Epidemiological study of viruses infecting western lowland gorillas

in Moukalaba-Doudou National Park (Gabon)

（ムカラバ国立公園（ガボン）におけるニシローランドゴリラに感染する

ウイルスの疫学的研究）

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Contents

Preface

The current doctoral thesis in based on the following papers:

1- C. Nze Nkogue, M. Horie, S. Fujita, M. Ogino, Y. Kobayashi, K. Mizukami, T. Masatani, S. Ezzikouri, A. Matsuu,T. Mizutani, M. Ozawa, O. Yamato, A. Ngomanda, J. Yamagiwa, K. Tsukiyama-Kohara, 2016. Molecular epidemiological study of adenovirus infecting western lowland gorillas and humans in and around Moukalaba-Doudou National Park (Gabon). Virus Genes 52, 671-678.

2- C. Nze-Nkogue, M. Horie, S. Fujita, E. Inoue, E-F. Akomo-Okoue, M. Ozawa, A. Ngomanda, J. Yamagiwa, K. Tsukiyama-Kohara, 2017. Identification and molecular characterization of novel primate bocaparvoviruses from wild western lowland gorillas of Moukalaba-Doudou National Park, Gabon. Infection, Genetics and Evolution 53, 30-37.

Dedication

To Mekui Mve, Mekui Mve Sow and Nze Majanga, with all my love.

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Summary

The screening of infectious agents (viruses, parasites or bacteria) in wildlife provides critical data regarding not only the presence of pathogens but also the diversity and the natural history of the target microbes. The emergence and re-emergence of diseases which originated from wildlife has emphasized the necessity for such pathogen assessment for the conservation of endangered animal populations, controlling the risk on the trade of wildlife or wildlife products, and preventing pathogen from spilling over into livestock or human population. In addition, the studies on infectious pathogens in the natural living great ape (gorillas, chimpanzees, etc.) populations has clarified the origins of some human viruses (HIV, HTLV, etc) as well as raising the concern about pathogen cross-species transmission between both hosts owing to the genetic relatedness between great apes and humans.

I conducted the surveillance of adenovirus and bocaparvovirus infection in wild western lowland gorillas in Moukalaba-doudou National Park (Gabon) in order to investigate the presence, genetic diversity, and evolutionary history of these viruses, and to assess a possible zoonotic transmission for the case of adenoviruses. Although

adenoviruses are widespread in humans and great apes, the data about the naturally occurring infections remain rare. On the other hand, the evolutionary study of adenoviruses infecting great apes has recently revealed that the *Human mastadenovirus B* (HAdV-B) originated from ancient gorillas and had experienced several cross-species (ape-ape and ape-human) transmission events. Bocaparvoviruses have been extensively studied because of their frequent association with respiratory illness and/or and gastroenteritis in humans. Although some bocaparvoviruses have been detected in non-human primates (gorillas and chimpanzees), the presence, diversity, and evolution of these viruses are not fully understood.

I detected adenoviral genes in fecal samples from gorillas and the local people living around the national park: the overall prevalence rates of adenovirus were 24.1% and 35.0% in gorillas and humans, respectively. Sequencing revealed that the adenoviruses detected in the gorillas were members of HAdV-B, HAdV-C, or HAdV-E, and those in the humans belonged to HAdV-C or HAdV-D. Although HAdV-C members were detected in both gorillas and humans, phylogenetic analysis revealed that the viruses detected in gorillas were genetically distinct from those detected in humans. On the other hand, HAdV-B was clustered with other gorilla adenoviruses together with human and

chimpanzee strains, which support the hypothesis of being a zoonotic virus. The HAdV-E was clustered with the chimpanzee strains. This result indicates the possibility of an ape-to-ape transmission of HAdV-E species because chimpanzees have been reported to be the most probable ancestor hosts of these viruses.

Regarding the bocaparvorirus infection, I detected two gorilla bocaparvovirus strains from 2 infant gorillas. Both strains clustered with viruses in the species *Primate bocaparvovirus 2* (with 86.0% nucleotide identity to a human bocavirus 2 isolate). The named *Gorilla bocaparvovirus 2* (GBOV2) of this study is the first non-human primate bocaparovirus within that species. The molecular evolutionary analyses of primate bocaparvoviruses revealed the presence of inter and intra-species recombination events which might lead to the emergence of new bocaparvovirus variants in human as well as in non-human primate population.

The description of several adenoviruses and the identification of novel bocaparvoviruses in wild western gorillas contribute to a better understanding of the genetic diversity of these viruses as well as clarifies their evolutionary processes. Although there is no evidence of gorilla-to-human interspecies transmission of the detected viruses in this study, I would advise the surveillance of these viruses in gorillas

as well as in humans (tourists, guides, local peoples, etc.) which potentially contact with these animals when eco-tourism, aiming the conservation of wildlife, is promoted.

Summary in Japanese

野生動物における病原体（ウイルス、寄生虫、細菌）の調査は、その病原体の存在のみならず、多様性および進化に関する知見を提供する。また、野生動物由来の新興および再興感染症が多数報告されており、野生動物における病原体の調査は、絶滅の危機に瀕した動物個体群の保全、野生動物あるいは野生動物由来産物の取引におけるリスク管理、さらにはヒトや家畜への病原体の伝播を防ぐために重要である。さらに、大型類人猿（ゴリラ、チンパンジーなど）の病原体に関する研究により、いくつかのヒトの病原ウイルス（ヒト免疫不全ウイルスやヒトT細胞白血病ウイルスなど）の起源が明らかとなった。

アデノウイルスはヒトおよび大型類人猿に広く感染が見られるが、野生の大型類人猿における報告は少ない。また、大型類人猿に感染するアデノウイルスの分子進化に関する研究により、ヒトマストアデノウイルスB（HAdV-B）はゴリラの祖先動物を起源とし、今日までに何回かの種間伝播（類人猿間および類人猿–ヒト間）を経てきたことが報告されている。ボカパルボウイルスはヒトにおいて呼吸器疾患や胃腸炎との関連が示唆されており、広く研究が行われている。しかし、非ヒト霊長類（ゴリラおよびチンパンジー）においてはいくつかのボカパルボウイルスが検出されているのみであり、その多様性や進化に

関する研究は十分に行われていない。本研究では、野生のゴリラにおけるアデノウイルスおよびボカパルボウイルスの感染状況、多様性およびその進化を理解するため、ムカラバ国立公園（ガボン）の野生ニシローランドゴリラにおいて、両ウイルスの感染状況を調査した。さらにアデノウイルスについては、ゴリラ-ヒト間伝播の可能性についても検討を行った。

本研究では、ゴリラおよび国立公園周辺に住む地域住民から採取した糞便試料からアデノウイルス遺伝子を検出した。ゴリラおよびヒトにおいてアデノウイルスの全体の検出率はそれぞれ 24.1%および 35.0%であった。塩基配列の解析から、ゴリラから検出されたアデノウイルスは HAdV-B、HAdV-C あるいは HAdV-E に、ヒトから検出されたウイルスは HAdV-C あるいは HAdV-D に分類された。HAdV-C はゴリラとヒトの両方から検出されたが、分子系統樹解析の結果、ゴリラから検出されたウイルスはヒトから検出されたものとは遺伝的に異なることが示唆された。一方、検出された HAdV-B はヒト由来株、チンパンジー由来株および、他のゴリラ由来株とクラスターを形成し、HAdV-B が過去に種間伝播を起こしたという過去の報告を支持する結果が得られた。また、HAdV-E はチンパンジー由来株とクラスターを形成した。チンパンジーはこの

ウイルスの起源となる宿主動物であると考えられており、このウイルスが種間伝播する可能性を示唆している。

ボカパルボウイルス感染について、申請者はゴリラの幼仔２個体の糞便試料から２つのゴリラボカパルボウイルス株を検出した。分子系統樹解析の結果、ゴリラより検出された２株は、霊長類ボカパルボウイルス２種のウイルスと同一のクラスターに属することが明らかとなった。これらのウイルスと最も近縁なウイルスはヒトボカウイルス２であり、86.0％の塩基配列の一致率を示した。これまでに非ヒト霊長類由来の霊長類ボカパルボウイルス２種に関する報告はなく、本研究において検出したウイルス（ゴリラボカパルボウイルス２、GBOV2）は、世界初の非ヒト霊長類に由来する霊長類ボカパルボウイルス２種に属するウイルスである。また、ウイルスゲノムの組換えに関する解析を行ったところ、これまでに報告されていない新たな種内・種間の遺伝子組換えパターンが検出された。このことから、ウイルスゲノムの組換えによって過去に新たなボカパルボウイルスが出現したことが示唆された。

本研究において、野生ニシローランドゴリラにおけるアデノウイルスおよびボカパルボウイルスの感染状況およびそれらの分子進化機構について新たな知

見が得られた。本研究ではゴリラ-ヒト間のウイルス伝播の証拠は得られていないが、野生動物の保全を目的としたエコツーリズムを開発する場合、ゴリラと接触の可能性があるヒト（観光客、ガイド、地元住民など）およびゴリラにおいて、今後も様々なウイルスの保有調査を実施することが推奨される。

List of abbreviations

°C. *Degrees Celcius*

µl. *microliter*

AdVs. *Adenoviruses*

bp. *base pair*

DPOL. *DNA polymerase*

G8. *Group 8*

GBOV. *Gorilla bocavirus*

GG. *Group Gentil*

HAdV. *Human adenovirus*

HBOV. *Human bocavirus*

HF. *High Fidelity*

HIV. *Human Immunodeficiency Virus*

HVR. *Highly Variable Region*

ICTV. *International Committee of Taxonomy for Viruses*

IUCN. *International Union for the Conservation of Nature*

MDNP. *Moukalaba-Doudou National Park*

min. *Minutes*

mM. *millimolar*

ng. *nanograms*

ORF. *Open Reading Frame*

PCR. *Polymerase Chain Reaction*

SIV. *Simian Immunodefiency Virus*

U. *Unit*

WHO. *World Health Organization*

µM. *micromolar*

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The tree was constructed based on the alignment of a 792-bp sequence of the hexon gene by using the neighbor-joining bootstrap-confirmed method in MEGA 5.05 software with 100 replicates. The names of simian isolates include the serotype nomenclature and the animal species of isolation (Ch: chimpanzee, Go: gorilla Bo: bonobo). Names of novel sequences obtained in this study are indicated with black dots.

Figure 1.4. Phylogenetic tree of the partial sequence of DPOL

The tree was constructed based on the alignment of AdV DPOL (539 bp) by using the neighbor-joining bootstrap-confirmed method in MEGA 5.05 software with 100 replicates. The names of simian isolates include the serotype nomenclature and the animal species of isolation (Ch: chimpanzee, Go: gorilla, Bo: bonobo). Names of novel sequences obtained in this study are indicated with black dots. Bootstrap values less than 90% are omitted. Scale bar, nucleotide substitutions per site.

Figure 1.5. Phylogenetic tree of the partial sequence of the hexon gene

The tree was constructed based on the alignment of a 792-bp sequence of the hexon gene by using the neighbor-joining bootstrap-confirmed method in MEGA 5.05 software with 100 replicates. The names of simian isolates include the serotype nomenclature and the

animal species of isolation (Ch: chimpanzee, Go: gorilla Bo: bonobo). Names of novel sequences obtained in this study are indicated with black dots.

Figure 1.6. Phylogenetic tree of partial hexon of HAdV-D

The tree was constructed based on the alignment of a 792-bp sequence of hexon gene by using the neighbor-joining bootstrap-confirmed method in MEGA 5.05 software with 100 replicates. The names of simian isolates include the serotype nomenclature and the animal species of isolation (Ch: chimpanzee, Go: gorilla Bo: bonobo). Names of novel sequences obtained in this study are indicated with black dots.

Figure 2.1. PCR strategy. **(A)** Schematic diagram of the PCR strategy. Scale, viral genome, and primers are shown. Gray arrows indicate primers used for detection and sequencing of bocaparvoviruses. The amplified regions are indicated by dashed gray lines. **(B)** Gel electrophoresis results showing the amplicon sizes of both positive samples.

