infiltration. Lesions within the gastrointestinal tract were not related to pox virus infection. Furthermore severe nematode infestation was observed within the proventriculus. Multifocal granulomatous lesions were noted around the cross-sections of the parasites involving the mucosa, submucosa, and glandular layers. Inflammatory cells, such as lymphocytes, macrophages, eosinophils and giant cells were found. Chronic myocarditis was characterized by mononuclear cell infiltration between the cardiac muscle bundles. Congestion, hemorrhage between muscle bundles, and focal bacterial pericarditis were also observed.

For polymerase chain reaction (PCR), DNA was extracted from homogenates of formalin-fixed paraffin embedded tissue (FFPE) of cutaneous nodules using a QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol [7]. The sample DNA was subsequently amplified using specific primers for *Avipoxvirus* 4b core protein gene

using; P1 forward (5′CAGCAGGTGCTAAACAACAA′3) and P2 reverse (5′CGGTAGCTTAACGCCGAATA′3) [12]. PCR amplifications were performed using Takara PCR Thermal Cycler Dice. The PCR was performed at 94°C for 1 min (denaturation), 60°C for 1 min (annealing), and 72°C for 1 min (extension) for 35 cycles after initial denaturation for 2 min at 94°C. The final extension step was for 5 min at 72°C. The PCR products were separated by electrophoresis on 1.2% agarose gel stained with ethidium bromide and were visualized under ultraviolet illumination [16].

Purification of DNA bands was performed using QIAquick Gel Extraction Kits® (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's recommendations. Purified PCR products were directly sequenced with DNA analyzer using the same primers as for the PCR reactions. DNA sequencing of PCR products was determined using an ALFred sequencer (Pharmacia Biotech, Tokyo, Japan). The sequences were analyzed with the aid of Genetyx-Mac (version 13.0.2; Genetyx Co., Tokyo, Japan). Evaluation of sequence specificity was initially performed using the program Nucleotide BLAST of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast/) through comparison of the sequences detected in the current study with that previously deposited in GenBank, on the basis of optimization of the BLAST program to search for and compare our sequences with the highly similar sequences (Megablast) in the GenBank database. Alignments of sequences were performed utilizing the MUSCLE algorithm. The phylogenetic tree was constructed using the neighbor-joining method, and the tree was drawn using NJplot [17]. The topological accuracy of the tree was estimated using the bootstrap method with 1,000 replicates [4].

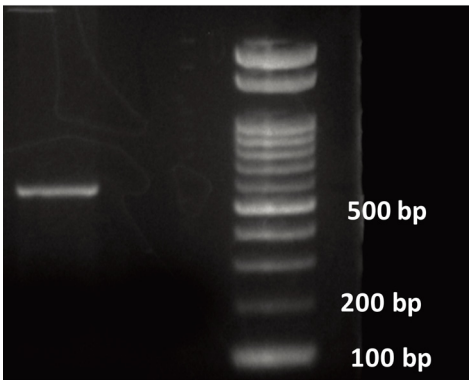


**Fig. 2.** Gel electrophoresis of the amplification product of the *Avipoxvirus* 4b core gene from crusted cutaneous lesions. A positive band of approximately 576 bp is detected.

PCR for avian pox virus 4b core gene yielded a 578-bp product (Fig. 2) as expected from DNA samples amplified using P1 and P2, as previously described [12]. The 4b core protein gene of avian poxvirus sequence obtained in the current study was deposited into the GenBank database under the accession number MG934698. Running our sequence on the BLAST tool of the GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) that was optimized for searching highly similar previously published *Avipoxvirus* sequences (Megablast option). The identified sequences were identical (100% homology) with those derived from the bald eagle (*Haliaeetus leucocephalus,* KR733092) from U.S.A.; Harris's hawk (*Parabuteo unicinctus*, KC693506) from Peru; red-tailed hawk (*Buteo jamaicensis,* KC018006) from Wisconsin, U.S.A.; and bald eagle (KC018003) from Florida, U.S.A.

