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Full Length Research Paper

Molecular characterization and phylogenetic analysis of Family Adenoviridae from marine environment as inferred from the hexon protein gene

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The marine environment, covering 70% of the planet's surface, is estimated to contain 10^{30} viruses. Despite their great importance in the marine environment, virtually very little is known about marine viral biodiversity or the evolutionary relationships of marine and non-marine viruses. The present study is aimed at molecular characterization and phylogenetic analysis of the viral Family Adenoviridae from the marine environment, based on the hexon protein gene. Briefly, 50 L of sea water was purified and concentrated by a combination of tangential flow filtration, flocculation and centrifugation. Metagenomic DNA was extracted from the viral particles and PCR amplification was performed using specific primers designed for hexon protein gene of adenoviruses. Positive amplicons were cloned and sequenced. Sequences were analysed using GeneTool, BioEdit, ExPASy, ClustalW, GeneDoc and Mega 5.0 programmes. Partial hexon protein amplicon of 254 bp fragment encoding hexon protein of 84 amino acids could be recovered from the metagenomc viral DNA (KJ958986). Phylogenetic relationship of the newly identified hexon protein gene was constructed employing neighbour joining method based on already available hexon protein sequences in GenBank. The results of phylogenetic analysis confirmed the basal placement of the newly identified hexon protein gene in the Adenoviridae family. The present study shows that the newly identified adenovirus from marine environment is more closely related to adenoviruses isolated from Ovine and Bovine sources than to other adenoviruses from human sources. The present study provides a clearer insight into adenoviridae present in the marine system.

Key words: Adenovirus, marine viruses, hexon protein, metagenomics, phylogeny.

INTRODUCTION

Viruses are the smallest sub-microscopic forms of life, attacking all organisms on the earth and the most abundant entity in sea water. Currently, best estimation of viral particles indicates concentrations of ~10^6 viruses ml^{-1} of surface seawater, ~3×10^6 viruses ml^{-1} in the deep sea to ~10^8 viruses ml^{-1} in productive coastal waters till now, the total estimation of viruses is about ~10^{30} in the oceans (Bergh et al., 1989; Suttle, 2005; Rohwer and Barott, 2013). With higher abundance of viruses it is also seen that their genome sequences are also remarkably diverse. Due to lack of standard culture techniques, our knowledge about viral world is limited (Fuhrman and Campbell, 1998; Paul and Sullivan, 2005). To overcome these difficulties, new culture-independent approaches came into light which are mainly based on molecular tools and done by amplification of conserved gene present in viruses (Nicholas and Paul, 2011).

Metagenomics is one of the prominent culture-independent approaches that have revealed an incredible amount of viral genetic diversity, particularly in the marine

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environment (Breitbart et al., 2002). Metagenomic study shows that approximately 75 % of the sequences in these viral metagenomes did not match any genes in the database (that is, E-value 00.001 to the non-redundant GenBank database) suggesting that most viral diversity remains uncharacterized (Breitbart and Forest, 2005). Studies have shown that some marine viruses show similarity with non-marine viruses (Forest et al., 2000). Analysis of the genome of Roseophage SIO1 (phage that infects the marine heterotrophic bacteria *Roseobacter* SIO67) has shown that marine and non-marine phages are genetically related, but basic life histories may be significantly different.

Family *Adenoviridae* includes a group of non-enveloped icosahedral viruses consisting of a protein coat or capsid, surrounding a DNA-protein core, ranging from 70 to 100 nm in diameter. The genome consists of double-stranded DNA ranging from 30 to 40 kb depending on the serotype (Bosch et al., 2006; Hundesa et al., 2006; Muscillo et al., 2008; Belsy et al., 2009). The viral proteins are essential for the infection process. Adenovirus with damaged DNA has been successfully shown to infect host cells (Ko et al., 2003; Jothikumar et al., 2005; Eischeid et al., 2009). Family *Adenoviridae* consists of four genera namely: Mastodenovirus, Aviadenovirus, Atadenovirus and Siadenovirus; however, only genera Mastadenovirus could affect humans (Pond, 2005).

Adenoviruses are able to withstand extreme conditions, including an acidic pH environment of 5-6 and are able to withstand even the harshness of lipid solvents. Hence, they can live outside the host for long periods of time compared to other viruses. Adenovirus has also shown to be resistant to both tertiary treatment and UV radiation (Pond, 2005). Human adenoviruses have been recently listed on the U.S. EPA Candidate Contaminant List (Ko et al., 2003; Griffin et al., 2003; Fon and Lipp, 2005; Xagoraraki et al., 2007) and is being suggested as an index of pollution of human origin in waters, based on its presence in greater numbers and high persistence in surface waters (Linden et al., 2007; Mahony, 2008). Furthermore, human adenoviruses have been shown to be prevalent in marine surface waters and in shellfishes namely: oysters and clams (Jiang et al., 2001; Pina et al., 1998; Bofill-Mas et al., 2006). However, it has been suggested that all viral genomes detected does not correspond to infectious viral particles and a high proportion of non-infectious viral particles may be expected in the environment (Aragao et al., 2010).