Figure 2.2. Genome organization of the gorilla bocaparvovirus 2. **(A)** The genome organization of GBOV2 is shown. The predicted ORFs are shown in boxes. The gray boxes are the regions shown in (B), (C), or (D). **(B)** A partial nucleotide alignment of the indicated viruses. The putative splice donor and acceptor are shown in boxes. **(C)** A

partial alignment of deduced amino acid sequences of NS1 proteins. The conserved ATP-binding Walker-Loop motif is indicated by the gray box. **(D)** An amino acid sequence alignment of partial VP1 proteins. The phospholipase A2 motif, which consists of the calcium binding region and catalytic residues, is indicated.

Figure 2.3. Phylogenetic tree based on the complete coding sequences of primate bocaparvoviruses. The tree was reconstructed based on a nucleotide alignment of the complete coding sequence of the indicated viruses using the maximum likelihood method with 1000 bootstrap replicates. The GBOV2 sequences determined in this study are indicated with black dots. Bootstrap values of >70% are indicated at nodes. Scale bar, nucleotide substitutions per site.

Figure 2.4. Phylogenetic tree based on the partial NS and VP2 genes of primate bocaparvoviruses. The tree was constructed based on the partial NS gene (486 b) (A) and VP gene (486 b) (B) using the maximum likelihood method with 1000 bootstrap replicates. The GBOV2 sequences obtained in this study are indicated with black dots. Bootstrap values of >70% are indicated at nodes. Scale bar, nucleotide substitutions per site.

Figure 2.5. Recombination analysis. **(A)** Breakpoint detection using DualBrothers applied to the complete coding sequences of representative primate bocaparvoviruses. (B–K) Phylogenetic trees were constructed based on partial alignments using the maximum likelihood method with 1000 bootstrap replicates. The nucleotide positions used for the phylogenetic inferences are indicated by blue letters. Bootstrap values of >70% are indicated at nodes

Figure 2.6. A representation of individual gorillas constituting the target group based on body size and age categories. The red circles show the infected infants.

Figure 2.7. Phylogenetic tree based on the complete NS1 sequence. The tree was constructed based on the nucleotide sequences of complete NS1 using the maximum likelihood method with 1000 bootstrap replicates. The GBOV2 sequences obtained in this study are indicated with black dots. Bootstrap values of >70% are indicated at nodes. Scale bar, nucleotide substitutions per site.

Figure 2.8. Phylogenetic tree based on the complete VP1 sequence. The tree was reconstructed based on the nucleotide sequences of complete NP1 using the maximum likelihood method with 1000 bootstrap replicates. The GBOV2 sequences determined in

this study are indicated with black dots. Bootstrap values of >70% are indicated at nodes. Scale bar, nucleotide substitutions per site.

Figure 2.9. Phylogenetic tree based on the complete NP1 sequence. The tree was constructed based on the nucleotide sequences of the complete NP1 gene using the maximum likelihood method with 1000 bootstrap replicates. The GBOV2 sequences obtained in this study are indicated with black dots. Bootstrap values of >70% are indicated at nodes. Scale bar, nucleotide substitutions per site.

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Chapter I: General introduction

Epidemiological investigations of microorganisms (bacteria, parasites, viruses) infecting wild animals contribute to the understanding of the diversity and the evolutionary history of the target microbes. For virus screening, two different approaches can be used: the antibody detection and the virus or viral nucleic acid detection (Lehmkuhl and Hobbs, 2008; Luebcke et al., 2006; Mouinga-Ondémé et al., 2010; Oberste et al., 2013; Wevers et al., 2011). Sero-epidemiological surveys inform about which virus the animal has been exposed to (past or ongoing infections) (Makuwa et al., 2006; Nidom et al., 2012; Rudicell et al., 2011; Starkman et al., 2003) and the virus isolation or viral nucleic acid detection informs about the ongoing infections (Gál et al., 2013; Wevers et al., 2010). The knowledge of the viral genetic sequence coupled with the phylogenetic characterization will contribute to the classification and the understanding of the diversity and the evolutionary history of the detected viruses.

Several molecular studies have revealed the close genetic relatedness between non-human primates and human viruses (Ahuka-Mundeke et al., 2010; Duncan et al., 2013; Leendertz et al., 2011; Njouom et al., 2010; Scuda et al., 2011). For example,

adenoviruses infecting human and apes cluster in the species Human adenvovirus B, C, E and F (Roy et al., 2009; Wevers et al., 2011). Similarly, primate bocaparvoviruses include human and non-human primate strains (Brožová et al., 2016; Kapoor et al., 2010a, 2010b; Sharp et al., 2010).

Additionally, approximately 75% of the diseases that have emerged or re-emerged over the past two decades have wildlife sources (Woolhouse et al., 2012) and great apes play a critical role regarding these zoonosis (Calvignac-Spencer et al., 2012). Ape-to-human zoonotic transmission is suggested for several viruses including the Human mastadenovirus-B (Hoppe et al., 2015), Ebolavirus (Leroy et al., 2011), the Human Immuno-Defiency virus (HIV)-I (Sharp and Hahn, 2010), the Human T-cell Leukemia virus (HTLV)-I (Junglen et al., 2010) or the spumavirus (Betsem et al., 2011).

Despite the extensive research on the viruses infecting wild apes, their diversity and evolutionary history remain poorly understood.

The object of this study is to investigate adenovirus and bocaparvovirus naturally occuring infections in wild gorillas using PCR in order to understand their diversity and evolution.

In the first part of this thesis, I targeted the masdenoviruses infecting wild gorillas of Moukalaba-Doudou NationalPark and human (local people leaving nearby the park and mainly composed by gorillas' trackers).

Mastadenoviruses infect human and non-human primates (Duncan et al., 2013; Roy et al., 2009) and the human and non-human primate strains belong to the Human adenovirus species A to G (Pantó et al., 2015; Roy et al., 2009). Additionally, a recent report has documented about the Human mastadenovirus B originating from gorillas (Hoppe et al., 2015), and switched to human population and chimpanzees (Hoppe et al., 2015) which highlights the ape-human and ape-ape cross-species transmission.

I detected adenoviral genes in fecal samples from gorillas and the local people living around the national park: the overall prevalence rates of adenovirus were 24.1% and 35.0% in gorillas and humans, respectively. Sequencing revealed that the adenoviruses detected in the gorillas were members of HAdV-B, HAdV-C, or HAdV-E, and those in the humans belonged to HAdV-C or HAdV-D. Although HAdV-C members were detected in both gorillas and humans, phylogenetic analysis revealed that the viruses detected in gorillas were genetically distinct from those detected in humans. On the other hand, HAdV-B was clustered with other gorilla adenoviruses together with human and chimpanzee strains, which support the hypothesis of being a zoonotic virus. The HAdV-E was clustered with the chimpanzee strains. This result indicates the possibility of an

ape-ape transmission of HAdV-E species because chimpanzees have been reported to be the most probable ancestor hosts of these viruses.

In the second part, I targeted bocaparvoviruses in wild gorillas. The viruses infecting human and non-human primates are grouped into 2 species named primate bocaparvoviruses. To date, 2 species of primate bocaparvoviruses have been described. Primate bocaparvovirus 1 and 2 (Allander et al., 2001; Arthur et al., 2009; Kapoor et al., 2010b, 2009). A captive gorilla and a captive chimpanzee have been reported to be infected by the members of the species Primate bocaparvovirus 1 (Brožová et al., 2016; Kapoor et al., 2010a); therefore the diversity of bocaparvoviruses infecting wild apes and their evolutionary history remain poorly understood.

I detected two gorilla bocaparvovirus strains from 2 infant gorillas. Both strains clustered with viruses in the species *Primate bocaparvovirus 2* (with 86.0% nucleotide identity to a human bocavirus 2 isolate). The named *Gorilla bocaparvovirus 2* (GBOV2) of this study is the first non-human primate bocaparovirus within that species. The molecular evolutionary analyses of primate bocaparvoviruses revealed the presence of inter and

intra-species recombination events which might lead to the emergence of new bocaparvovirus variants in human as well as in non-human primate population.

The findings of this study contribute to a better understanding of the genetic diversity of mastadenoviruses and bocaparvoviruses infecting gorillas and provide insights to the evolutionary history of primate bocaparvoviruses.

Chapter II: Molecular epidemiological study of adenovirus infecting western lowland gorillas and humans in and around Moukalaba-Doudou National Park (Gabon)

Abstract

Adenoviruses are widespread in human population as well as in great apes, although the data about the naturally occurring adenovirus infections remain rare. I conducted the surveillance of adenovirus infection in wild western lowland gorillas in MDNP (Gabon), in order to investigate naturally occurring adenovirus in target gorillas and tested specifically a possible zoonotic transmission with local people inhabiting the vicinity of the park. Fecal samples were collected from western lowland gorillas and humans, and analyzed by PCR. I detected adenoviral genes in samples from both gorillas and the local people living around the national park respectively: the overall prevalence rates of adenovirus were 24.1% and 35.0% in gorillas and humans, respectively. Sequencing revealed that the adenoviruses detected in the gorillas were members of *Human mastadenovirus B* (HAdV-B), HAdV-C, or HAdV-E, and those in the humans belonged to HAdV-C or HAdV-D. Although HAdV-C members were detected in both gorillas and humans, phylogenetic analysis revealed that the viruses detected in gorillas are genetically distinct from those detected in humans. The HAdV-C constitutes a single host lineage which is compatible with the host-pathogen divergence. However, HAdV-B

and HAdV-E are constituted by multiple host lineages. Moreover there is no evidence of zoonotic transmission thus far. Since the gorilla-to-human transmission of adenovirus has been shown before, the current monitoring should be continued in a broader scale for gaining more insights about naturally occurring adenoviruses which would be helpful for the safe management of gorilla population and human health.

II.1. Introduction

Adenoviruses (AdVs) are non-enveloped icosahedral double-stranded DNA viruses. They belong to the family of *Adenoviridae*, which is divided into 5 genera: *Mastadenovirus*, *Atadenovirus*, *Aviadenovirus*, *Siadenovirus*, and *Ichtadenovirus*. Members of species belonging to genera *Mastadenovirus* and *Atadenovirus* are known to infect mammalian hosts (Lehmkuhl and Hobbs, 2008; Pantó et al., 2015). Mastadenoviruses infecting primates encompass 7 Human mastadenovirus species (HAdV-A to G), the accepted species *Simian mastadenovirus A* and candidate species SAdV-B to G (Chen et al., 2011), and further not yet classified mastadenoviruses (Chen et al., 2011; Gál et al., 2013; Pantó et al., 2015). That classification into species or subgroups is based on hemagglutination features, DNA (Deoxyribonucleic acid homology, and genomic organization (Robinson et al., 2013).There are currently over 60 HAdV types with HAdV-D containing the most members (Robinson et al., 2013).

Adenoviruses were first isolated from humans and identified as the causative agent of epidemic febrile respiratory disease among military recruits in the 1950s (Hilleman and H., 1954; Rowe, et al., 1953). It is estimated that more than 90% of the human population

is seropositive for one or more serotypes of adenoviruses (D'Ambrosio et al., 1982; Wadell et al., 1987). The molecular biology of human-derived adenoviruses has been characterized extensively for the species C group, for which HAdV *2* (HAdV-2) and HAdV-5 serve as prototypes (Fields, et al., 1996). Adenoviruses cause a variety of nonlethal infectious diseases in humans, and lethal disseminated adenovirus infection occurs in immunosuppressed patients (Fields, et al., 1996).