A homology of 99% was detected between the sequences of the present isolate and those isolated from the white-tailed eagle (AB576861) from Betsukai, Hokkaido, Japan; crested serpent eagle (*Spilornis cheela*, HQ441566) from Taiwan, and red kite (*Milvus milvus*, KC018010) from Spain.

A 96% similarity was observed between the detected sequence and the previously published 4b sequences of *Avipoxvirus* from the white-tailed sea eagle (KF956003) from Germany; Eurasian crane (*Grus grus*, KF956000) from Germany, black-browed albatross (*Thalassarche melanophris,* AM050392) from the U.K.; common murre (*Uria aalge*, KC017986) from Midway Islands, U.S.A.; southern giant petrel (*Macronectes giganteus,* KC017981) from Antarctica; and Eurasian eagle owl (*Bubo bubo*, KC017984 and KC017983) from South Korea.

Three major strongly supported clades (A, B and C) were detected in the phylogenetic tree. The sequence of the *Avipoxvirus* isolated from the Steller's sea eagle in the current study was located within clade A with other birds of prey from Spain, Peru, U.S.A., and Taiwan (Fig. 3).

The present study aimed to report a poxvirus infection in a Steller's sea eagle. Pox virus infection has been prevalent in commercial chickens as well as in many wild birds. The infection may be primary, secondary, or combined [1]. The cause of death of the present case was not clear e.g. pox infection, nematodal gastric ulceration or electrocution. It is possible that, the pox lesions on the medial canthi and maxillary rhamphotheca might negatively affect the vision of this eagle, and thereby impaired its predation capability that leads to emaciation. In addition, impaired vision might increase the possibilities of getting injuries (like laceration at right wing) and electrocuted by the power lines. On the other hand, parasitic infestation is a common finding in wildlife birds that the severe parasitic infestation in the present case may be just a consequence of immunocompromising due to malnutrition. This is the first study to report an *Avipoxvirus* infection in a Steller's sea eagle. The histological examination showed the presence of Bollinger bodies within vacuolated epidermal cells consistent with avian pox particles [19]. The vacuolization was attributed to the fatty nature of inclusion bodies, which includes the virus particles [1]. The pathological lesions were similar to pox lesions seen in avian species including a white-tailed sea eagle [16], peregrine falcons (*Falco peregrinus*) [3], red-tailed hawk [5], Spanish imperial eagle (*Aquila adalberti*) [8], and crows [6]. In the present case, the cutaneous dry form of avian pox is similar to that in raptors [14]. Avian poxviruses induce intracytoplasmic, lipophilic inclusion bodies (Bollinger bodies) that infect epithelial cells of the integument, resulting in hyperplasia of the affected cells. A cutaneous form is characterized by variously sized nodular proliferations of unfeathered skin around the eyes, beak, nares, and legs [14].

The 4b core protein was reported as a highly conserved gene in the *Avipoxvirus* genome [2]. There were 447 nucleotide positions in the final dataset. Evolutionary analyses were conducted using the MEGA7 software [10]. The Avipox 4b gene sequence under accession number LC055564 isolated from the large-billed crow from Asahikawa, Hokkaido, Japan, was used as out-group for the current evolutionary analysis.