Recent research is focused on using adenoviruses for cancer treatment for effective gene delivery and expression since they demonstrate several advantages over other vectors (Kanera and Hemminki, 2004; Rein et al., 2007, 2011). After subjecting the virus to extensive engineering to remove the genes which control viral replication and thereby also creating room to insert genes of therapeutic interest, adenoviruses become a promising agent for killing tumor cells.

The present study is aimed at molecular characterization and phylogenetic analysis of the viral family *Adenoviridae* from the marine environment, based on the hexon protein gene. The study also analyzes the genetic diversity and phylogenetic relationship of the newly identified adenovirus to the already identified adenoviruses from other sources. This is the first report of metagenomic characterization of adenoviruses from the Cochin Barmouth Region, India.

MATERIALS AND METHODS

Sample collection

About 50 L of surface sea water sample was collected in polyethylene containers from the Cochin Barmouth region, India (at latitude 9° 58' 0" North and longitude 76° 15' 0" East) during September 2013 (Figure 1).

Purification and concentration of samples

The water samples were immediately brought to the lab and filtered through 2 mm mesh and 0.4 µm to remove larger particles. This filtered sample was concentrated using tangential flow filtration system (QuixStandBenchtop System, GE healthcare). At each stage, the fractions were checked for the presence of viral particles using epiflourescence microscopy using SYBR Green stain (Invitrogen).

Flocculation of viral particles

The concentrated samples were further concentrated by flocculation method following previously explained procedure for marine samples by John et al. (2011). Briefly, the viral particles in the retentate were concentrated using 1 ml Fe solution (10 g $Fecl_3$ l^{-1}). After addition of $FeCl_3$ solution, shaking was repeated several times and kept for 1 h at room temperature to allow Fe-virus flocculate formation. Filtrate was collected in 10 ml tubes and centrifuged at 14,000 rpm for 15 min at 4°C. The pellets were then dissolved in freshly prepared 0.2 M Ascorbate-0.1M EDTA-Mg buffer (pH 6-7) and stored in the dark at -20°C until use.

Extraction of viral nucleic acids

Viral nucleic acids were extracted using QIAamp MinElute Virus Spin Kit (QIAGEN) following the manufacturer's protocol and was stored at -20°C. Viral nucleic acids were quantified and qualified by spectrophotometry at 260 nm and 280 nm and agarose gel electrophoresis.

PCR amplification

PCR amplification of 1 µl of viral nucleic acid (~200 ng)

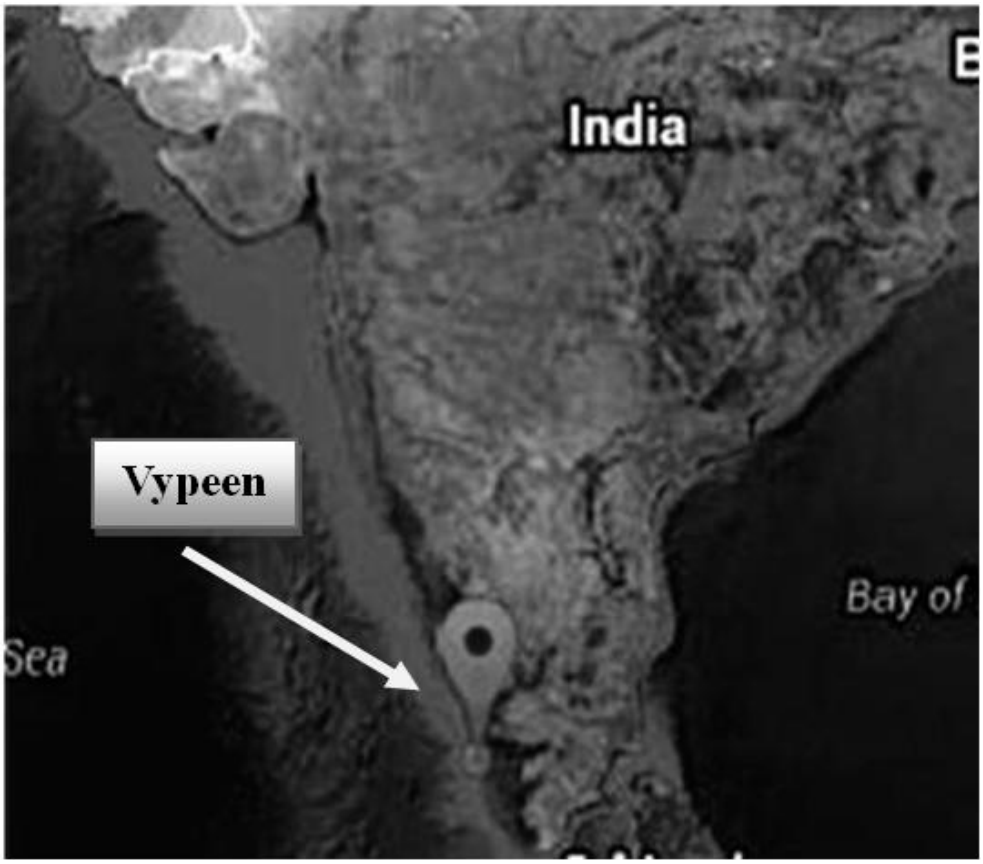

Figure 1. Sampling site – Cochin Barmouth Region, Vypeen, India.

was performed in a 25 µl reaction volume containing 1× standard Taq buffer (10 mMTris-HCl, 50 mMKCl, pH 8.3), 1.5-3.5 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM of each primer and 1U Taq DNA polymerase. The primer pairs used were Adeno- F (5'-gccgcagtggtcttacatgcacatc-3') and Adeno- R (5'-cagcacgccgcggatgtcaaagt-3'). The thermal profile used for the PCR amplification was 94°C for 2 min followed by 35 cycles of 94°C for 15 s, 60°C for 30 s and 68°C for 30 s and a final extension at 68°C for 10 min. PCR products were analyzed by electrophoresis in 1.5 % agarose gel in TBE buffer, stained with ethidium bromide and visualized under UV light.