The first description of a simian adenovirus in the literature was of a chimpanzee AdV (Rowe et al., 1956), today known as SAdV-21 within the species *Human mastadenovirus B*. Later, when investigating chimpanzees suffering from kuru, four novel apes AdVs were discovered (Rogers, et al., 1967). Ape AdVs have been detected or isolated from African apes including chimpanzees, bonobos and gorillas (Duncan et al., 2013; Hoppe et al., 2015; Roy et al., 2009; Seimon et al., 2015; Wevers et al., 2011, 2010). Gorilla adenoviruses have been proposed to be members of HAdV-B, C, E, and F (Duncan et al., 2013; Hoppe et al., 2015; Roy et al., 2009; Seimon et al., 2015; Wevers et al., 2011, 2010). A recent report confirmed that the species HAdV-B which includes viruses from mixed host origin (Wevers et al., 2011), originated from gorillas and have switched to humans

and to chimpanzees during 2 different host switch events (Hoppe et al., 2015). Serological surveys have found that anti-AdV antibodies were prevalent in 96% of mountain gorillas, suggesting that AdVs are circulating among these animals (Whittier, 2009). In addition, Hoppe et al. recently reported a high prevalence of AdV in wild apes including gorillas (45 to 100%) (Hoppe et al., 2015). Because AdVs are shed in the feces and saliva of infected animals (Roy et al., 2009), these viruses could possibly be transmitted among host animals via the fecal-oral route and inhalation of aerosols (Fong et al., 2010).

Comprehensive studies are still needed to clarify the origin and the diversity of adenoviruses spread in human and non-human primate populations. Thus, to fill the gap, understanding the evolution pattern of AdVs spread in non-human primates and in people frequently coming in contact with these animals is critical. In this study, I investigated AdV infection in 2 habituated western lowland gorilla groups in MDNP. In addition, I assessed AdV infection in the local people living around the national park to evaluate potential zoonotic transmissions.

II.2. Materials and Methods

II.2.1. Sample collection and preparation

The study site (MDNP) is located in the south-western part of Gabon (Fig. 1.2). MDNP has been reported to have a high gorilla density (more than 3 gorillas per square kilometer) (Ando et al., 2008), and the absence of hunting pressure from local villagers makes it a major habitat for western lowland gorillas in central Africa. From December 2010 to November 2011, during tracking, we collected 112 fresh fecal samples from 2 wild gorilla groups, which were named as Group Gentil (GG) and Group 8 (G8). GG and G8 had been habituated to human observers since 2003 (Ando et al., 2008) and 2011, respectively. During the study period, GG consisted of 20–21 individuals, including 1 adult (expected age ≥13 years old) male, 6 adult (≥10 years old) females, 10 young (4–6 years old) males, and 3 young females, and all members were individually identified. In contrast, G8 was estimated to consist of 8–12 individuals, including 1 adult male, 2 adult females, 5–8 young males and females. GG was mainly sampled near the village Doussala, in the ancient plantations, where the forest has been formerly used in various crop fields, while G8 was found far from the village in the primary forest (Fig. 1.2). In

addition to the gorilla samples, 20 fecal samples were collected from villagers, including trackers working for the habituation of gorillas. Upon collection, each fecal specimen was immediately placed into a tube containing 2 ml of RNAlater (Ambion, Austin, TX, USA). The tubes were kept at room temperature for at most 20 days at the field camp until the samples were transported to the laboratory in Libreville, the capital city of Gabon. At the laboratory, the tubes were stored at –20°C until DNA extraction.

II.2.2. DNA extraction and PCR

Total DNA was extracted from the sample by using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. I used the following primer sets for nested PCR: (1) 4431-s/4428-as and 4428-s/4429-as (Table 1.3), targeting the HAdV DPOL gene (Wevers et al., 2010) and (2) AdhexF1/AdhexR1 and AdhexF2/AdhexR2, targeting loop 1—encompassing the hypervariable region (HVR1–6)—of the hexon gene of mastadenoviruses (Lu and Erdman, 2006). PCR for the DPOL gene was performed in a total volume of 20 µl containing 10 µl of 2×GoTaq Green Master Mix (Promega, Madison, WI, USA), 20 pmol of each primer, and 50 ng of DNA template. The following cycling conditions, slightly modified from Wevers et al. (Wevers et al.,

2010) were used: 95°C for 2 min; 35 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min; and a 7-min final extension step at 72°C. PCR amplification of the hexon gene (HVR1–6) was performed in a total volume of 50 µl containing 200 µM of each dNTP, 20 pmol of each primer, 1.25 U of PrimeSTAR GXL polymerase (TaKaRa, Tokyo, Japan), and 50 ng of DNA template. The cycling conditions were as follows: 98°C for 3 min; 35 cycles of 98°C for 10 s, 45°C for 1 min, and 72°C for 2 min; and a final extension of 72°C for 7 min. For the nested reaction, 2 µl of the first PCR was amplified as above. Amplified products were separated on 1.5% agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions; the amplicons were then directly sequenced with the primers for the second PCR.

II.2.3. BLAST search

BLAST searches were carried out in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the determined nucleotide sequence as a query in the BLASTN program. The queries with at least 90% identity with the deposited adenovirus gene sequences were considered for AdV species identification.

II.2.4. Sequencing and phylogenetic analysis

Twenty-four of the 27 positive samples (DNA quantity ≥ 5 ng/µl), were subjected to direct sequencing of DPOL gene fragments. Six samples were selected randomly for cloning and sequencing of DPOL and hexon HVR1–6 gene fragments. The PCR products were cloned into plasmid vector pCR-Blunt II-TOPO using the Zero Blunt TOPO PCR cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Plasmid extraction was carried out using the Wizard Miniprep Kit (Promega), and the extracted plasmids were sequenced by Big Dye terminator cycle sequencing (Applied Biosystems, Foster City, CA, USA).

The hexon HVR1–6 and DPOL gene sequences were edited and aligned using GENETYX software version 12.0 (Genetyx Co., Tokyo, Japan) and MEGA software version 5.05 (Tamura et al., 2011). The nucleotide sequences of DPOL (528-bp, corresponding to the position 29,200-29,727 in the reference simian adenovirus 21) and 782-bp fragments of the hexon gene (corresponding to the position 18, 867-19,635 in the reference simian adenovirus 21) were aligned using MUSCLE, with the default parameters for gap opening and gap extension. These alignments were used for

phylogenetic analyses. Phylogenetic trees were constructed using the maximum likelihood method in MEGA 5.05 (Tamura et al., 2011). A statistical test for the phylogeny was computed by means of bootstrapping. Percentages of 100 bootstrap replicates at the node were calculated to ensure the reliability of the trees.

II.2.5. **Nucleotide sequence accession numbers**

Preliminary names were given to candidate novel HAdVs following the method used by Wevers et al.(Wevers et al., 2010). The gorilla adenoviruses detected in this study were named as follows: Gorilla gorilla AdV B11-B23 (KM886307- KM886309, KM886311, KM886325- KM886328, KM886331- KM886335), Gorilla gorilla AdV C10-C18 (KM886310, KM886320- KM886324, KM886329), and Gorilla gorilla AdV E1 (KM886330). The sequences used as references for phylogenetic analysis are listed in Table 1.4.

II.3 Results

II.3.1. Detection of AdV genes in western lowland gorillas in MDNP

To survey AdV infection in gorillas in MDNP, i collected fecal samples from 2 gorilla groups (GG: well-habituated group, G8: newly habituated group), and analyzed them by nested PCR targeting the DPOL and hexon genes.The DPOL and hexon genes were detected in both groups (Table 1.1). The overall prevalence of AdV in the gorilla population was 24.1% (27/112): of the 86 samples from GG, 21 were positive for both genes, 4 were positive only for the DPOL gene, and 1 sample was positive only for the hexon gene. In contrast, only 1 of the 26 samples was positive for both tested genes in G8 (Table 1.1). These data suggest that AdVs are naturally circulating among gorillas in MDNP. To confirm the detected AdV species, I further determined the nucleotide sequences of the amplicons and determined the species of the detected AdVs by BLAST searches. Of the tested samples, 16 belonged to HAdV-B; 10 to HAdV-C; and 1 to HAdV-E.

II.3.2. Detection of AdV genes in local people living around the national park

The prevalence of AdVs in well-habituated gorillas (30.2% in GG group) was higher than that of newly habituated ones (3.8% in G8 group), raising 2 possibilities either the AdVs in gorillas are derived from humans during the habituation process or AdVs are ubiquitous in the environment in and around areas of human habitation. Therefore, I screened the local people (village Doussala in Fig. 1.2) for AdV infection. The prevalence in the local people was 35.0 % (7/20): 2 samples were positive for both DPOL and hexon genes, and 5 were positive only for the hexon gene (Table 1.2). These results revealed that the local people including trackers were also infected with AdVs.

I sequenced the detected virus genes and identified the species of AdVs: 1 sample was infected with HAdV-C, and the others harbored HAdV-D.

II.3. 3. Phylogenetic analysis

HAdV-C genes were detected in both gorillas and humans in MDNP, suggesting zoonotic transmission of AdV between the human and gorilla populations. To investigate this possibility, as well as to gain insights into the genetic diversity of adenoviruses in MDNP, I performed phylogenetic analyses.

In gorillas, in the tree based on the DPOL gene, the 14 AdV genes identified in this study were divided into 2 groups; they clustered with SAdV-28.2, SAdV-46, SAdV-47, and gorilla AdV strains 6589 and 6575, which are representative strains of HAdV-B in gorillas, and unidentified simian adenoviruses recently described (Hoppe et al., 2015) (Fig. 1.3 and 1.5). Nine AdV genes were clustered with simian AdV-45 and simian AdV-43, which are representative strains of HAdV-C in gorilla and new unidentified simian adenoviruses (Hoppe et al., 2015) (Fig.1.3 and 1.5). In contrast, one AdV gene clustered with SAdV-26 and chimpanzee AdV strain Y25, which are chimpanzee-specific strains belonging to HAdV-E (Fig. 1.3 and 1.5). In the hexon gene-based trees, five HAdV-B (Figure 1.6.A), 1 HAdV-C (Figure 1.6.B), and 1 HAdV-E (Fig. 1.4) strains were identified among those isolated from gorillas. HADV-E is divided into 4 groups (Fig. 1.4): 2 groups of human origin and 2 of simian origin. The HAdV-E detected in gorillas in this study belonged to the Human mastadenovirus E of simian origin (Fig. 1.4).

In the case of humans, the tree based on the DPOL gene showed 1 AdV gene clustered with HAdV-1 (HAdV-C), which is genetically different from the strains detected in gorillas (Fig. 1.2 and 1.4.B), and 1 clustered with the human AdV type 53 and human

AdV type 8, which belong to the HAdV-D (Fig. 1.3 and 1.7). The hexon gene-based tree showed that the detected viruses belonged to 4 different serotypes in HAdV-D group (Fig.1.7). The HAdV-D seems to be exclusively limited to the human population as reported earlier (Hoppe et al., 2015).

II. 4. Discussion

In this study, I detected several species of AdVs in western lowland gorillas in MDNP as well as in local people residing nearby. Interestingly, the positive rate in the well-habituated group (30.2%) was higher than that of the newly habituated group (3.8%). In addition, members of HAdV-C were detected in both gorillas and humans. However, the phylogenetic analyses revealed that the AdVs detected from gorillas are genetically distinct from those from local people living around the national park. Therefore, gorilla viruses and human viruses may have been separately circulating in each population in this region, and transmission between human and animals does not seem to happen easily in both directions, although I cannot exclude the possibility that I just missed zoonotically transmitted AdVs in this study. The difference in the prevalence between groups GG and G8 may be attributed to the quality of samples, because samples from GG might have

been fresher than the ones from G8; GG was sampled while following animals, but G8 was sampled on trails, sometimes without observing the animals. In contrast, AdVs were reported to be transmitted between humans and non-human primates, indicating that AdVs have zoonotic potential (Hoppe et al., 2015; Wevers et al., 2011) despite the belief that AdVs have co-evolved with their hosts and are usually not transmitted to other species.

Adenovirus infections have been reported in high prevalence in wild gorillas' populations as well as in other great apes (Hoppe et al., 2015; Seimon et al., 2015; Wevers et al., 2011). In this study, the overall prevalence of AdV infection in gorillas was 24.1%, which is lower than the previously reported figure of 44.9% in free-ranging gorillas in Congo Republic (Seimon et al., 2015) or of 48 % in free-ranging gorillas in Loango National Park (Gabon) (Hoppe et al., 2015). These differences might be due to the quality of the samples and/or sensitivity of the PCR. In addition, the PCR systems used in this study targeted the conserved DPOL gene of mastadenovirus or the hypervariable region of the hexon gene, but in some samples, only 1 of the 2 genes was amplified. This shows that my PCR system might not be able to amplify all AdV genes or that the samples could

have been partially degraded, or simply natural differences (Hoppe et al., 2015). Alternatively, AdV prevalence in the gorillas included in this study was low. Further systematic studies are needed to assess these possibilities.

