Research Article

Circulation of A2 subclade of Avipoxviruses in pigeons of Andaman and Nicobar Islands: First report from India

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Abstract

Genus *Avipoxvirus*, an important member of the family *Poxviridae*, has twelve species which have been recognized by the International Committee on Taxonomy of Viruses (ICTV). *Fowlpox* virus and *Pigeonpox virus* are two important species which may affect other species of birds besides chickens and fowls. For that reason, accurate identification of species, clades and subclades of Avipoxvirus is extremely important to design and implement adequate control strategies. The investigation was carried in a pigeon colony having the symptoms of pox. A fragment of viral pan-genus 4b (P4b) gene was amplified and sequenced. As an equivocal and prototype species, sequence information of fowl pox virus isolated from an infected bird was also generated. The generated sequence information was compared with those of previously reported strains. It was found that both fowl pox and pigeon pox virus belonged to clade A and there was circulation of A1 and A2 subclades in chickens and pigeons, respectively. From India, one report showed circulation of A1 and A3 subclades in Indian chickens and pigeons, respectively since other reports were based on clinical symptoms, histopathological examination and comparison of sequence information with available sequence information without distinct demarcation of clade and subclade of Avipoxvirus. The present report describes findings of the novel A2 subclade of Avipoxvirus and existence of pigeon pox in Andaman and Nicobar Islands for the first time.

Keywords: Fowl pox, Pan-genus 4b locus, Pigeon pox, Clade and subclade

Introduction

POX is a very well-known disease for its wide host range since pox viruses may infect humans, animals, avian species as well as invertebrates. This double stranded DNA virus with a genome size of 250-400 kb is very much prevalent in avian species that involves nearly 374 species of 23 orders which virtually includes domestic as well as wild birds but

not seen in kingfishers and other related species due to their less species richness (contributes 1.7% of avian species) or less contact with other species of birds[1]. Avian pox viruses belong to the genus *Avipox* virus under the family *Poxviridae* within the subfamily *Chordopoxvirinae*[2]. A total of 12 species have been recognized by the 'International Committee on Taxonomy of Viruses' besides a proposal of two more species[3] using two main criteria viz. phylogenetic distance and natural host[4]. Pigeon is the natural host for pigeon pox virus albeit may affect and cause mild disease in chickens, turkeys, ducks and goose[5,6]. Like other avian pox viruses, pigeon pox occurs in two different forms; cutaneous and diphtheritic forms. The former manifestation occurs from mechanical injury and through bite of arthropod vectors. The clinical sign is characterized by proliferative nodular lesions on featherless parts of the body. Diphtheritic form occurs due to inhalation or ingestion of virus and there is probability of high mortality due to obstruction of oropharynx[7,8].

There is re-emergence of Avipox virus in different avian species[9] and some of the reports have been made based on clinical signs and histopathological examinations[10-12]. Few recent reports of Avipox virus in domestic and wild birds have been made based on phylogenetic signalling of Pan-genus 4b (P4b) gene locus[9,13,14]. P4b is the most preferable molecular marker for detection of avian poxviruses as it is highly conserved among all poxviruses[14]. Till date, there is only one report on subclades of circulating Avipox virus from mainland of India[9]. Therefore, through this communication we report for the first time phylogenetic position of pigeon pox virus from Andaman and Nicobar Islands, which is separated from mainland of India by natural geographical barrier along with a claim of circulation of a unique clade which is yet to be reported from India.

Materials and Methods

Ethical permission

Ethical clearance to carry out the study was approved by the Institute Animal Ethics Committee of ICAR-Central Island Agricultural Research Institute (ICAR-CIARI), Port Blair, Andaman and Nicobar Islands, India (Approval letter no. ICAR-CIARI/IAEC/2021/209 dated 13.06.2021).

Study area and sample collection

A colony of pigeons (n=60) in a residential complex of Garacharma, Port Blair situated in South Andaman was seen to be affected with wart like lesions in the month of June, 2021. Observations on clinical symptoms, gross lesions and morbidity and mortality of the birds were recorded. For confirmation and genetic characterization of the virus, nodular wart lesions were collected. Nodular lesions were excised from infected birds and collected in separate vials containing 0.5 ml phosphate buffer saline (pH 7.2). Two randomly chosen samples from infected pigeons were collected. In addition, for characterizing Avipox virus in chickens, biological material (nodular wart lesion, one sample) was collected from an organized poultry farm in South Andaman.

Nucleic acid extraction, amplification, and sequencing of P4b gene fragment

For extraction of DNA, QIAamp MinElute Virus kit (QIAGEN GmbH, Germany) was used. For amplification of partial fragment of P4b gene, primer design was adopted from Huw Lee and Hwa Lee[15] with minor modifications. The primer sequences were as follows:

Forward: 5' CAGCAGGRGCTAAACAACAA 3’

Reverse: 5’ CGGTAGCTTAACGCCGAAAA 3’

PCR amplification was carried out in a 25 µl reaction mixture containing 10X PCR buffer, 25 mM $MgCl_2$, 10 mM dNTPs, 1.5 U Taq DNA polymerase, 10 pM of each primer and 25 ng of DNA template. Cycling conditions remained the same as described earlier[14]. The PCR product was run through 1.2% w/v agarose gel and visualized in a Gel Documentation system (Labmate Asia Pvt. Ltd., Chennai, India). PCR products were directly sent to a commercial company (Eurofins Scientific India Pvt. Ltd., Bengaluru, India) for generation of sequence information on both ends.