Sequencing

Sequencing was performed for both PCR products and cloned plasmids. In the case of PCR products, the samples were subjected to ExoSap treatment for purification and the purified samples were sequenced at Scigenom, India, using both gene specific forward and reverse primers.

pGEMT cloning and plasmid sequencing

The positive PCR amplicons were cloned onto pGEM-T Easy vector (Promega) following manufacturer's protocol using *E. coli* JM109 strain. Positive clones were screened using vector specific and gene specific primers. Plasmids were extracted using GenElute Plasmid MiniPrep kit (Sigma) from positive clones and were sequenced using vector specific forward and reverse primers at Scigenom, India.

Sequence analysis

The sequences were edited, assembled and analysed using GeneTool and BioEdit software. Gene translation and prediction of deduced proteins were performed with ExPASy (http://www.au.expasy.org/). The nucleotide sequence homology and the translated amino acid sequence comparisons were performed using BLAST algorithm (BLASTn, BLASTp and tBLASTx) of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast). The multiple sequence alignments of nucleotide and amino acid sequence were performed with sequences retrieved from NCBI and multi-aligned using ClustalW and GeneDoc computer programmes. Phylogenetic and molecular evolutionary analyses were conducted by the Neighbor-Joining (NJ) and Maximum-Likelihood (ML) methods

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tgccgcagtggtcttacatgcacatcgcggggcggcatacagccgaactggctgtcggtgtg
   P  Q  W  S  Y  M  H  I  A  G  R  H  T  A  E  L  A  V  G  V
gaaatcgggggtggtcacgggcaaagcgtgatcaatcggcgtcggtcggtccacacggac
 E  I  G  GG  H  G  Q  S  V  I  N  R  RR  S  V  H  T  D
accacgggcctcttgctgcgctgcgaacttgtccagcaactcattcgcacggtcgatcgt
 T  T  G  L  LL  R  C  E  L  V  Q  Q  L  I  R  T  V  D  R
ctcatgtggacgccgtccccattgcccgcgcatatcgctgtcttcaggaactttgacatc
 L  M  W  T  P  S  P  L  P  A  H  I  A  V  F  R  N  F  D  I
cgcggcgtgctg
 R  G  V  L
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Figure 2. Nucleotide and amino acid sequences of the partial hexon protein gene of Marine Adenovirus (KJ958986).

using MEGA version 5 (Tamura et al., 2011).

RESULTS AND DISCUSSION

Adenoviruses were first isolated and characterized in 1953 and was named “adenoviruses”, after the original tissue (adenoids) in which they were discovered. Till date, adenoviruses occur worldwide and have been isolated from a large number of different species, majority being from vertebrates including every major class from fishes to mammals. So far, more than 100 members of the adenovirus group have been identified that infect a wide range of vertebrate hosts and count to 100 serotypes, 57 being in humans. The adenoviruses isolated from mammals, especially those from humans, are well characterized. All of these viruses isolated so far contain a linear, double-stranded DNA (dsDNA) genome encapsidated in an icosahedral protein shell with fiber proteins of varying lengths.

Adenoviruses, due to their high prevalence and fairly conserved organization of the central part of their genomes, offer one of the best models for studying the molecular evolution of DNA viruses on a larger time scale. Adenoviruses have been shown to be prevalent in marine surface waters, including marine organisms, which may or may not be infectious (Jiang et al., 2001; Bofill-Mas et al., 2006; Aragao et al., 2010). Although the frequency of reports on marine adenoviruses is increasing worldwide, there are no molecular data concerning the adenoviruses of the Indian Coast. The present study is aimed at molecular characterization and phylogenetic analysis of a marine adenovirus from the Indian Coast, based on the hexon protein gene. This study also analyzes the genetic diversity and phylogenetic relationship of the newly identified adenovirus to the already identified adenoviruses from other sources. This is the first report of metagenomic characterization of adenoviruses from the Cochin Barmouth Region, India.