I detected members of 3 species: HAdV-B, HAdV-C, and HAdV-E in western lowland gorillas in MDNP; these AdV species have been reported earlier (Duncan et al., 2013; Hoppe et al., 2015; Seimon et al., 2015; Wevers et al., 2011) in western lowland gorillas as well as in other gorilla sub-species in sub-Saharan Africa. The gorilla adenoviruses of this study mainly belong to the HAdV-B (59%). This confirms the gorilla as the major host of HAdV-B in sub-Saharan Africa. Based the hexon tree (Fig.1.6 A), the new virus named Gorilla gorilla adenovirus B19, together with the human mastadenovirus B isolates 6560 and 6674 constitute a single clade probably originating from gorillas. The pattern observed within the species Human mastadenovirus C (Fig. 1.6 B) is compatible with the host-pathogen divergence as previously reported (Hoppe et al., 2015; Roy et al., 2009; Wevers et al., 2011). All the lineages in HAdV-C are host specific (Hoppe et al., 2015). The only member of HAdV-E detected in this study clusters with chimpanzee strains (Fig. 1.4). This finding supports previous report describing the non-

human primate AdVs members of the HAdV-E to originate from chimpanzees (Hoppe et al., 2015). I can suspect the Gorilla gorilla adenovirus E1 of this study to be the result of chimpanzee-to-gorilla transmission, as chimpanzees and gorillas are living sympatrically in MDNP. Broader screening would clarify the evolution of viruses belonging to HAdV-E.

On the other hand, the adenovirus-like genes detected in the human population around MDNP are mainly members of the HAdV- D (85.71%) which confirms that the species HAdV-D originated in humans (Hoppe et al., 2015) and so far has been exclusively human specific. Therefore, 4 different serotypes were detected in this study; highlighting the diversity of adenoviruses circulating in the target human population. Further systematic studies should clarify the the circulation of AdVs in human population.

Taken together, my results show that AdVs are naturally present among gorillas and humans in MDNP in Gabon. Although there is no evidence of zoonotic transmission of AdVs in this region, my data shows de feasibility of monitoring viral agents in wild habituated gorillas (Gilardi et al., 2015) and in local people living nearby for the safe management of wild gorilla populations and human health, as well as for understanding

the evolution of virus. Since the zoonotic transmission of adenovirus already occurred during hominin evolution, assessing the zoonotic transmission of that virus in the context of habituation sites such as MDNP is recommended.

Table 1.1. Detection of adenovirus *DPOL* and hexon genes in samples from gorilla groups in MDNP

Gorilla groups	No. of tested samples	No. of positive samples in PCR (%)	Species No. of samples		
			B	C	E
GG	86	26 (30.2%)	16	9	1
G8	26	1 (3.8%)	0	1	0
Total	112	27 (24.1%)	16	10	1

Table 1.2. Adenovirus infection in humans

Sample ID	PCR DPOL	PCR hexon
H1	HAdV-C	HAdV-C
H2		HAdV-D
H3		
H4		
H5		
H6		HAdV-D
H7		
H8		HAdV-D
H9		
H10		
H11		HAdV-D
H12		HAdV-D
H13		
H14		
H15		
H16		
H17	HAdV-D	HAdV-D
H18		
H19		
H20		

Table 1.3. Primers and probe sequences for amplification of *DPOL* and hexon genes

Primer set abbreviation	Targeted gene	Name of primer	Sequence 5'-3'	PCR length
DPOL-cons	*DPOL* 1st round	4431-s	GTnTwyGAyAThTGyGGhATGTAyGC	
		4428-as	GAGGCTGTCCGTrTC(n/I)CCGTA[#]	956
	2nd round	4428-s	CGGACGCCTCTGyTGGAC(n/I)AA	
		4429-as	GGCCAGCACrAA(n/I)GArGC	650
HVR($_{1-6}$)	Hexon 1st round	AdhexF1	TICTTTGACATICGIGGIGTICTIGA	
		AdhexR1	CTGTCIACIGCCTGRTTCCACA	850
	2nd round	AdhexF2	GGYCCYAGYTTYAARCCCTAYTC	774
		AdhexR2	GGTTCTGTCICCCAGAGARTCIAGCA	

Table 1.4.: Adenoviruses, accession number and hosts

Adenovirus	Abbreviation	GenBank accession number/reference	Host	Wild	Captive[a]
HAdV-B of this study					
Gor. gorilla adenovirus B11	Ggor AdV B11	**KM886307**	Gorilla	+	
Gor. gorilla adenovirus B12	Ggor AdV B12	**KM886308**	Gorilla	+	
Gor. gorilla adenovirus B13	Ggor AdV B13	**KM886309**	Gorilla	+	
Gor. gorilla adenovirus B14	Ggor AdV B14	**KM886311**	Gorilla	+	
Gor. gorilla adenovirus B15	Ggor AdV B15	**KM886314**	Gorilla	+	
Gor. gorilla adenovirus B16	Ggor AdV B16	**KM886315**	Gorilla	+	
Gor. gorilla adenovirus B17	Ggor AdV B17	**KM886317**	Gorilla	+	
Gor. gorilla adenovirus B18	Ggor AdV B18	**KM886319**	Gorilla	+	

Gor. gorilla adenovirus B19	Ggor AdV B19	**KM886325**	Gorilla	+
Gor. gorilla adenovirus B20	Ggor AdV B20	**KM886326**	Gorilla	+
Gor. gorilla adenovirus B21	Ggor AdV B21	**KM886327**	Gorilla	+
Gor. gorilla adenovirus B22	Ggor AdV B22	**KM886328**	Gorilla	+
HAdV-C of this study				
Gor. gorilla adenovirus C10	Ggor AdV C10	**KM886310**	Gorilla	+
Gor. gorilla adenovirus C11	Ggor AdV C11	**KM886312**	Gorilla	+
Gor. gorilla adenovirus C12	Ggor AdV C12	**KM886313**	Gorilla	+
Gor. gorilla adenovirus C13	Ggor AdV C13	**KM886320**	Gorilla	+
Gor. gorilla adenovirus C14	Ggor AdV C14	**KM886321**	Gorilla	+
Gor. gorilla adenovirus C15	Ggor AdV C15	**KM886322**	Gorilla	+

Gor. gorilla adenovirus C16	Ggor AdV C16	**KM886323**	Gorilla	+
Gor. gorilla adenovirus C17	Ggor AdV C17	**KM886324**	Gorilla	+
Gor. gorilla adenovirus C18	Ggor AdV C18	**KM886329**	Gorilla	+
HAdV E of this study				
Gor. gorilla adenovirus E1	Ggor AdV E1	**KM886330**	gorilla	+
Reference sequences used for phylogeny				
Gorilla gorilla adenovirus 1	Ggor AdV1	**Wevers et al.,2011**	Gorilla	+
6588 Gor. gor. adenovirus	6588 Ggor AdV	**Wevers et al.,2011**	Gorilla	+
6575 Gor. gor. adenovirus	6575 Ggor AdV	**Wevers et al.,2011**	Gorilla	+
Human adenovirus type 18	HAdV-A-18	**GU191010**		
Human adenovirus type 31	HAdV-A-31	**AM749299**		
Human adenovirus type 1	HAdV-C	**AF534906**		
Human adenovirus D-8	HAdV D-8	**AB448767**		
Human adenovirus D-53	HAdV D-53	**FJ169625**		
Human_adenovirus_D_strain_human/CA		**HQ007053**		

Human_adenovirus_D_isolate_hu4555_UG_	**KF976533**
Human_adenovirus_26_:_BP-2_	**AB330107**
Human_adenovirus_62	**JN162671**
Human_adenovirus_69	**JN226748**
Human_adenovirus_29	**JN226754**
Human_adenovirus_D	**KF976527**
Human_adenovirus_54	**AC000006**
Human_adenovirus_9	**NC010956**
Human_adenovirus_10	**NC012959**
Human_adenovirus_15	**AJ854486**
Human_adenovirus_13	**AB330091**
Human_adenovirus_17	**AB562586**
Human_adenovirus_19_	**JN226747**
Human_adenovirus_20_	**HQ910407**
Human_adenovirus_22_	**JQ326209**
Human_adenovirus_22_	**JN226749**
Human_adenovirus_23_	**FJ619037**
Human_adenovirus_24_	**KF279629**
Human_adenovirus_25_	**JN226751**

Human_adenovirus_27_		**JN226752**
Human_adenovirus_28_		**JN226753**
Human_adenovirus_30_		**FJ824826**
Human_adenovirus_32_		**JN226755**
Human_adenovirus_33_		**JN226756**
Human_adenovirus_36_		**JN226758**
Human_adenovirus_37_		**GQ384080**
Human_adenovirus_38_		**AB448778**
Human_adenovirus_39_		**JN226759**
Human_adenovirus_42		**JN226760**
Human_adenovirus_43		**JN226761**
Human_adenovirus_44		**JN226762**
Human_adenovirus_45		**JN226763**
Human adenovirus type 4[c]	HAdV-4	**AY594253**
Human adenovirus type 4[d]	HAdV-4	**AY594254**
Human adenovirus type 4[e]	HAdV-4	**AY599835**
Human adenovirus -E	HAdV-E	**X74508**
Human adenovirus-E	HAdV-E	**AY487947**
Human adenovirus-E	HAdV-E	**EF371058**

Human adenovirus type 4[f]	HAdV-4	**AY599837**		
Human adenovirus type 4[g]	HAdV-4	**KF006344**		
Human adenovirus F-40	HAdV F-40	**NC_001454**		
Human adenovirus F-41	HAdV-41	**DQ 315364**		
Human adenovirus G- 52	HAdV G-52	**DQ 923122**		
Simian adenovirus 1	SAdV-1	**AY771780**	OWM[b]	
Simian adenovirus 3	SAdV-3	**AY598782.1**	OWM[b]	
Simian adenovirus 28.2	SAdV -28.2	**FJ025915**	Gorilla	+
Simian adenovirus 46	SAdV-46	**FJ025930**	Gorilla	+
Simian adenovirus 45	SAdV-45	**FJ025901**	Gorilla	+
Simian adenovirus 48	SAdV-48	**HQ241818.1**	OWM[b]	+
Simian adenovirus 24	SAdV-24	**AY530878.1**	Chimpanzee	+
Simian adenovirus 37.2	SAdV-37.2	**FJ025919**	Bonobo	+
Simian adenovirus 38	SAdV-38	**FJ025922**	Chimpanzee	+
Simian adenovirus 30	SAdV-30	**FJ025920**	Chimpanzee	+
Bovine adenovirus B	BAdV-B-3	**AC000002**		
Unidentified simian adenovirus strain u5753		**LN829111**	Chimpanzee	+
Unidentified simian adenovirus strain u7312		**LN829046**	Gorilla	+
Unidentified simian adenovirus strain u7304		**LN829045**	Gorilla	+

Unidentified simian adenovirus strain u7289	**LN829041**	Gorilla	+
Unidentified simian adenovirus strain u7283	**LN829040**	Gorilla	+
Unidentified simian adenovirus strain u7280	**LN829039**	Gorilla	+
Unidentified simian adenovirus strain u7264	**LN829038**	Gorilla	+
Unidentified simian adenovirus strain u7261	**LN829037**	Gorilla	+
Unidentified simian adenovirus strain u6208	**LN829036**	Chimpanzee	+
Unidentified simian adenovirus strain u7259	**LN829034**	Bonobo	+
Unidentified simian adenovirus strain u7258	**LN829033**	Bonobo	+
Unidentified simian adenovirus strain u7257	**LN829032**	Bonobo	+
Unidentified simian adenovirus strain u7256	**LN829031**	Bonobo	+
Unidentified simian adenovirus strain u7255	**LN829030**	Bonobo	+
Unidentified simian adenovirus strain u7254	**LN829029**	Bonobo	+
Unidentified simian adenovirus strain u7253	**LN829028**	Bonobo	+
Unidentified simian adenovirus strain u7252	**LN829027**	Bonobo	+
Unidentified simian adenovirus strain u7251	**LN829026**	Bonobo	+
Unidentified simian adenovirus strain u7250	**LN829025**	Bonobo	+
Unidentified simian adenovirus strain u7248	**LN829024**	Bonobo	+
Unidentified simian adenovirus strain u7246	**LN829023**	Bonobo	+
Unidentified simian adenovirus strain u7244	**LN829022**	Bonobo	+