Bioinformatics analysis

Generated sequence information was evaluated, aligned, and compared with the available sequence information using Basic Local Alignment Search Tool (BLASTn) implemented in NCBI (ncbi.nlm.nih.gov). Currently, a total of five genetic lineages of Avipox virus have been reported as clades (A-E) and clade A is further subdivided into seven subclades (A1-A7). Accession numbers of clades and subclades of Avipox virus used in the present study have been presented in Supplementary Table 1. Nucleotide variation of Andaman isolates of pigeon pox and fowl pox with different clades and subclades of the genus *Avipoxviruses* was carried out in MEGA X[16]. Phylogenetic analysis based on nucleotide sequence information was performed by using neighbour joining method[17] implemented in MEGA X with 1000 bootstrap replications[16]. For phylogenetic analysis, we trimmed extra and ambiguous positions from our sequences and GenBank retrieved sequences to make a homogeneous length of 410 bp dataset. To visualize the genetic relationships among different sequences, median-joining networks were constructed in PopART ver. 1.7[18].

Results

In the pigeon colony of 60 birds, morbidity rate was 31.66 % with a mortality rate of 16.66 %. The affected birds had nodular lesions on the eye lids, beak as well as on the face. The affected eyes were totally closed (Figure 1). The clinical symptoms observed were loss of appetite, dyspnoea, gasping and dullness. In an organized poultry farm in South Andaman, the morbidity in chicken was 20.34 % and mortality was 2.77 %. To rule out the possibility of infection with avian influenza, serum samples from infected birds were screened for detection of Bird Flu antibody using Bird Flu antibody ELISA test kit (Life Technologies, New Delhi, India) and all the samples were found negative for avian influenza.

DNA extracted from the nodular lesions after amplification with P4b gene fragment specific primer set yielded amplicon of 574 bp (Figure 2). Subsequently, sequence information generated in this study was submitted to GenBank with accession numbers OK483026-27 for pigeon pox and OK483028 for fowl pox.

Further, analysis on pairwise nucleotide variation revealed that, Andaman isolates of pigeon pox (OK483026-27) had variation of 0-1.22 with clade A, 0.311-0.323 with clade B, 0.317-0.320 with clade C and 0.344 with single isolate of clade D and 0.326 with single isolate of clade E (Figure 3). When compared with different sub-clades of A, Andaman isolate of pigeon pox exhibited no nucleotide variation with clade A2 and the highest nucleotide variation with clade A4 (Figure 4).

Phylogenetic analysis of data revealed that the Andaman isolates of Avipox virus (OK483026-28) belonged to clade A. Andaman isolates of pigeon pox (OK483026-27) were homologous to Indian isolates of Avipox reported elsewhere (DQ873811, MF496043, HM481408-9, NC_043178) and Avipox isolated from ostrich of unknown geographical location (AY530305). On the contrary, Andaman isolate of fowl pox virus shared cluster with

turkey pox and sparrow pox characterized from Germany (AY530304, AY530307) and fowl pox reported from China (KX196452) and had complete homology with Avipox virus reported from Germany (AY530304, AY530307) (Figure 5,6). Further analysis of data revealed that the Andaman isolates of pigeon pox (OK483026-27) belonged to the A2 sub clade and Andaman isolate of fowl pox was under A1 sub clade (Figure 7,8).

Discussion

Avian pox is a deadly viral disease and affects 374 avian species from 23 orders[1,19]. Avipoxvirus infection causes huge economic losses to poultry industry as it impairs growth, egg production and causes blindness and significant mortality to the poultry birds[5]. Moreover, it also reduces mating success; therefore, is considered as a risk factor for the conservation of endangered bird species[20]. Therefore, genetic diversity of Avipoxviruses needs to be understood to design and implement adequate control strategies[21]. Considering the fact, in the present study, genetic lineage of Avipoxvirus has been worked out by pan-genus 4b core protein using primers described[15] elsewhere with minor modification in the sense primer.

Avian pox is known to occur in two different forms i.e. dry or cutaneous form and diphtheric form. The latter form is less common and former one is more common in birds[2]. The cutaneous form of pox is characterized by wart like growth on feather free areas of birds like legs, feet, beak or eyes. Wart like growths are generally 1-5 mm in diameter and birds may not exhibit any clinical signs and symptoms. But in severe cutaneous lesions, there may be impairment of vision, loss of appetite and difficulty in breathing[22,23]. In the present study, the birds suffered from loss of appetite and lost their vision since lesions were seen on the eyes. The mortality rate was quite high (16.66 %) due to severity of the lesions.

In this study, we have characterized pigeon pox and fowl pox on the basis of sequence information and phylogenetic signalling of pan-genus P4b locus which is well known pre-requisite for characterization of Avipox viruses[24,25]. On the basis of sequence information, a total of five genetic lineages of Avipox virus have been reported as clades (A-E). Fowl, turkey, albatross, pigeon, ostrich, sparrow and falcon pox viruses come under clade A. Clade B includes canary, great tit, stone curlew and houbara pox viruses. Clade C comprises of macaw, parrot and agapornis pox viruses[8,26]. Later, Bányai *et al*.[21] and Manarolla *et al*.[27] described two unique clades designated as clade D and E. Our findings on clustering of pigeon pox and fowl pox in clade A (Figure 5,6) is in agreement with the findings of Jarmin *et al*.[8].