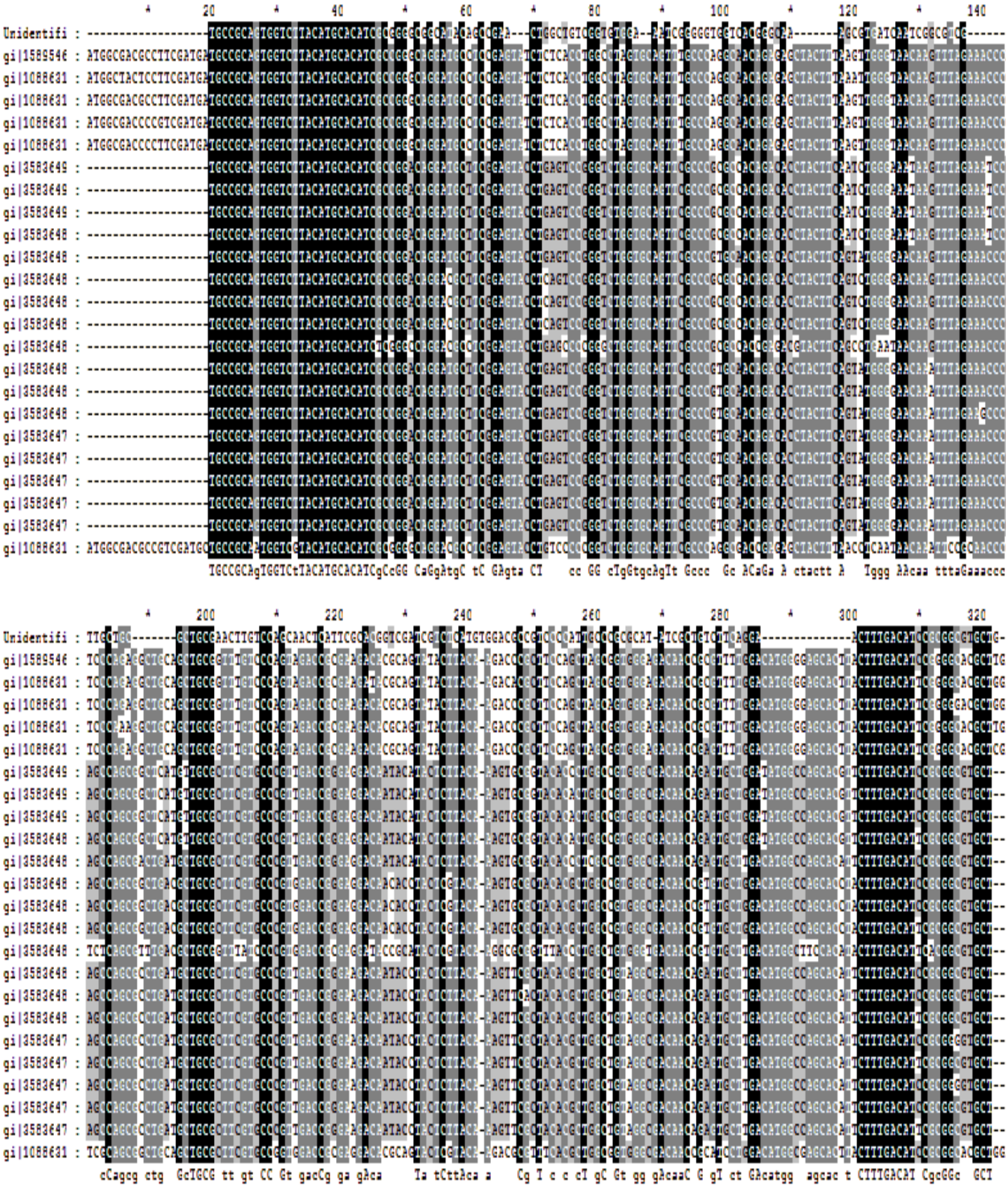
In this study, 50 L of initial sample was concentrated down to 12 ml using a combination of tangential flow filtration and flocculation method. Epiflourescence microscopy confirmed the presence and concentration of viral particles by these methods. Number of viral like particles was found to be higher in the final sample after concentration, indicating that the viral purification protocol from seawater was efficient. Also, viral nucleic acids could be successfully isolated from the sample using QIAamp MinElute virus spit kit (Qiagen). The quality of viral nucleic acid obtained (A260/A280) was found to be 1.7 and the concentration obtained was 450 ng/µl, as inferred from spectrophotometry. Diluted viral nucleic acid was subjected to PCR amplification using gene specific primers.

A 254 bp fragment encoding 84 amino acids and possessing homology to the hexon protein gene of family *Adenoviridae* could be obtained from the metagenomic viral DNA (Figure 2). The sequence was submitted in NCBI GenBank under the accession number KJ958986. Translation of the nucleotide sequence was performed using GeneTool and ExPaSy programmes. Results of BLASTn, BLASTp and tBLASTx analysis of the sequences showed similarity to the already submitted hexon protein gene of adenovirus from various sources, thereby confirming the sequence to belong to Family Adenoviridae.

Analysis of the sequence suggested that the hexon coding sequence was unlike any other known adenovirus hexon sequence so far reported. The sequence showed similarity of 97-100% for query coverage of 12-11% respectively in the case of BLASTn, whereas, BLASTp showed only 67-80% for query coverage of 17% for an E-value ranging from 0.001-0.007. Sources of adenovirus that showed similarity to the newly identified marine adenovirus includes Simian, Ovine, Bovine, Human, Equine, Canine, Bat, Caprine, Alpaca, Cynomolgus and Sea Lion.