Unidentified simian adenovirus strain u7243	**LN829021**	Bonobo	+
Unidentified simian adenovirus strain u7242	**LN829020**	Bonobo	+
Unidentified simian adenovirus strain u7241	**LN829019**	Bonobo	+
Unidentified simian adenovirus strain u7239	**LN829018**	Bonobo	+
Unidentified simian adenovirus strain u7237	**LN829017**	Bonobo	+
Unidentified simian adenovirus strain u7236	**LN829016**	Bonobo	+
Unidentified simian adenovirus strain u7231	**LN829015**	Bonobo	+
Unidentified simian adenovirus strain u7230	**LN829014**	Bonobo	+
Unidentified simian adenovirus strain u7229	**LN829013**	Bonobo	+
Unidentified simian adenovirus strain u7228	**LN829012**	Bonobo	+
Unidentified simian adenovirus strain u7227	**LN829011**	Bonobo	+
Unidentified simian adenovirus strain u7226	**LN829010**	Bonobo	+
Unidentified simian adenovirus strain u7225	**LN829009**	Bonobo	+
Unidentified simian adenovirus strain u7224	**LN829008**	Bonobo	+
Unidentified simian adenovirus strain u7315	**LN829004**	Gorilla	+
Unidentified simian adenovirus strain u7287	**LN828995**	Gorilla	+
Unidentified simian adenovirus strain u7273	**LN828990**	Gorilla	+
Unidentified simian adenovirus strain u7268	**LN828988**	Gorilla	+
Unidentified simian adenovirus strain u7265	**LN828987**	Gorilla	+

Unidentified simian adenovirus strain u6776	**LN828984**	Gorilla	+
Unidentified simian adenovirus strain u6588	**LN828983**	Gorilla	+
Unidentified simian adenovirus strain u6575	**LN828982**	Gorilla	+
Unidentified simian adenovirus strain u6565	**LN828981**	Gorilla	+
Unidentified simian adenovirus strain u6560	**LN828980**	Gorilla	+
Unidentified simian adenovirus strain u3135	**LN828979**	Chimpanzee	+
Unidentified simian adenovirus strain u6211	**LN828978**	Chimpanzee	+
Unidentified simian adenovirus strain u5052	**LN829047**	Gorilla	+
Unidentified simian adenovirus strain u7297	**LN829044**	Gorilla	+
Unidentified simian adenovirus strain u7296	**LN829043**	Gorilla	+
Unidentified simian adenovirus strain u7294	**LN829042**	Gorilla	+
Unidentified simian adenovirus strain u6480	**LN829007**	Gorilla	+
Unidentified simian adenovirus strain u5855	**LN829006**	Gorilla	+
Unidentified simian adenovirus strain u7317	**LN829005**	Gorilla	+
Unidentified simian adenovirus strain u7314	**LN829003**	Gorilla	+
Unidentified simian adenovirus strain u7311	**LN829001**	Gorilla	+
Unidentified simian adenovirus strain u7306	**LN829000**	Gorilla	+
Unidentified simian adenovirus strain u7302	**LN828999**	Gorilla	+
Unidentified simian adenovirus strain u7295	**LN828997**	Gorilla	+

Unidentified simian adenovirus strain u7293	**LN828996**	Gorilla	+
Unidentified simian adenovirus strain u7278	**LN828994**	Gorilla	+
Unidentified simian adenovirus strain u7276	**LN828993**	Gorilla	+
Unidentified simian adenovirus strain u7275	**LN828992**	Gorilla	+
Unidentified simian adenovirus strain u7274	**LN828991**	Gorilla	+
Unidentified simian adenovirus strain u7270	**LN828989**	Gorilla	+
Unidentified simian adenovirus strain u7262	**LN828986**	Gorilla	+
Unidentified simian adenovirus strain u7260	**LN828985**	Gorilla	+
Unidentified simian adenovirus strain u7310	**LN828977**	Gorilla	+

aCaptive: zoo animals bOWM: Old world monkey c: strain RI-67, d: vaccine strain, e: strain NHRC 42606, f: strain NHRC 3, g: strain GZ01