This has been further observed that, clade A is subdivided into seven subclades (A1-A7) and clade B is having three subclades (B1-B3)[8,27,28]. A1 subclade has been reported from chicken (China, Tanzania, Portugal, Nigeria, UK, France, Europe, Hungary, Egypt and USA), turkey (Egypt Iran, USA and Italy), parrot (Chile), macQueens bustard (UAE), blue-eared pheasant (Hungary) and paradise shelduck, variable oystercatcher, black robin, shore plover (New Zealand)[9]. A2 subclade has been reported from pigeon (Egypt, UK and South africa), turkey (Mozambique, UK and Italy), dove (Hungary), Indian peafowl (Hungary), macQueens bustard (UAE), grey partridge (Italy), gyrfalcon (Italy), as well as booted eagle, red kite, red-legged partridge (Spain)[9]. A3 subclade has been reported from feral pigeons (South Africa), oriental turtle dove (South Korea) and great bustard (Spain), A4 subclade has been reported from red-footed falcon and peregrine falcon (Hungary), A5 subclade has been reported from red head duck and trumpeter swan (USA), A6 has been reported from goose and mourning dove (USA) and A7 subclade has been seen in hawk and red kite (Spain)[9]. Among B clade, B1 subclade from canary (USA) and golden eagle (Spain), B2 subclade from European

starling (USA) and B3 subclade from American crow and house finch (USA) have been reported[9].

As far as the Indian scenario is concerned, several studies have described Avian pox infection including the circulation of virus in pigeon. Reports are available from Northern, Eastern, Western and Southern parts of India[9-12,14]. In only one study by Sahu *et al*[9]. circulation of subclades of A in pigeons and chickens was evaluated and it reported the circulation of A1 and A3 subclades of Avipox in Indian chickens and pigeons, respectively[8]. However, the present study reports the circulation of A2 subclade of Avipox virus in pigeon which is the first report from India. Difference in genotype may be due to the natural geographical barrier between the Andaman Islands and mainland India (Supplementary Figure 1).

During the present investigation, morbidity and mortality due to pigeon pox was more compared to fowl pox in chickens. This may be attributed to immediate isolation of infected chickens from the rest of the flock and also for providing palliative treatment with oral antibiotic and topical application of antiseptic ointment to check secondary infection. But such approaches are not possible to be implemented in the semi domesticated pigeon.

Animals and human play no role to spread pigeon pox and fowl pox infection. For controlling pox infection in pigeons and chickens, regular vaccination is advised. During the time of the outbreak, segregation of the infected bird and its palliative treatment with oral and topical antibiotic is advocated along with disinfection of the premises with commonly used disinfectants to prevent the spread of the virus. This is extremely important looking into the recent outbreak of monkeypox in humans in several countries.

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Figure legends

Figure 1. Pigeon showing lesion on the eye, on the beak and inside buccal cavity

Figure 2. Amplification of partial fragment of P4b core protein of pox virus (Lanes 1 & 2= Amplified products of pigeon pox and fowl pox, respectively, Lane M= 100 bp DNA ladder, arrows from the bottom 100, 200, 300 and 400 bp)

Figure 3. Pairwise nucleotide sequence variation of Andaman isolates of pigeon pox with A-E clades of Avipox virus (NV= Nucleotide variation). The prototype strains used are as follows; clade A: Fowlpox (KX196452, OK483028), Quailpox (NC_043178), Turkeypox (AY530304), Ostrichpox (AY530305), Falconpox (AY530306), Sparrowpox (AY530307), Indian little brown dovepox (HM481408), Common wood pigeonpox (HM481409), Pigeonpox (MF496043, DQ873811); clade B: Canarypox (AY318871, AY530309), Great titpox (AY453173, AY453174, AY453175), Stone curlewpox (AY530310); clade C: Agapornispox (AY530311), Macawpox (AM050382), Fowlpox in parrot (AM050383); clade D: Fowlpox in quail (GQ180200) and clade E: Turkeypox (KP728110)

Figure 4. Pairwise nucleotide sequence variation of Andaman isolates of pigeon pox with A1-A7 subclades of Avipox virus (NV= Nucleotide variation). The prototype strains used are as follows; subclade A1: Fowlpox (AM050377, GQ180207); subclade A2: Fowlpox (GQ180204); subclade A3: Great bustardpox (KC017974); subclade A4: Woodpecker finchpox (KC017949); subclade A5: Wood duckpox (KC017996); subclade A6: Mourning dovepox (KC018000) and subclade A7: Goshawkpox (KC018008)

Figure 5. Phylogenetic analysis of clades of Avipox virus based on nucleotide sequence on P4b gene fragment to work out the phylogenetic signalling of Andaman isolates of pox virus isolated from pigeon and fowl. The evolutionary distances were worked out using Neighbour joining method with 1000 bootstrap replications.

Figure 6. Network analysis of clades of Avipox virus based on nucleotide sequence on P4b gene fragment to work out the positioning of Andaman isolates of pox virus isolated from pigeon and fowl.

Figure 7. Phylogenetic analysis of subclades of Avipox virus based on nucleotide sequence on P4b gene fragment to work out the phylogenetic signalling of Andaman isolates of pox virus isolated from pigeon and fowl. The evolutionary distances were worked out using Neighbour joining method with 1000 bootstrap replications.

Figure 8. Network analysis of subclades of Avipox virus based on nucleotide sequence on P4b gene fragment to work out the positioning of Andaman isolates of pox virus isolated from pigeon and fowl.

Figures

Figure 1.