Multiple alignment performed for the nucleotide sequence and amino acid sequence of the hexon protein gene (KJ958986) showed the presence of conserved regions with the sequences (Figure 3a and b). The phylogenetic relationships of adenoviruses from various sources were also established, based on nucleotide sequence and amino acid comparison of the hexon

a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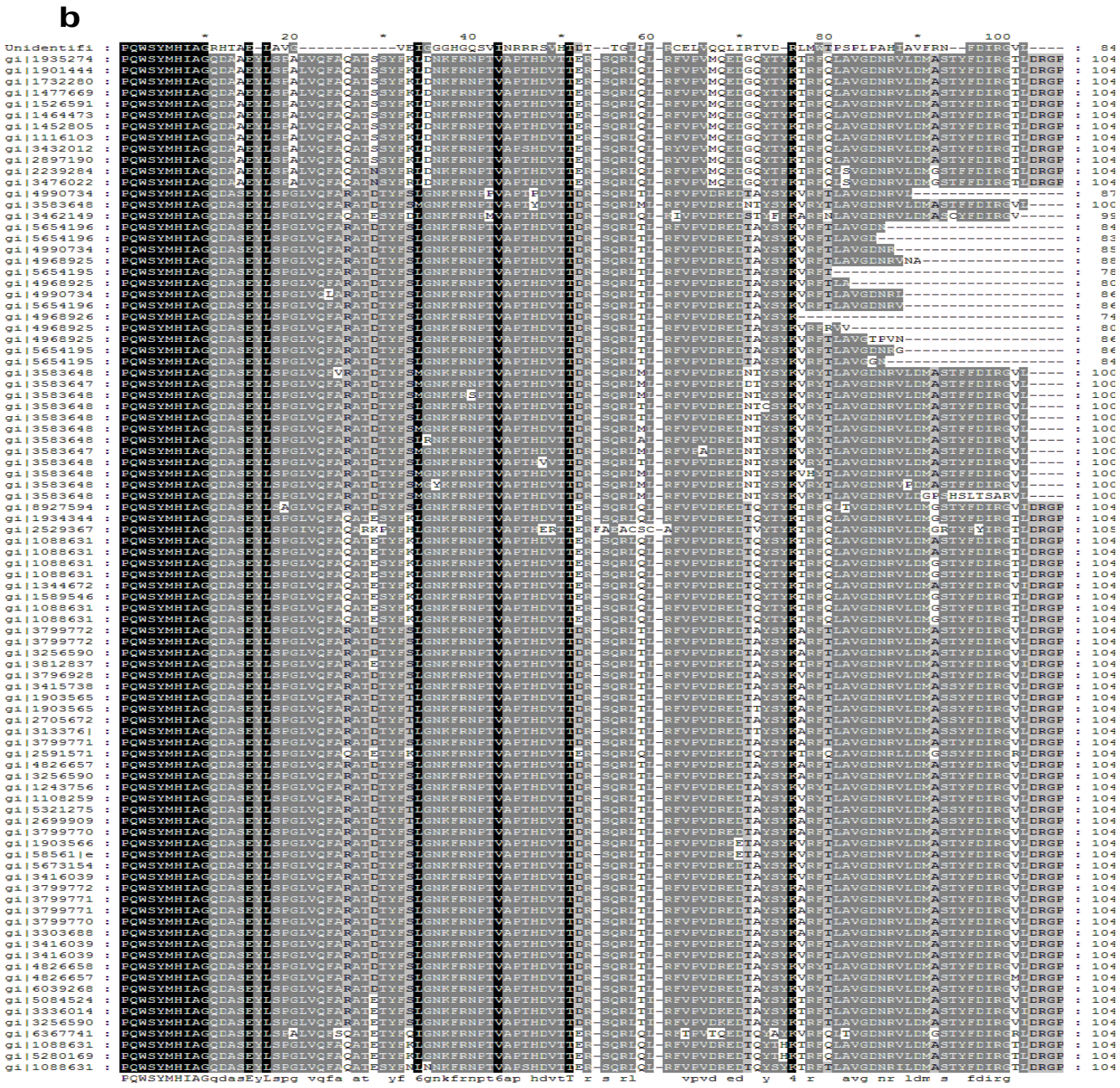
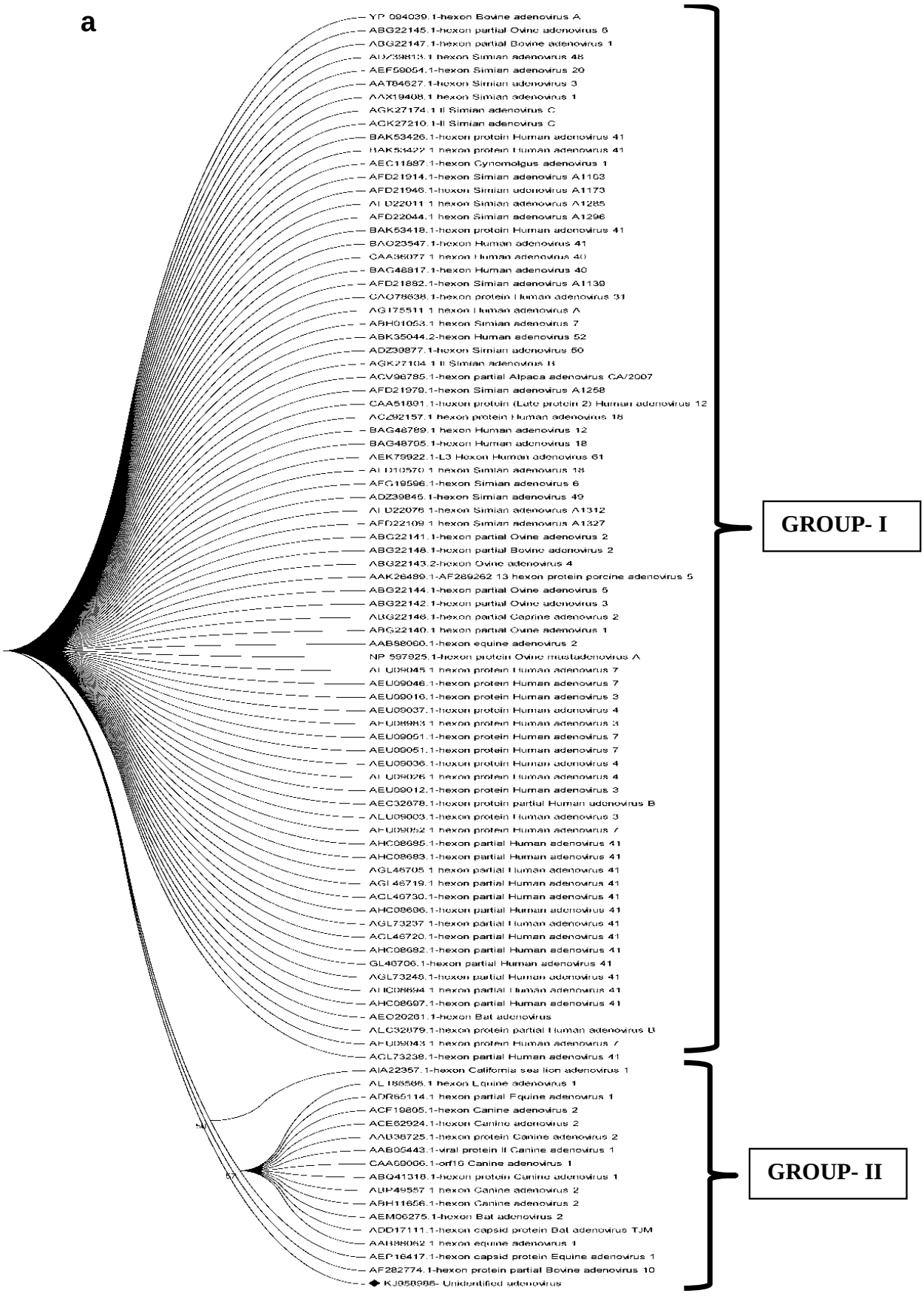