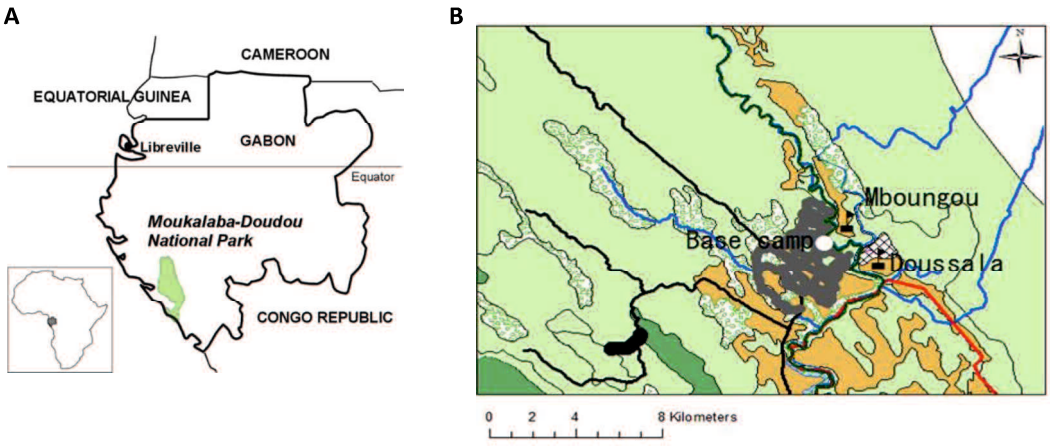

Fig.1.1. Location features of the sampling area

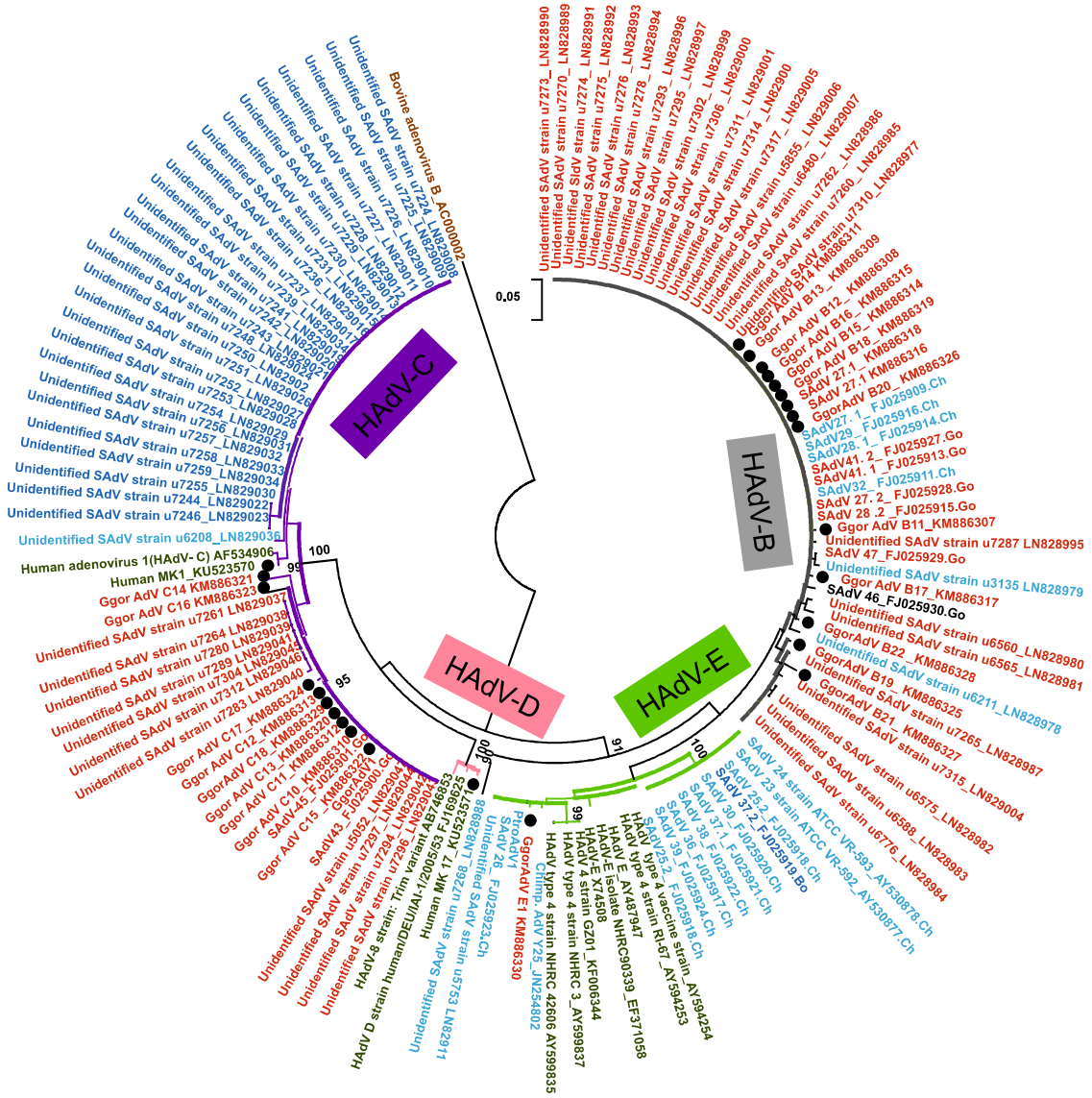

Fig.1.2. Phylogenetic tree of adenovirus (AdV) DPOL

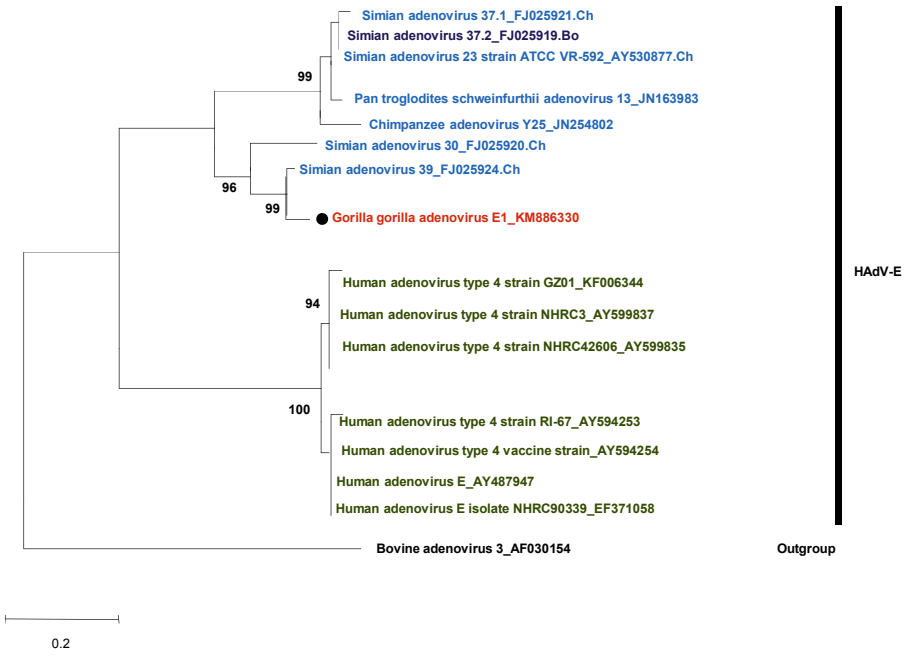

Figure 1.3. Phylogenetic tree of the adenovirus hexon gene loop 1 of HAdV-E

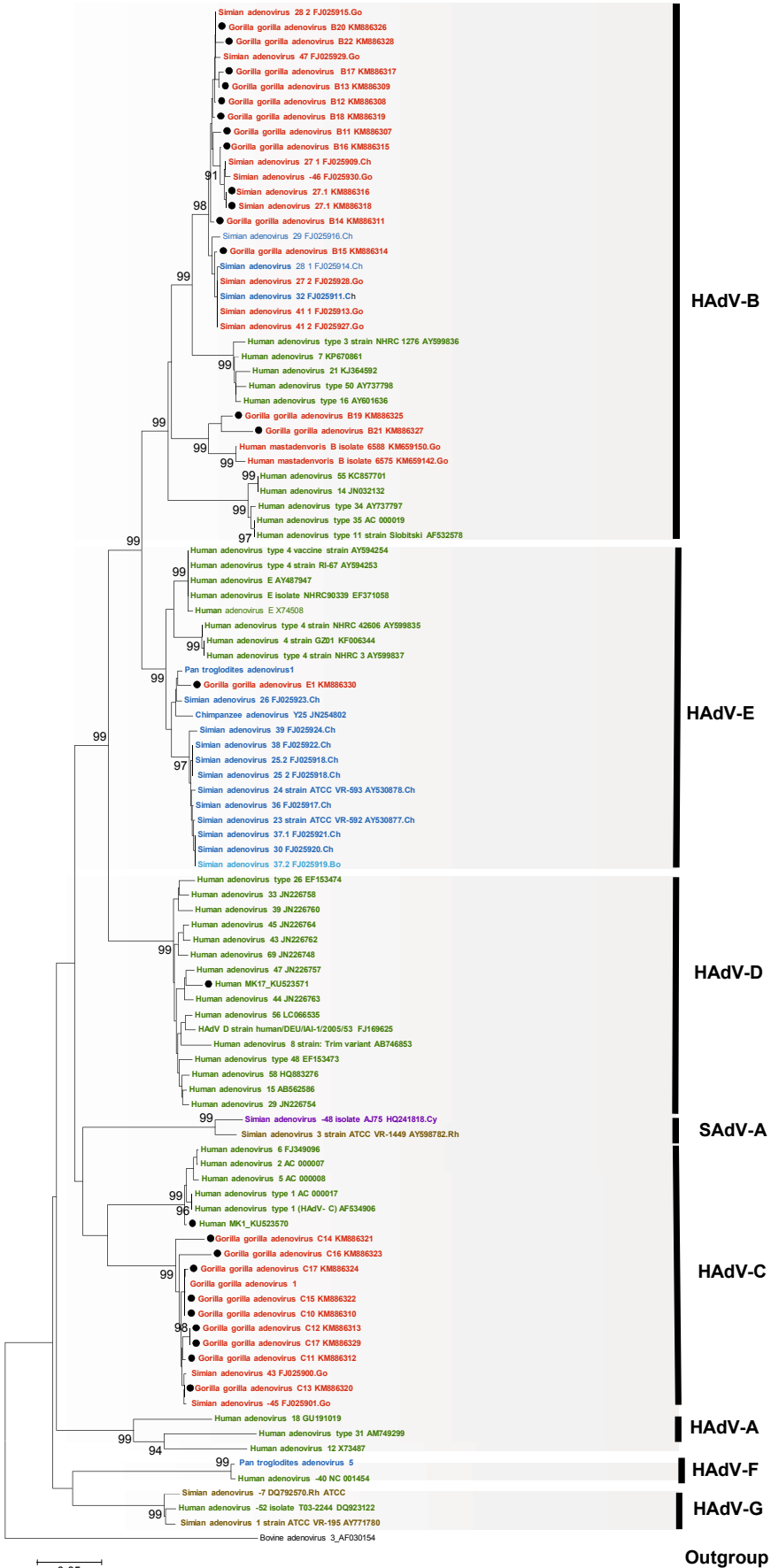

Figure 1.4. Phylogenetic tree of the partial sequence of DPOL

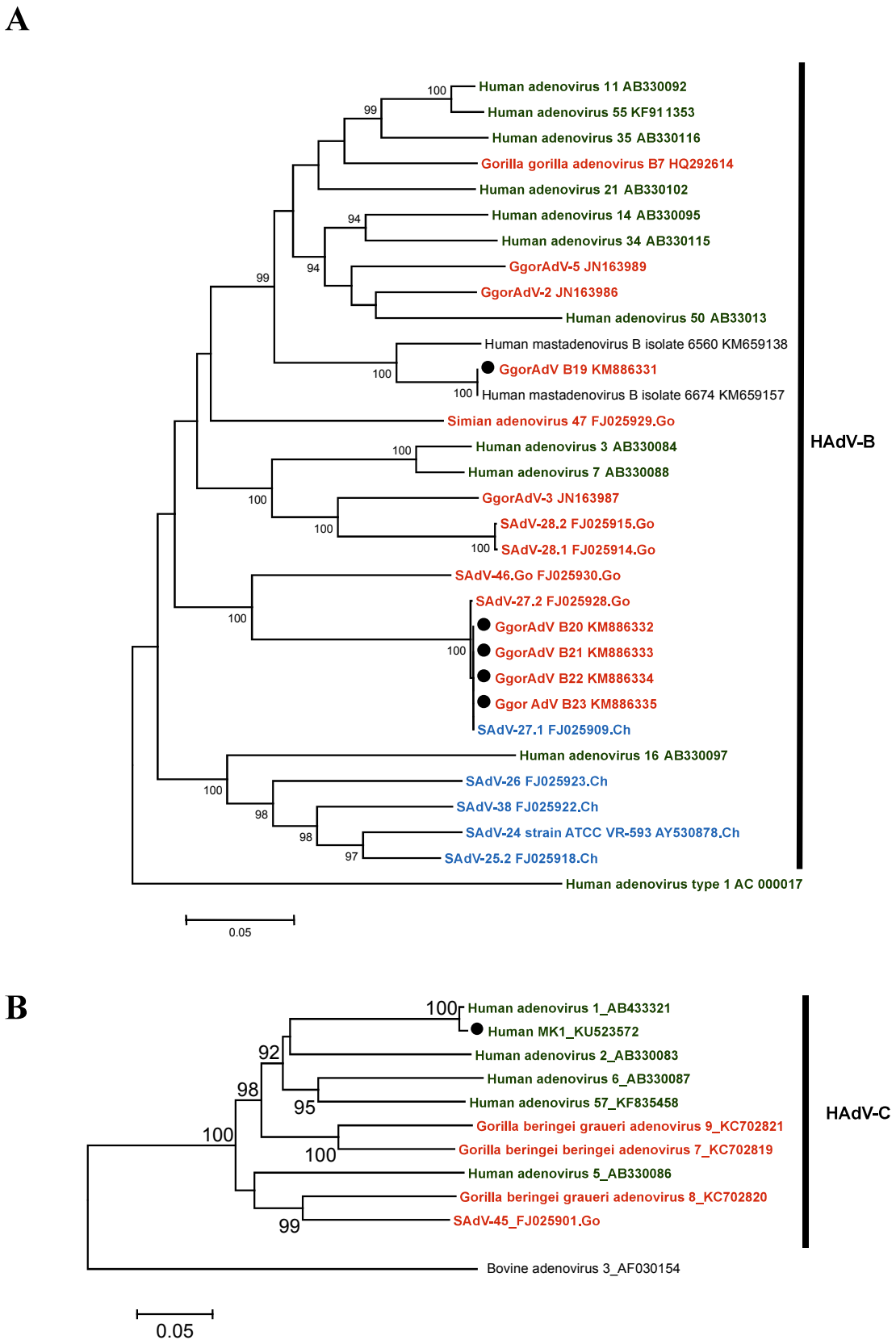

Fig. 1.5. Phylogenetic tree of the partial sequence of the hexon gene

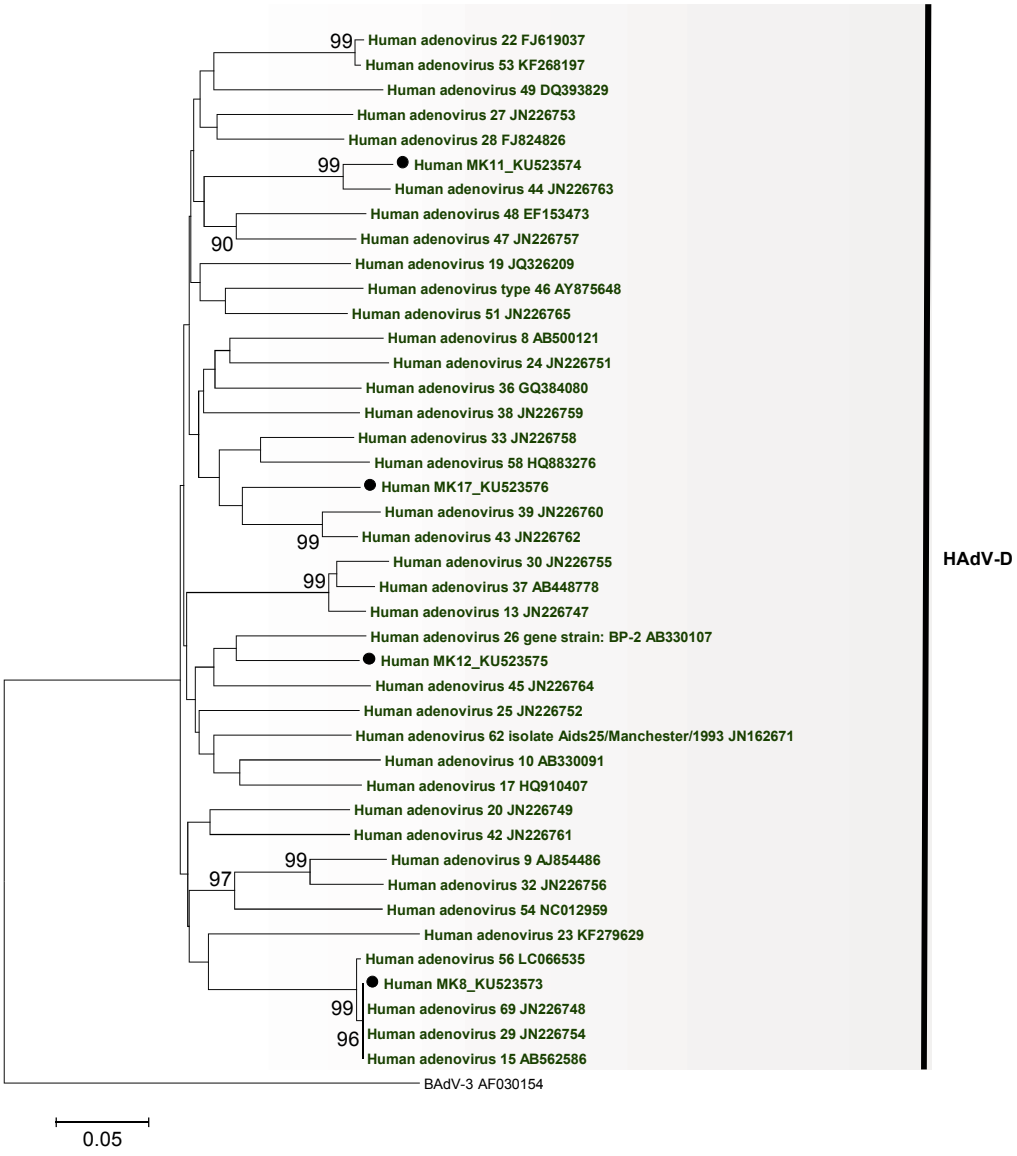

Figure 1.6. Phylogenetic tree of partial hexon of HAdV-D

Chapter III: Identification and molecular characterization of novel primate bocaparvoviruses from wild western lowland gorillas of Moukalaba-Doudou National Park, Gabon

Abstract

Bocaparvoviruses have been studied extensively owing to their ability to cause respiratory illness or gastroenteritis in humans. Some bocaparvoviruses have been detected in non-human primates (gorillas and chimpanzees), but the diversity and evolution of these viruses are not fully understood. In this study, I collected 107 fecal samples from wild western lowland gorillas in MDNP in Gabon to investigate the presence of bocaparvoviruses. Using a combination of pan-bocaparvovirus PCR and individual identification by microsatellite genotyping, I found that two samples from two apparently healthy infant gorillas were positive for bocaparvovirus. Sequencing and phylogenetic analyses revealed that the two gorilla bocaparvovirus strains are nearly identical and are closely related to viruses in the species *Primate bocaparvovirus 2* (with 86.0% nucleotide identity to a human bocavirus 2 isolate). To my knowledge, this is the first report showing the presence of a non-human primate bocaparovirus within *Primate bocaparvovirus 2*. My findings provide novel insights into the diversity and evolution of bocaparvoviruses and highlight the importance of surveying these viruses for the safe management of gorilla-based ecotourism.