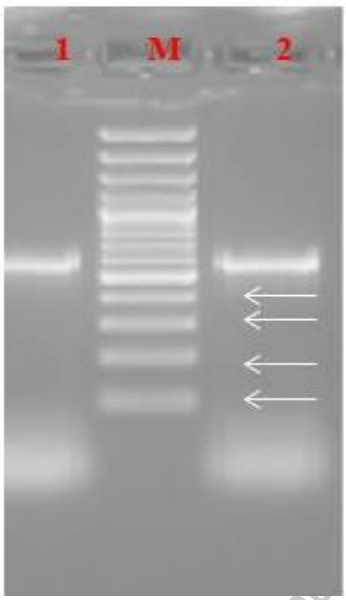
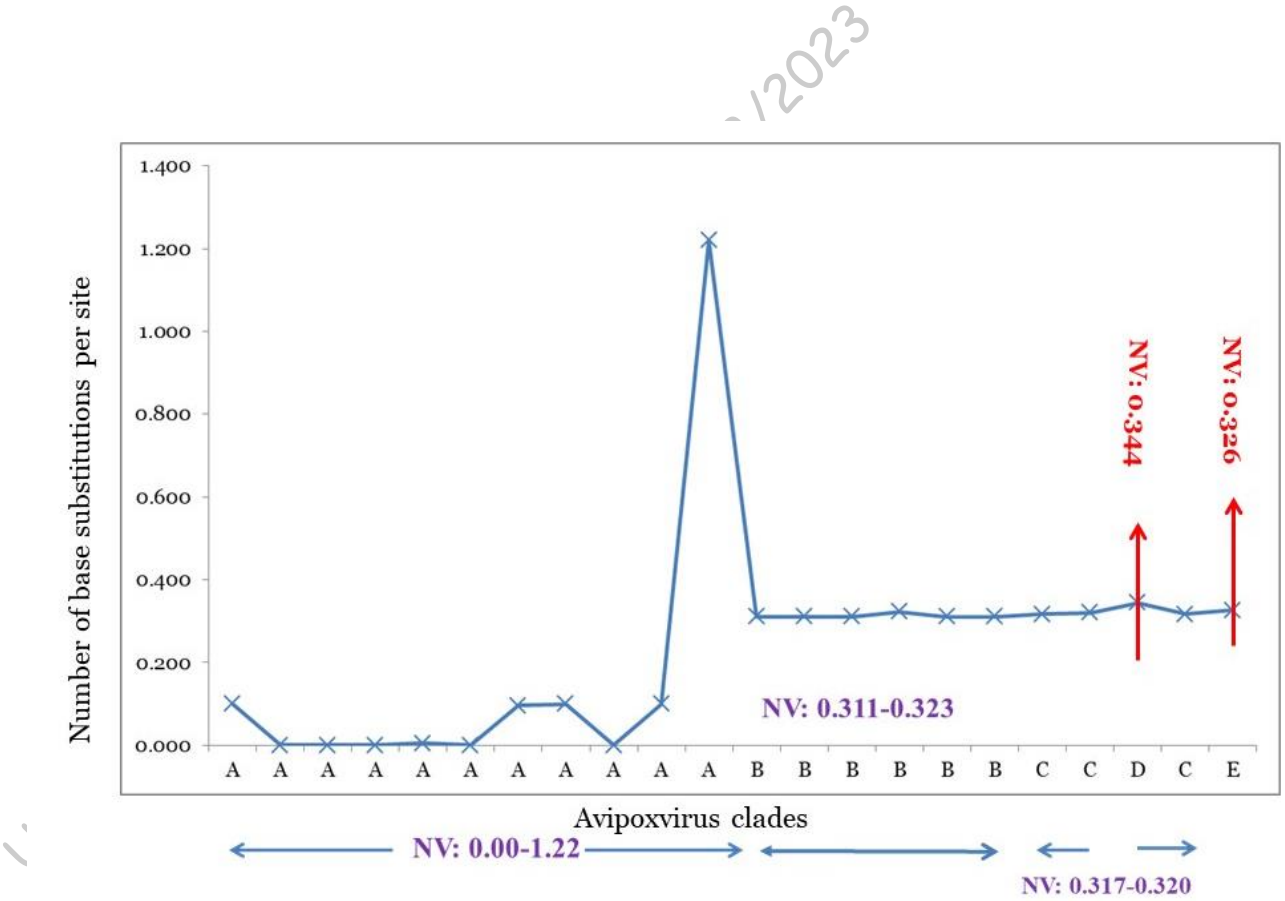


Figure 2.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1.000
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0.200
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A A A A A A A A A A A B B B B B B C C D C E
Avipoxvirus clades
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NV: 0.344
NV: 0.326
NV: 0.00-1.22
NV: 0.317-0.320

Figure 3.