Figure 3. Multiple alignments of (a) nucleotide sequence, and (b) deduced amino acid sequence of the hexon protein gene of Marine Adenovirus (KJ958986) with other adenoviruses obtained using GeneDoc programme Version 2.7.0. The alignment was performed with ClustalW and edited with GeneDoc software. The three levels of shading indicate different degrees of conservation. Black background and white letters corresponds to 100% conservation, gray background and white letters corresponds to 80% conservation, and gray background and black letters corresponds to 60% conservation.

protein genes. For a robust and reliable phylogenetic analysis, two methods were used, namely: maximum likelihood and neighbor joining methods. Neighbor joining tree was constructed using bootstrap test by 1000 replicates (Figure 4a and b).

Analysis of the results of the ML tree constructed using the nucleotide sequence showed that the tree could be broadly divided into two clusters. Cluster 1 included


a
YP 094039.1-hexon Bovine adenovirus A
ABG22145.1-hexon partial Ovine adenovirus 6
ABG22147.1-hexon partial Bovine adenovirus 1
AID39813.1 hexon Simian adenovirus 48
AEF59054.1-hexon Simian adenovirus 20
AAT84627.1-hexon Simian adenovirus 3
AAX19408.1 hexon Simian adenovirus 1
AGK27174.1 II Simian adenovirus C
AGK27210.1-II Simian adenovirus C
BAK53426.1-hexon protein Human adenovirus 41
BAK53422.1 hexon protein Human adenovirus 41
AEC11887.1-hexon Cynomolgus adenovirus 1
AFD21914.1-hexon Simian adenovirus A1163
AFD21946.1-hexon Simian adenovirus A1173
AID22011.1 hexon Simian adenovirus A1285
AFD22044.1 hexon Simian adenovirus A1296
BAK53418.1-hexon protein Human adenovirus 41
BAO23547.1-hexon Human adenovirus 41
CAA36077.1 hexon Human adenovirus 40
BAG48817.1-hexon Human adenovirus 40
AFD21882.1-hexon Simian adenovirus A1139
CAO78638.1-hexon protein Human adenovirus 31
AGI75511.1 hexon Human adenovirus A
ABH01053.1 hexon Simian adenovirus 7
ABK35044.2-hexon Human adenovirus 52
ADZ39877.1-hexon Simian adenovirus 50
AGK27104.1 II Simian adenovirus B
ACV96785.1-hexon partial Alpaca adenovirus CA/2007
AFD21979.1-hexon Simian adenovirus A1258
CAA51891.1-hexon protein (Late protein 2) Human adenovirus 12
ACZ92157.1 hexon protein Human adenovirus 18
BAG48789.1 hexon Human adenovirus 12
BAG48705.1-hexon Human adenovirus 18
AEK79922.1-L3 Hexon Human adenovirus 61
AID10570.1 hexon Simian adenovirus 18
AFG19596.1-hexon Simian adenovirus 6
ADZ39845.1-hexon Simian adenovirus 49
AID22076.1-hexon Simian adenovirus A1312
AFD22109.1 hexon Simian adenovirus A1327
ABG22141.1-hexon partial Ovine adenovirus 2
ABG22148.1-hexon partial Bovine adenovirus 2
ABG22143.2-hexon Ovine adenovirus 4
AAK26489.1-AF289262 13 hexon protein porcine adenovirus 5
ABG22144.1-hexon partial Ovine adenovirus 5
ABG22142.1-hexon partial Ovine adenovirus 3
ABG22146.1-hexon partial Caprine adenovirus 2
ABG22140.1 hexon partial Ovine adenovirus 1
AAB58060.1-hexon equine adenovirus 2
NP 597925.1-hexon protein Ovine mastadenovirus A
AIU09045.1 hexon protein Human adenovirus 7
AEU09046.1-hexon protein Human adenovirus 7
AEU09016.1-hexon protein Human adenovirus 3
AEU09037.1-hexon protein Human adenovirus 4
AHU08983.1 hexon protein Human adenovirus 3
AEU09051.1-hexon protein Human adenovirus 7
AEU09051.1-hexon protein Human adenovirus 7
AEU09036.1-hexon protein Human adenovirus 4
AIU09026.1 hexon protein Human adenovirus 4
AEU09012.1-hexon protein Human adenovirus 3
AEC32878.1-hexon protein partial Human adenovirus B
ALU09003.1-hexon protein Human adenovirus 3
AHU09052.1 hexon protein Human adenovirus 7
AHC08685.1-hexon partial Human adenovirus 41
AHC08683.1-hexon partial Human adenovirus 41
AGL46705.1 hexon partial Human adenovirus 41
AGL46719.1 hexon partial Human adenovirus 41
ACL46730.1-hexon partial Human adenovirus 41
AHC08606.1-hexon partial Human adenovirus 41
AGL73237.1-hexon partial Human adenovirus 41
ACL46720.1-hexon partial Human adenovirus 41
AHC08682.1-hexon partial Human adenovirus 41
GL46706.1-hexon partial Human adenovirus 41
AGL73248.1-hexon partial Human adenovirus 41
AIC08694.1 hexon partial Human adenovirus 41
AHC08697.1-hexon partial Human adenovirus 41
AEO20261.1-hexon Bat adenovirus
ALC32879.1-hexon protein partial Human adenovirus B
AHU09043.1 hexon protein Human adenovirus 7
ACL73238.1-hexon partial Human adenovirus 41
AIA22357.1-hexon California sea lion adenovirus 1
ALI86586.1 hexon Equine adenovirus 1
ADR65114.1 hexon partial Equine adenovirus 1
ACF19805.1-hexon Canine adenovirus 2
ACE62924.1-hexon Canine adenovirus 2
AAU38725.1-hexon protein Canine adenovirus 2
AAB05443.1-viral protein II Canine adenovirus 1
CAA59006.1-orf18 Canine adenovirus 1
ABQ41318.1-hexon protein Canine adenovirus 1
AUP49557.1 hexon Canine adenovirus 2
ABH11656.1-hexon Canine adenovirus 2
AEM06275.1-hexon Bat adenovirus 2
ADD17111.1-hexon capsid protein Bat adenovirus TJM
AAH88062.1 hexon equine adenovirus 1
AEP16417.1-hexon capsid protein Equine adenovirus 1
AF282774.1-hexon protein partial Bovine adenovirus 10
KJ958986- Unidentified adenovirus
54
57
GROUP- I
GROUP- II