III.1. Introduction

Bocaparvoviruses belong to the genus *Bocaparvovirus* of the sub-family *Parvovirinae* and the family *Parvoviridae*. Currently, 12 species have been reported in the genus: *Carnivore bocaparvovirus 1–3*, *Pinniped bocaparvovirus 1* and *2*, *Primate bocaparvovirus 1* and *2*, and *Ungulate bocaparvovirus 1–5* (Cotmore et al., 2014; ICTV, 2016). Further viruses, genetically related, recently described have not been classified yet (Guo et al., 2016; Lanave et al., 2015; Lau et al., 2017; Yang et al., 2016). Members of *Primate bocaparvovirus 1* and *2* are known to infect human and non-human primate hosts (Allander et al., 2005; Arthur et al., 2009; Brožová et al., 2016; Kapoor et al., 2010a, 2010b, 2009; Sharp et al., 2010). The first primate bocaparvovirus was detected in humans in 2005 from pooled nasopharyngeal aspirate specimens by large-scale molecular virus screening (Allander et al., 2005). Since then, many molecular epidemiological studies have suggested that human bocaviruses (HBOV) are associated with respiratory or gastrointestinal illnesses (Ahn et al., 2014; Arthur et al., 2009; Jin et al., 2011; Lu et al., 2015; Medici et al., 2012; Nunes et al., 2014; Phan et al., 2012). Additionally, some bocaparvoviruses have been detected from non-human primates, such as gorillas (Kapoor et al., 2010a, Sharp et al., 2010) and chimpanzees (Brožová et al., 2016; Sharp et al., 2010). Currently, *Primate bocaparvovirus 1* can be classified into four genotypes, including human and non-human primate bocaparoviruses: HBOV1, HBOV3, gorilla bocavirus 1 (GBOV1), and primate bocaparvovirus 1 isolate CPZh2 (Allander et al., 2005; Arthur et al., 2009; Brožová et al., 2016; Cotmore et al., 2014; Kapoor et al., 2010a, 2010b). *Primate bocaparvovirus 2* includes only two genotypes: HBOV2 and HBOV4 (Cotmore et al., 2014; Kapoor et al., 2009, 2010b; Khamrin et al., 2013).

Bocaparvoviruses are small non-enveloped viruses with a linear single-stranded DNA genome of approximately 4.9–5.5 kb. The viral genome contains three major open reading frames (ORFs); the left ORF encodes a non-structural protein (NS1) involved in replication (Allander et al., 2005), the right ORF encodes the viral capsid proteins (VP1 and VP2), and the middle ORF is a unique feature of bocaparvoviruses in the family *Parvoviridae* and encodes the highly phosphorylated non-structural protein NP1. Although primate bocaparvoviruses have been detected in diseased and healthy gorillas and chimpanzees (Brožová et al., 2016; Kapoor et al., 2010a), the clinical significance or symptoms in apes is unknown thus far. Additionnally, bocaparvovirus-like genes have been found in healthy free-ranging gorillas (prevalence: 36%) and chimpanzees (prevalence: 73%) (Sharp et al., 2010); However, the diversity and evolution of bocaparvoviruses infecting wild apes remain poorly understood.

In this study, I have investigated the bocaparvovirus infection in wild western lowland gorillas (Gorilla gorilla gorilla) in MDNP, Gabon. I have detected a novel bocaparovovirus genotype and characterized the nearly complete genome of two novel gorilla bocaparvoviruses that form a single cluster within the species Primate bocaparvovirus 2.

III.2. Material and methods

III.2.1. Sample collection and DNA extraction

Between November and December 2014, 107 fresh fecal samples were collected from wild western lowland gorillas subjected to habituation in MDNP, Gabon (Ando et al., 2008). During the sample collection period, the target gorilla group included one adult male or silverback (estimated age, >13 years old), four adult females (estimated age, >10 years old), two sub-adults (estimated age, >6 years old), eight juveniles (estimated age, 4–6 years old), and three infants (estimated age, <3 years old) (Table 2.1). Opportunistically, other individual wild gorillas sharing the same home range with the target group were sampled. Gorillas were followed daily as part of the habituation process and fecal samples were collected on trails when following the animals. To detect bocaparvoviruses, few grams of the feces were taken using a sterile plastic stick and preserved in 5 ml of RNAlater (Ambion, Austin, TX, USA). In addition, for individual genetic identification (see the below section "Individual genetic identification"), the surface of each feces, which contains gorilla-derived tissues, were scratched using a cotton swab. The swabs were preserved in 1 ml of lysis buffer (0.5% SDS, 100 mM

EDTA, 100 mM Tris-HCl, and 10 mM NaCl) until DNA extraction. The following information: date, time, GPS coordinates, and fecal diameter were recorded upon collection. To prevent cross contamination, fecal collection were performed using disposable plastic sticks and protective gloves were used once. DNA was extracted from both samples using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions.

III.2.2. Individual genetic identification

To identify gorilla individuals, multiplex PCR was performed using QIAGEN Multiplex PCR Kits (QIAGEN), as described by (Inoue et al., 2013), with host DNA extracted from the swab samples of feces from gorillas. One primer set was used for six autosomal microsatellite loci (D7s817, D1s550, vWF, D1s2130, D7s794, and D6s1056 (Inoue et al., 2013). Genotype data for target individuals were already available and genetic variation at these six loci was sufficient to identify individuals (Inoue et al., unpublished results). After PCR to amplify these microsatellite loci, genotypes were determined using Peak Scanner V1.0 (Applied Biosystems, Foster City, CA, USA).

III.2.3. PCR detection of bocaparvovirus

The samples were tested for the presence of bocaparvovirus DNA by hemi-nested PCR using the primers PanBov F1 (5′TAATGCAYCARGAYTGGGTIGANCC-3′) and PanBov-R1 (5′-GTACAGTCRTAYTCRTTRAARCACCA-3′) for the first round of PCR

and PanBov-F2 (5′-GCAYCARGAYTGGGTIGANCCWGC-3′) and PanBov-R1 for the second round of PCR, according to Kapoor et al., 2010a. The first-round PCR was performed in a total volume of 20 µl containing 0.4 U of Phusion High-fidelity DPOL (Thermo-Scientific, Waltham, MA, USA), 200 µM each dNTP, 0.5 µM primers, Phusion HF buffer, and 1 µl of gorilla fecal DNA. The second round was performed in 50 µl with the same composition, using 1 µl of the first round PCR product as the template. The PCR strategy and the expected size of bands are shown in Fig. 2.1A-B. The amplicons were directly purified using the Innuprep PCR pure kit (Analytik Jena, Jena, Germany) or gel extracted using the NucleoSpin ® gel and PCR clean-up kit (Macherey–Nagel, Düren, Germany) according to the manufacturer's instructions.

III.2.4. Sequencing of the gorilla bocaparvoviruses

To determine the nearly complete genome sequence of the detected bocaparvoviruses, additional primers were designed (Table 2.2) and PCR and sequencing were performed using a primer walking approach (by direct-sequencing). Each PCR fragment was read at least twice with both forward and reverse primers. GENETYX version 12.0 (Genetyx Co., Tokyo, Japan) was used to edit and assemble the nearly complete genome sequence. Annotations were performed using Geneious R9.1 (Kearse et al., 2012) with the built-in ORF finder of NCBI.

III.2.5. Phylogenetic analysis

The phylogenetic relationships among primate bocaparvoviruses were inferred using the sequence data obtained in this study and reference sequences listed in Table 2.3. The sequences were aligned using MUSCLE in MEGA version 7.0 (Kumar et al., 2016)

with default parameters for gap opening and gap extension. Phylogenetic trees were constructed in MEGA 7.0 (Kumar et al., 2016) using the maximum likelihood method with 1000 bootstrap replicates based on the models, which were chosen using the Find DNA/Protein models function in MEGA 7.0. The chosen models are listed in Table 2.4.

III.2.6. Recombination analysis

To identify potential recombination sites and possible nucleotide sequence topologies within the primate bocaparvoviruses, a nucleotide alignment of 7 strains representing the 7 primate bocaparvovirus genotypes reported to date (HBOV1–4, GBOV1, CPZh2, and GBOV2 of this study) was obtained using MAFFT version 7 (Katoh and Standley, 2013). A DualBrothers analysis was conducted using a built-in algorithm in Geneious R9.1 (Kearse et al., 2012) with default settings. The DualBrothers analysis is a recombination detection algorithm based on a phylogenetic dual multiple change-point model (Minin et al., 2005); it allows the detection of spatial variation in the phylogenetic tree topology and spatial variation in nucleotide substitution events. Phylogenetic reconstructions of the regions showing different topologies I performed using MEGA7.0 (Kumar et al., 2016), as described above (Fig. 2.5 A-K).

III.3. Results

III.3.1. Detection of bocaparvoviruses in gorillas

A PCR using pan-bocaparvovirus primers targeting a 244-bp fragment of the conserved NS1 gene, was performed on healthy gorillas of MDNP. Among 107 fecal samples, I detected bands of the expected size from 2 samples. I determined the nucleotide

sequences of the DNA fragments, confirming they were partial bocapavovirus DNAs (data not shown). Interestingly, the nucleotide sequences were not identical to the counterparts of the known gorilla bocaparovirus GBOV1 (accession number: HM145750.1), and thus I tentatively named these viruses gorilla bocaparvovirus 2 (GBOV2). GBOV2 showed 86-85.0% nucleotide and 93-87.1% amino acid identity with HBOV2 strains.

To identify which gorilla individual(s) shed the viruses, I determined the microsatellite genotypes of 107 samples. The microsatellite analysis showed that the 107 samples were derived from 26 individuals (Table 2.1). The two positive samples belonged to two different infants (approximately 2 years old). Therefore, the two strains were designated GBOV2/GAB1 and GBOV2/GAB2.

III.3.2. Sequence analysis of the gorilla bocaparvoviruses

I subsequently determined the nearly complete genome sequences of GBOV2/GAB1 (4872 nucleotides) and GBOV2/GAB2 (4880 nucleotides) (Fig. 2.1B). Pairwise alignment of the two nucleotide sequences showed that they differ at only one nucleotide (position 135). The GC contents of the viruses were 40.9%. The genome organization was similar to those of other known primate bocaparvoviruses (Fig. 2.2A). The nearly complete genome contained three major ORFs. The left ORF encoded the putative NS1, consisting of 640 amino acids. Note that the splicing site in the NS gene was conserved in GBOV2 (Fig. 2.2B), suggesting that they also encode a spliced protein, i.e., NS2. The right ORF encoded the putative capsid protein VP1 (667 amino acids) and truncated capsid protein VP2 (538 amino acids). The middle ORF encoded the putative NP1 (216 amino acids). The putative viral proteins of GBOV2 shared similar features to

those of closely related HBOVs, including the ATP-binding Walker-Loop (at amino acid positions 423–430) in the predicted NS1 protein (Fig.2.2C) and the phospholipase A2 in the unique region of VP1 (VP1u) (Fig.2.2D). The GBOV2 genome map is shown in Fig. 2.2A-D. The nucleotide sequences were deposited in GenBank (accession numbers GBOV2/GAB1: KY581736; GBOV2/GAB2: KY581737).