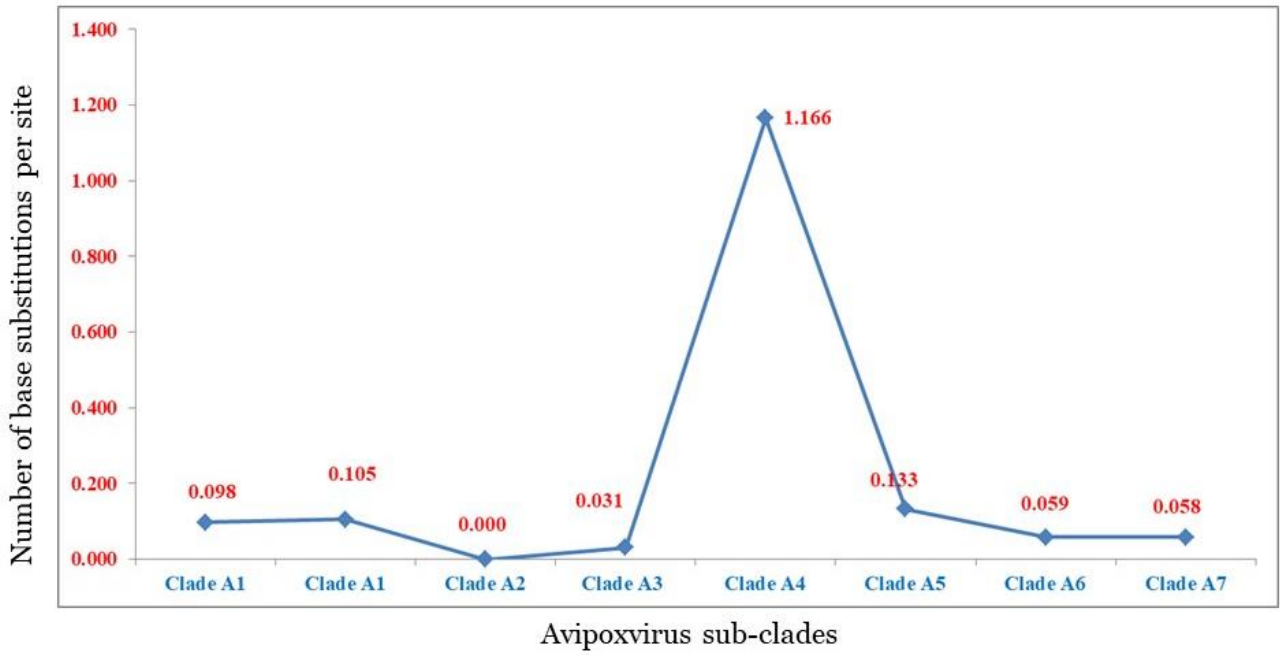
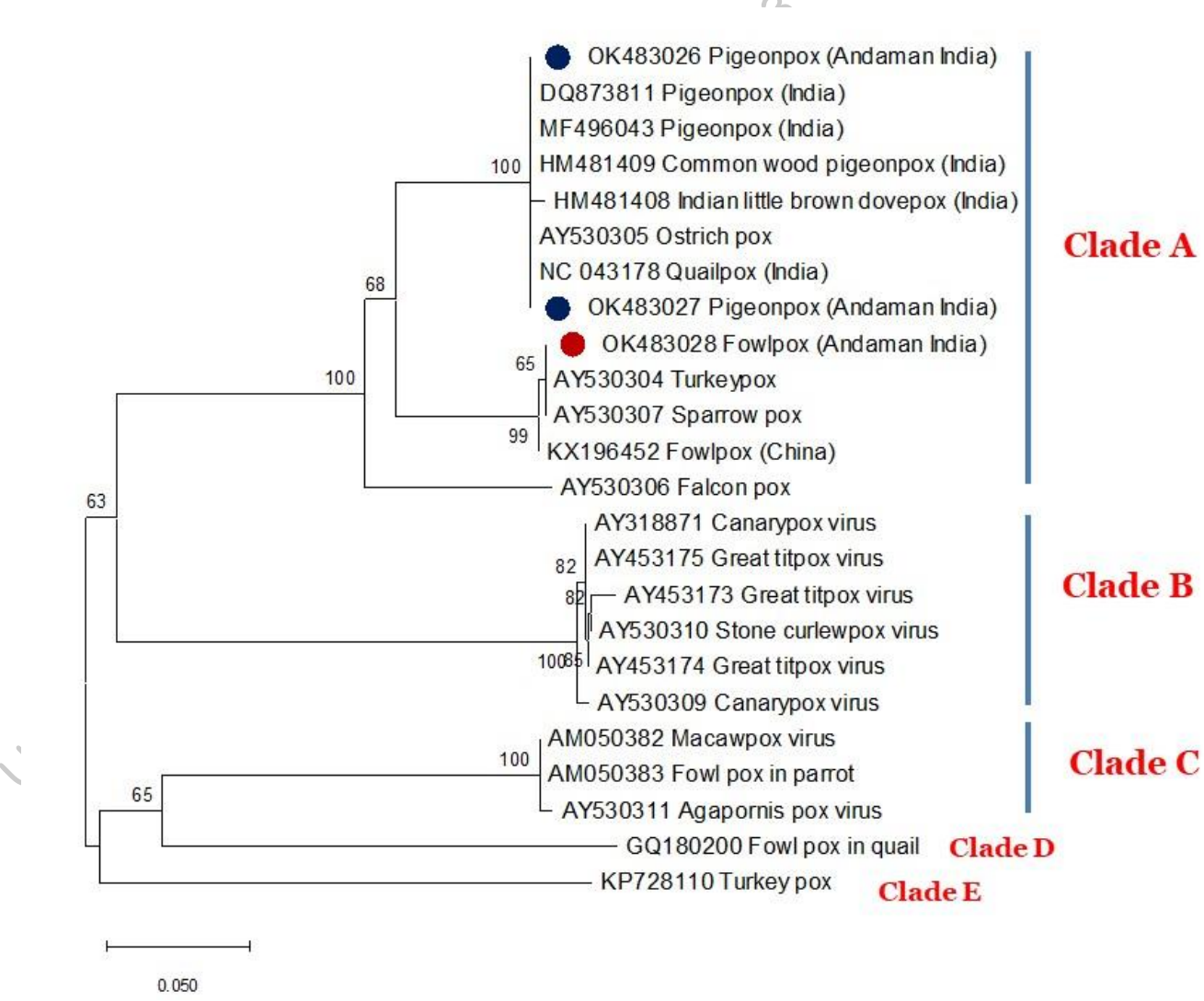


Figure 4.