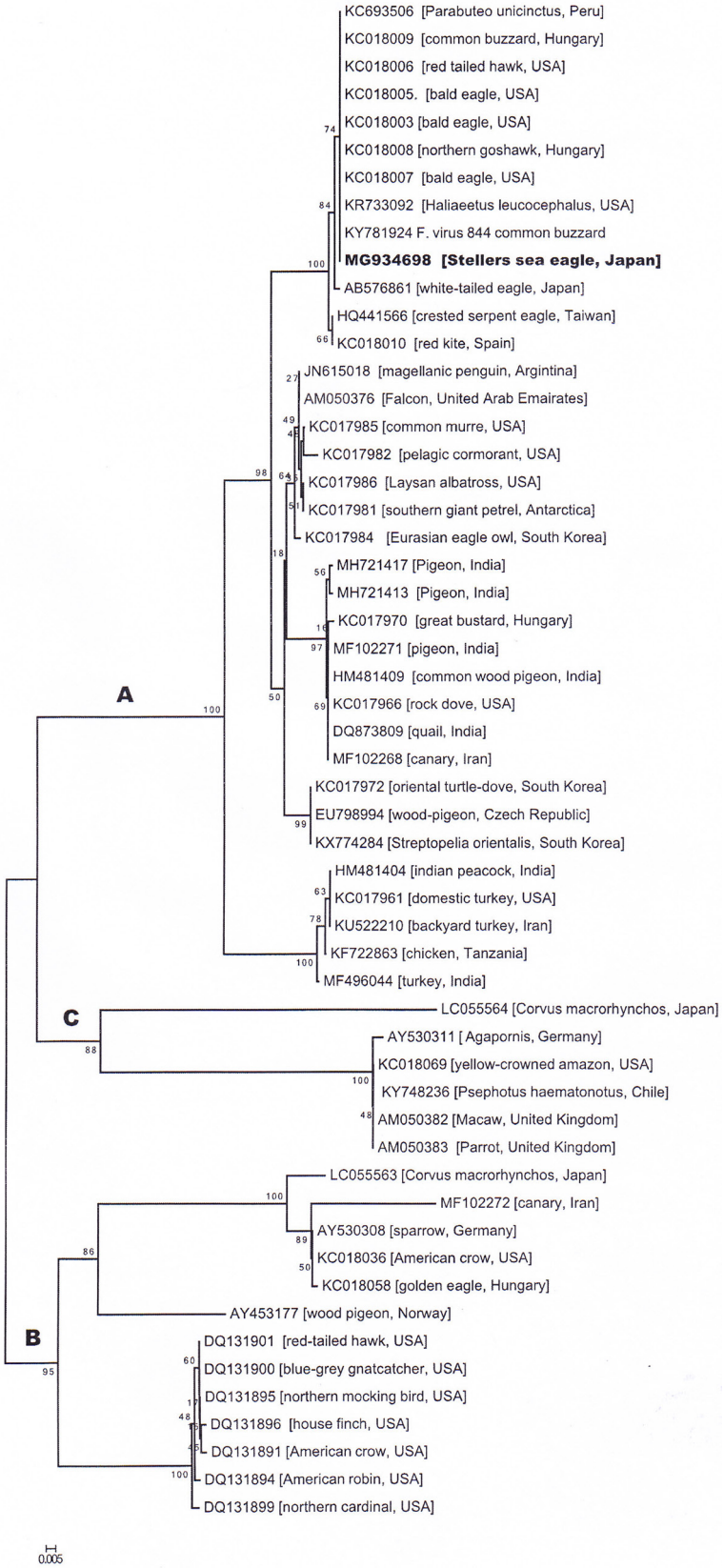


**Fig. 3.** Phylogenetic tree of the *Avipoxvirus* 4b core gene sequence. Sequences from various avian hosts are represented on the cladogram by accession numbers in GenBank and their geographic locations. The black square indicates the sequence obtained from the present case of a Steller's sea eagle (accession number MG934698). Bootstrap values were calculated with 1,000 replicates and were located on the nodes or the bifurcation points of the tree branches. The scale bar represents the genetic distances among different taxa included in the neighbor-joining tree. The *Avipoxvirus* 4b gene sequence under accession number LC055564 isolated from a large-billed crow (*Corvus macrorhynchos*) was utilized as out-group.

The relative evolution data showed that the nucleotide sequence detected in this study was clustered in clade A with the poxviruses detected from raptors and some other bird species. The sequences derived from the bald eagle (KR733092) from U.S.A.; Harris's hawk (KC693506) from Peru, red-tailed hawk (KC018006) from Wisconsin, U.S.A.; and bald eagle (KC018003) from Florida, U.S.A., had a 100% homology. Saito *et al.* (2009) previously reported an *Avipoxvirus* infection in a white-tailed sea eagle in Japan. A phylogenetic tree was constructed based on nucleotide sequences, and the virus was clustered in clade A.

The pathological and molecular findings of the present case were consistent with *Avipoxvirus* infection in eagles [16].

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