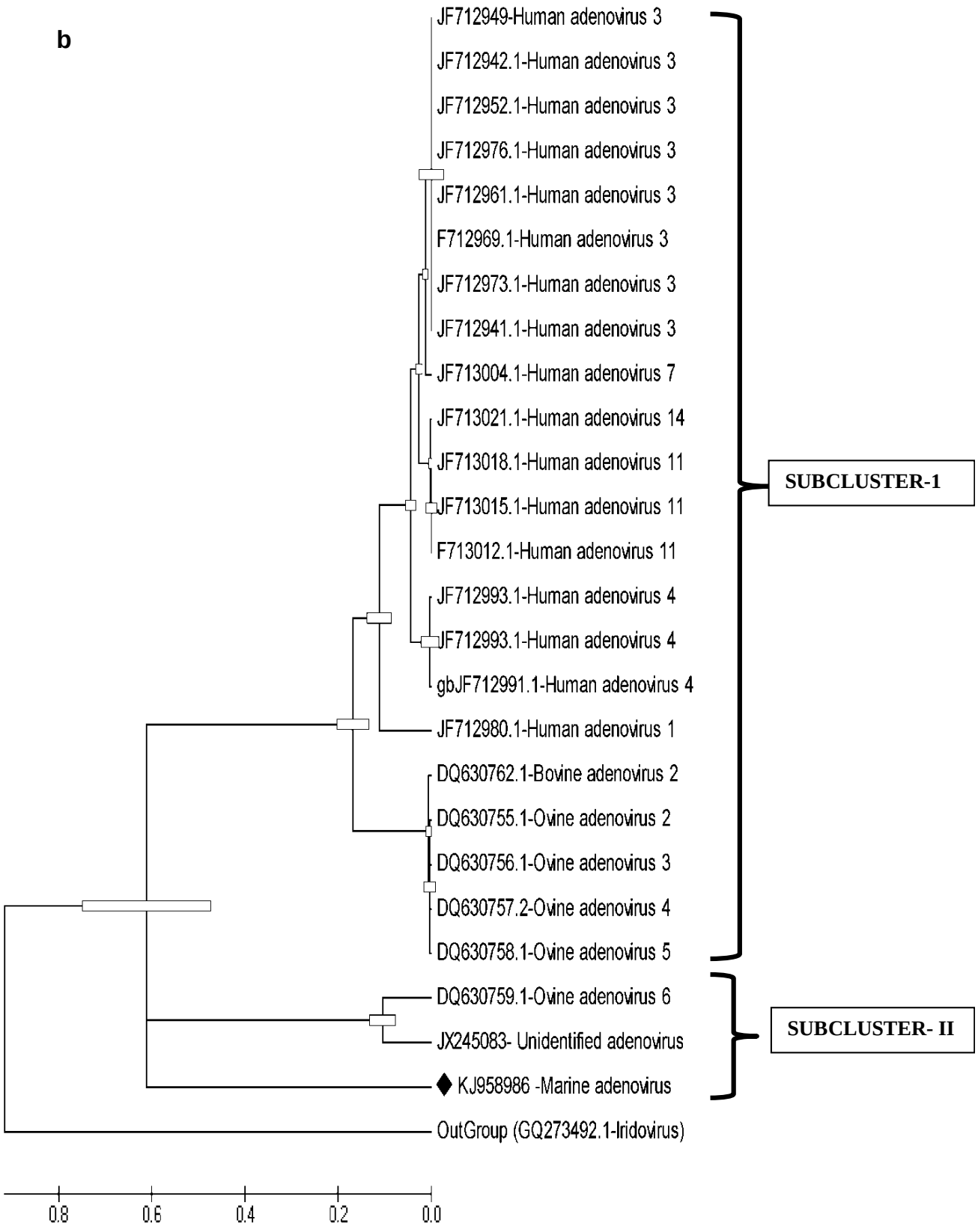


Figure 4. Bootstrapped (a) Neighbor-Joining Tree and (b) Maximum Likelihood Tree obtained using MEGA version 5.0 illustrating relationships between the nucleotide and deduced amino acid sequence of the hexon protein gene of Marine Adenovirus (KJ958986) with other adenoviruses. Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original nucleotide sequences for the NJ tree.

adenoviruses isolated from various sources and cluster 2 is an outgroup. The present sequence was found to belong to cluster 1, thereby confirming the sequence identity. Cluster 1 could again be divided into 2 subclusters. Subcluster 1 contains human, bovine and canine adenoviruses, and subcluster 2 consists of an ovine adenovirus, an unidentified adenovirus and the adenovirus from the present study.

Analysis of the results of the NJ tree constructed using the amino acid was worth noticing. The tree could be broadly divided into two clusters. Cluster 1 includes adenoviruses isolated from various sources namely: human, bovine, ovine, simian, alpaca and bat; whereas, cluster 2 consists of equine, canine, bat, California sea lion and the newly identified adenovirus. The results of phylogenetic analysis based on the hexon protein sequences showed light onto the basal placement of marine adenoviruses in the family. It is shown that the marine adenovirus of the present study is more similar to the adenoviruses isolated from organisms other than from humans. It was really interesting to see that the adenovirus isolated from sea lion also belonged to cluster 2.

Conclusion

Adenovirus has been considered as a key organism for uncovering new knowledge and providing insight into multiple aspects of animal cell biology and human antiviral defences. It also serves for adenovirus-based gene transduction vectors with engineered receptor-binding domains that promote infection of specific cell types. The viral proteins are potent engineered molecular tools for exploring these cellular processes. In this study, the hexon protein gene of an apparently novel marine adenovirus present in the marine waters off Cochin, India has been reported. The hexon coding sequence, coupled with bioinformatic analysis, demonstrated that the reported marine adenovirus is different from all previously characterized adenoviruses, and might be a novel marine adenovirus. The present study also reports the results of phylogenetic analyses based on the nucleotide and amino acid sequence data of hexon protein of family *Adenoviridae*. This is the first report of a marine adenovirus strain identified and characterized from the Cochin Barmouth region, India. The identified hexon protein showed less similarity to the already deposited hexon protein sequences of family *Adenoviridae* in GenBank. Multiple alignments showed the presence of conserved regions within the sequence. The phylogenetic analysis showed that the hexon protein of adenoviruses diverged from a common ancestral sequence into two major groups. The results of phylogenetic analysis based on the hexon protein sequences confirmed the basal placement of marine adenovirus in the family *Adenoviridae*. Molecular phylogenetic arrangements as inferred from the present study suggest the presence of single origin for all the viruses belonging to family Adenoviridae. Increasing number of research in the fundamental aspects of adenovirus biology itself indicates the potential scope of the organism and endorses that adenovirus research continues to be a productive and rewarding area of discovery.

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