OK483026 Pigeonpox (Andaman India)
DQ873811 Pigeonpox (India)
MF496043 Pigeonpox (India)
HM481409 Common wood pigeonpox (India)
HM481408 Indian little brown dovepox (India)
AY530305 Ostrich pox
NC 043178 Quailpox (India)
OK483027 Pigeonpox (Andaman India)
OK483028 Fowlpox (Andaman India)
AY530304 Turkeypox
AY530307 Sparrow pox
KX196452 Fowlpox (China)
AY530306 Falcon pox
AY318871 Canarypox virus
AY453175 Great titpox virus
AY453173 Great titpox virus
AY530310 Stone curlewpox virus
AY453174 Great titpox virus
AY530309 Canarypox virus
AM050382 Macawpox virus
AM050383 Fowl pox in parrot
AY530311 Agapornis pox virus
GQ180200 Fowl pox in quail
KP728110 Turkey pox
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68
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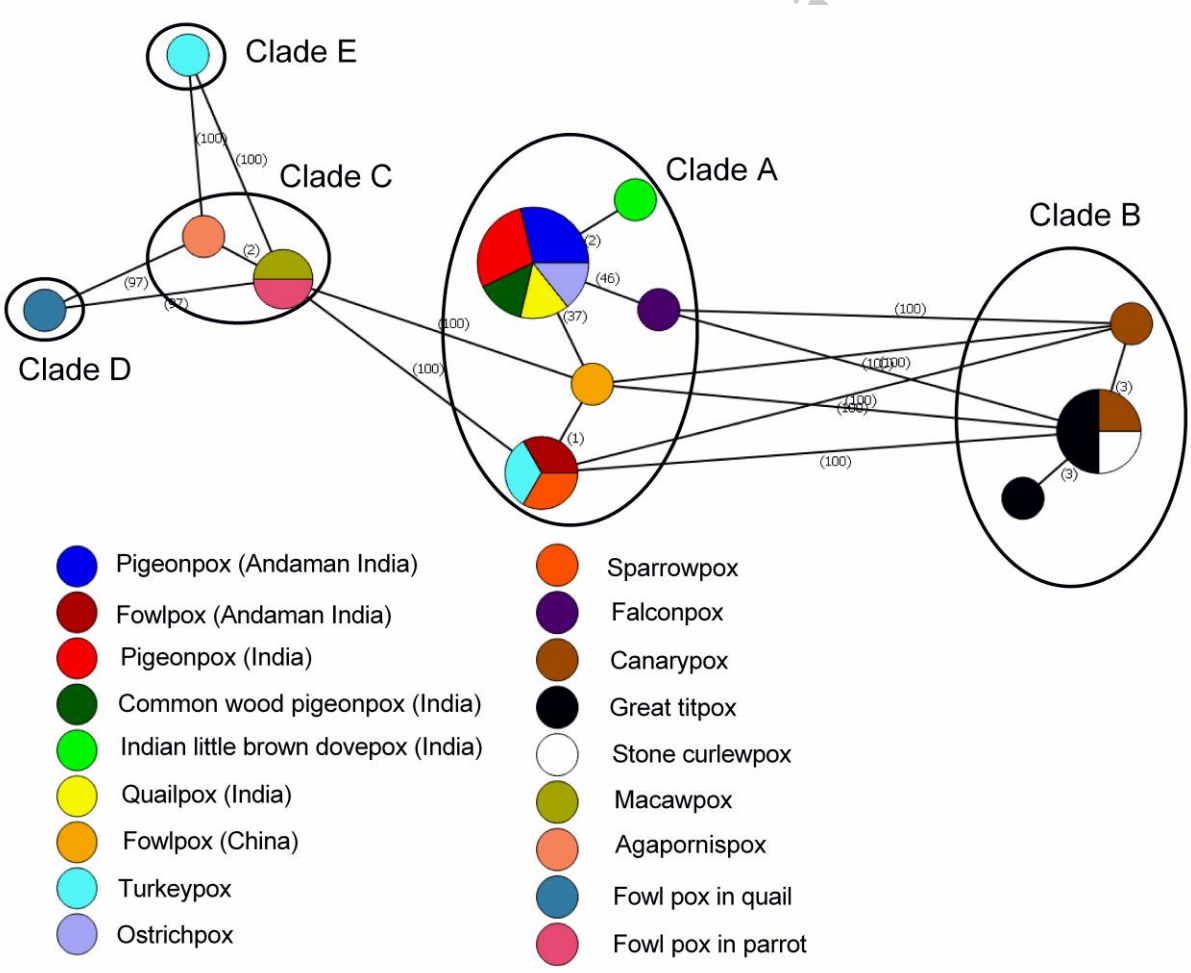
Figure 5.


Clade E
Clade C
Clade A
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(3)
Pigeonpox (Andaman India)
Fowlpox (Andaman India)
Pigeonpox (India)
Common wood pigeonpox (India)
Indian little brown dovepox (India)
Quailpox (India)
Fowlpox (China)
Turkeypox
Ostrichpox
Sparrowpox
Falconpox
Canarypox
Great titpox
Stone curlewpox
Macawpox
Agapornispox
Fowl pox in quail
Fowl pox in parrot

Figure 6.

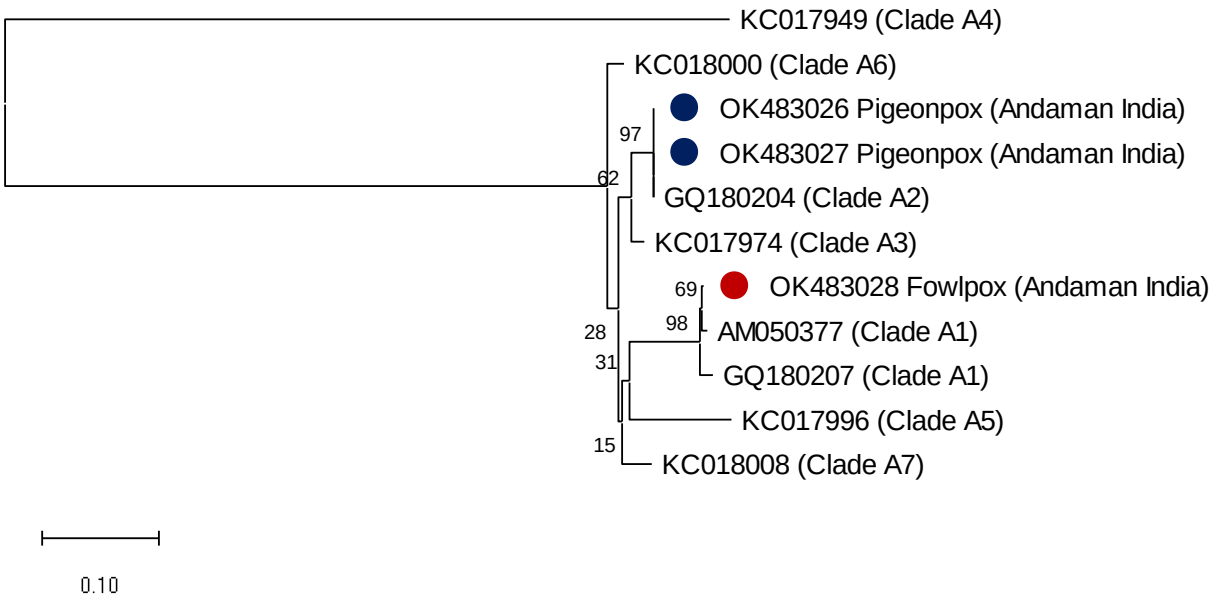


Figure 7.

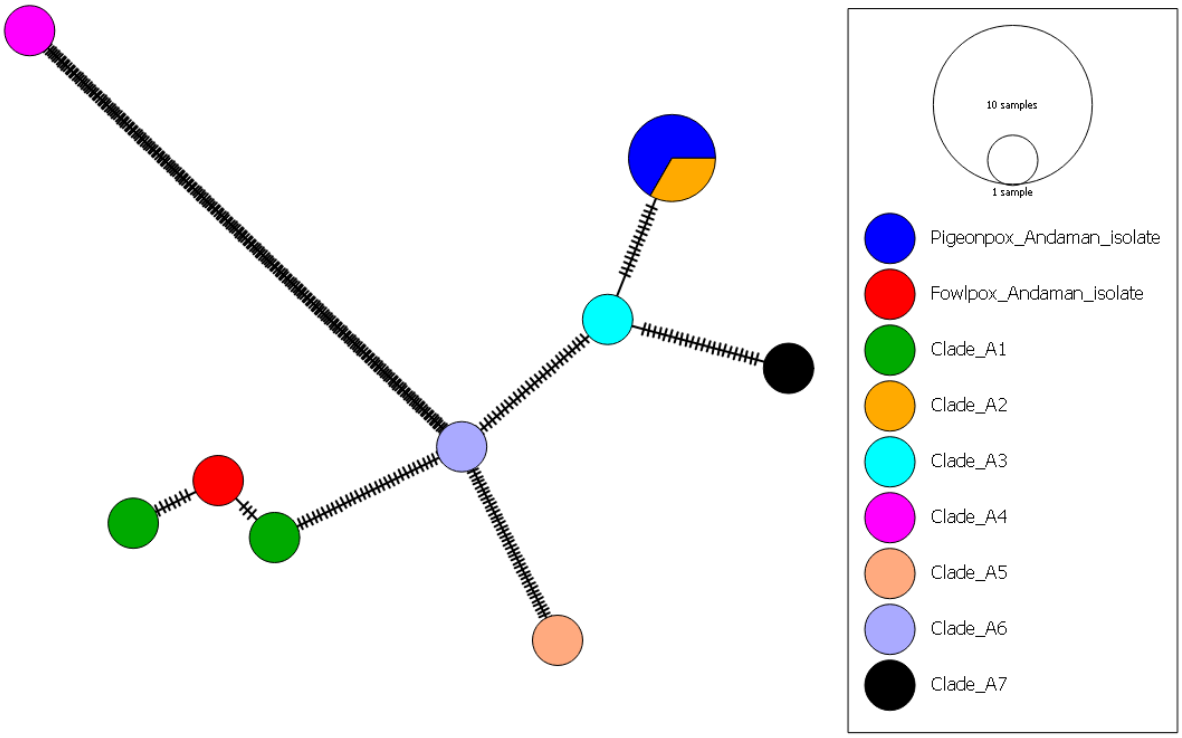
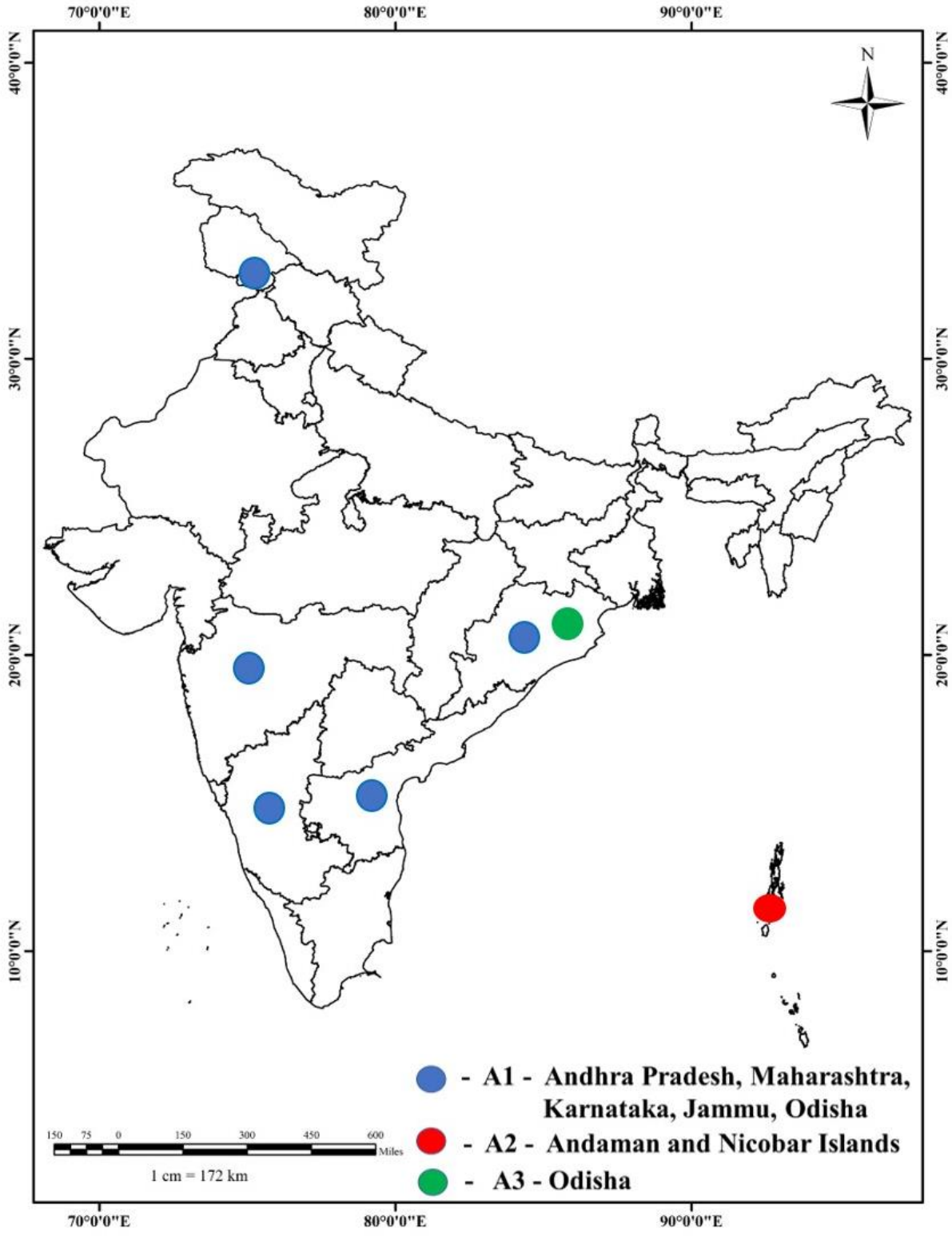


Figure 8.

Supplementary Table 1. Accession Numbers of clades and subclades of Avipox virus

Sl No.	Accession Number (Clade/Subclade)	Country	Host species
1.	DQ873811 (A)	India	Pigeon
2.	MF496043 (A)	India	Pigeon
3.	HM481409 (A)	India	Common wood pigeon
4.	HM481408 (A)	India	Indian little brown dove
5.	AY530305 (A)	-	Ostrich
6.	NC_043178 (A)	India	Quail
7	AY530304 (A)	Germany	Turkey
8	AY530307 (A)	Germany	Sparrow
9	KX196452 (A)	China	Chicken
10	AY530306 (A)	United Arab Emirates	Falcon
11	AY318871 (B)	USA: New Jersey	Canary
12	AY453175 (B)	-	Great tit
13	AY453173 (B)	-	Great tit
14	AY530310 (B)	United Arab Emirates	Stone Curlew
15	AY453174 (B)	-	Great tit
16	AY530309 (B)	-	Canary
17	AM050382 (C)	United Kingdom	Macaw
18	AM050383 (C)	United Kingdom	Parrot
19	AY530311 (C)	Germany	Agapornis
20	GQ180200 (D)	Italy	Quail
21	KP728110 (E)	Hungary	Turkey
22	AM050377 (A1)	United Kingdom	Chicken
23	GQ180207 A1)	Italy	Pheasant
24	GQ180204 (A2)	Italy	Grey Partridge
25	KC017974 (A3)	Spain	Great bustard
26	KC017949 (A4)	Ecuador: Galapagos Islands	Woodpecker finch
27	KC017996 (A5)	USA: Wisconsin	Wood duck
28	KC018000 (A6)	USA: Wisconsin	Mourning dove
29	KC018008 (A7)	Hungary	Northern goshawk

- = Not mentioned



Supplementary Figure 1. Map of India showing locations from where different subclades A1, A2 & A3 of Avipox virus were reported. Source file of Indian boundaries (.shp file) was downloaded from Survey of India official website. Further output map was created in ArcGIS 10.5v platform.