III.3.3. Phylogenetic analysis

To gain insight into the genetic relationships among primate bocaparvoviruses, I performed phylogenetic analyses using the nearly complete genome sequences (Fig. 2.3), NS gene (Fig. 2.7), NP gene (Fig. 2.8), and VP gene (Fig.2.9). Phylogenetic analysis based on the nearly complete genome, NS, VP, and NP genes (Fig. 2.3 and 2.7- 2.9) consistently indicated that the two GBOV2 strains form a well-supported cluster within the Primate bocaparvovirus 2 group.

Because only partial sequences were available for some non-human primate bocaviruses, I performed phylogenetic analyses using 486 nucleotides of partial NS genes (Fig.2. 4A) and partial VP genes (Fig. 2.4B). Phylogenetic analysis based on the partial VP genes showed that GBOV2 strains also form a well-supported cluster within Primate bocaparvovirus 2. Interestingly, GBOV2 strains clustered with a chimpanzee strain found in Cameroon (HQ113150.1, Bocavirus chimpanzee/PT-LM1861/CMR isolate PT-LM1861) in the partial VP tree, but not in the partial NS tree.

III.3.4. Recombination analysis

I next performed a recombination analysis and identified possible breakpoints at several positions (Fig. 2.5A). Thus, to assess the topologies of each of the regions, I reconstructed phylogenetic trees using each of the nucleotide alignments (Fig. 2.5B-K).

Within Primate bocaparvovirus 2, GBOV2 clustered with HBOV2 in the trees based on the alignments of nucleotide positions 1500–2336 and 2337–2640 (Figures 5 E-F), but not in the remaining trees (Fig. 2.5B–2.5D; 2.5G–2.5K), suggesting that there may have been an intra-species recombination event(s) during the evolution of those viruses. In addition, interestingly, I found that the chimpanzee strain CPZh2 was located in a cluster formed by the members of Primate bocaparvovirus 2 and HBOV3 (Figure 2.5H).

III.4. Discussion

In this study, I detected two bocaparvoviruses from the feces of two habituated infant gorillas in MDNP, Gabon. I further determined the nearly complete genome sequences of the two strains, named GBOV2/GAB1 and GBOV2/GAB2. The strains were nearly identical and clustered with members of Primate bocaparvovirus 2. Based on the criteria of the International Committee of Taxonomy for Viruses (ICTV) (Cotmore et al., 2014), GBOV2 strains were proposed to be classified as belonging to Primate bocaparvovirus 2 because the deduced amino acid sequence of GBOV2 NS1 showed 93.0–91.0% identities with HBOV2 NS1 sequences (Table 2.5). To my knowledge, all gorilla bocaparvoviruses reported to date belong to the species Primate bocaparvovirus 1. Therefore, this is the first report of a non-human primate bocaparvovirus in Primate bocaparvovirus 2.

At least two bocaparovirus species are circulating in wild gorillas. Only two previous studies have reported the presence of bocaparvoviruses in diseased captive and healthy free ranging gorillas (Kapoor et al., 2010a; Sharp et al., 2010), including 1 GBOV1 strain and 2 gorilla bocaparvovirus-like sequences that are genetically similar to GBOV1. Therefore, I cannot further evaluate the differences among gorilla viruses, including the differences in geographical distribution and epidemiology of bocaparvoviruses in non-human primates. Further molecular epidemiological studies are needed to understand these issues.

Based on microsatellite genotyping, the two gorillas in which GBOV2 was detected were both infants. Fewer samples were collected from infants than from adults or juveniles, suggesting that the higher incidence in infants was not due to sampling bias. Although the sample size was small to conclude a high incidence in infants, this infection pattern was consistent with that of human bocaparvoviruses, which show a relatively high incidence in children between 12 and 24 months (Ahn et al., 2014). HBOV2 and HBOV4 have been detected in children of less than three years old suffering from acute gastroenteritis (Arthur et al., 2009) and bronchopneumonia (Song et al., 2010). Adult humans seem to be less susceptible to bocaparvovirus infection, probably owing to immunity acquired during early childhood (Ahn et al., 2014).

Importantly, the two infant gorillas in this study were apparently healthy. In various animals and humans, bocaparvovirus infection are mainly associated to respiratory and gastro-intestinal illnesses (Arthur et al., 2009; Lu et al., 2015). In non-human primates, however, its clinical significance or symptomatology remains unclear. CPZh2 was also detected from an apparently healthy chimpanzee (Brožová et al., 2016). Although GBoV1 was identified from gorillas suffered from diarrhea, its etiological relationship is unclear

(Kapoor 2010a). Further epidemiological studies are required to assess the clinical significance of bocaparvoviruses in non-human primates.

Previous studies have suggested that multiple recombination events may have driven the evolution of primate bocaparvoviruses (Babkin et al., 2013; Brožová et al., 2016; Chieochansin et al., 2010; Fu et al., 2011; Kapoor et al., 2009, 2010b; Khamrin et al., 2013; Tyumentsev et al., 2014; Zehender et al., 2010). Here, i newly identified intra- and inter-species recombination events among primate bocaparvoviruses (Fig.2. 5). Within Primate bocaparvovirus 2, i observed differences between the topologies of some trees; the closest relative of GBOV2 was HBoV2 in some trees (Fig.2.5E and F), but not all, trees (Fig.2. 5 B-D, G, I). Thus, members of Primate bocaparvovirus 2 may have experienced an intra-species recombination event during their evolution as reported elsewhere (Kapoor et al., 2010b). Interestingly, i also found evidence for inter-species recombination of bocaparvoviruses. In the tree based on nucleotide positions 3069–3374 (Fig.2.5H), the chimpanzee strain CPZh2, a member of Primate bocaparvovirus 1, is clustered with members of Primate bocaparvovirus 2 and HBoV3, which was previously reported to be possibly a recombinant of HBoV1 and HBOV2 lineages (Arthur et al., 2009; Chieochansin al., 2010; Wang et al. 2011). This observation suggests an inter-species recombination event between members of Primate bocaparvovirus 1 and 2. Thus, the short segment of the CPZh2 strain may be derived from a Primate bocaparvovirus 2 virus.

In the phylogenetic analysis, i also found that a chimpanzee bocaparovirus strain, PT-LM1861/CMR isolate PT-LM1861, was located in different clusters in the partial NS tree and partial VP tree. These results suggest that the virus may be a recombinant virus, like HBoV3 (Sharp et al., 2010). Alternatively, the chimpanzee may have been co-

infected with two bocaparvovirus strains. Further sequence analyses are needed to determine the precise evolutionary history of these viruses.

III. 5. Conclusions

The discovery of the GBOV2 in wild infant gorillas suggests a high diversity of bocaparvoviruses in primates; there may be many undiscovered non-human primate bocaparvoviruses belonging to Primate bocaparvovirus 2. These findings emphasize the importance of assessing bocaparvovirus infections in wild non-human primates for a better understanding of the evolution and the safe management of the ape population.

Table 2.1. Bocavirus infection prevalence in gorillas in MDNP

Individual ID	Number of samples tested	Number of individuals tested	Number of positive samples	Number of positive individuals
Silverback	7	1	0	0
Adult female	20	4	0	0
Sub-adults	8	2	0	0
Juveniles	43	8	0	0
Infants	4	3	2	2
Others	25	8	0	0
Total	107	26	2	2

Table 2.2. Primers used for sequencing the gorilla bocaparvovirus 2

Primer name	Primer sequence	Targeted nucleotide position*
P1-Fouter	5'-GGCGAGTGAACATCTCTGGG-3'	206–225
P2-Router	5'-AAGGTCACCTCGCTTGTCTC-3'	827–846
P3-Finner	5'-TGGTGAGTGACACTATGGCC-3'	242–261
P4-Rinner	5'-AGCCATGTAGCATGCTTTGC-3'	491–510
P5-Router	5'-CTCCAAATGTTTGTGGTAGTTGC-3'	1878–1900
P6-Finner	5'-CTGCTCCTGTAATTAGAGCTTTTTC-3'	266–290
P7-Rinner	5'-GTACACAGCAGATGGCATGC-3'	1461–1480
P8-Router	5’-GACGAKTGGCGGMTSTC-3'	2573–2589
P-9-Finner	5'-GGCGCAAGGTTGATAGAAC-3'	1294–1312
P10-Fouter	5’-GGATTTGTGTTTAACGATTGCAGAC-3'	1609–1633
P11-Router	5'-ATGACCATGGTGTGCTGACA-3'	3695–3714
P12-Finner	5’-GGACTCTTTGTCCTACACATTCAC-3'	2021–2044
P13-Rinner	5’-GAAGTGTTTGGTTGAGGTTCA-3'	3401–3421
P14-Fouter	5'-AGACGATTGGTCTCTTGGTG-3'	3251–3270
P15-Router	5'-TCTGGTTATTGGATATCTGTCCC-3'	4639–4661
P16-Finner	5'-TTGGTGGCATTATTGGCTC-3'	3265–3283
P17-Fouter	5'-AAACCAACAAGCTGGATGAC-3'	4293–4312
P18-Router	5’-GTAMCGGCTAGGTTCGAGAC-3'	5047-5066
P19-Rinner	5'-CTAGGTTCGAGACGGCAAC-3'	5165-5183

*Reference position from GenBank database accession number GU048662.

Table 2.3. Bocavirus sequences used for the phylogenetic inference

Accession number	Name	Abbreviation	Host	Wild	Captive**
KY581736	Gorilla bocaparvovirus 2*	GBOV2	Gorilla	+	
KY581737	Gorilla bocaparvovirus 2*	GBOV2	Gorilla	+	
HM145750.1	Bocavirus gorilla	GBOV1	Gorilla		+
KT223502.1	Primate bocaparvovirus 1 CPZh2	CPZh2	Chimpanzee		+
HQ113149.1	Bocavirus_gorilla/GG-CP1426/CMR	GBOV_GG_CP1426_CMR	Gorilla	+	
HQ113150.1	Bocavirus_chimpanzee_PT-LM1861/CMR_isolate_PT-LM1861	CPZ_PT_LM1861_CMR_PT-LM1861	Chimpanzee	+	
HQ113151.1	Bocavirus_chimpanzee_PT-LM1861/CMR_isolate_PT-BQ2392	CPZ_PT_LM1861_CMR_PT-BG1861	Chimpanzee	+	
GU048662.1	Human_bocavirus_2_strain_CU47TH	HBOV2_CU47TH	Human		
FJ973560.1	Human_bocavirus_2b_NI	HBOV2b_NI213	Human		
JQ964116.1	Human_bocavirus_isolate_Rus-Nsc10-N386	HBOV_Rus-Nsc10-N386	Human		
FJ948860.1	Human_bocavirus_2_strain_W298	HBOV2_W298	Human		
EU082213.1	Human_bocavirus_2_strain_W153	HBOV2_W153	Human		
EU082214.1	Human_bocavirus_2_strain_W208	HBOV2_W208	Human		
KM624025.1	Human_bocavirus_strain_LZFB080	HBOV_LZFB080	Human		
GU301644.1	Human_bocavirus_2_isolate_LZ53819	HBOV2_LZ53819	Human		
FJ973558.1	Human_bocavirus_2a_TU_strain_HBoV2A	HBOV2a_TU	Human		
JQ964115.1	Human_bocavirus_isolate_Rus-Nsc10-N751	HBOV_Rus-Nsc10-N751	Human		
GU048664.1	Human_bocavirus_2_strain_CU1557UK	HBOV2_CU1557UK	Human		
FJ170280.1	Human_bocavirus_2_isolate_UK-648	HBOV2_UK_648	Human		
GU048663.1	Human_bocavirus_2_strain_CU54TH	HBOV2_CU54TH	Human		
FJ375129.1	Human_bocavirus_isolate_SH3	HBOV_SH3	Human		
FJ170279.1	Human_bocavirus_2_isolate_PK-2255	HBOV2_PK2255	Human		

JX257046.1	Human_bocavirus_2_strain_BJQ435	HBOV2_BJQ435	Human
GU301645.1	Human_bocavirus_2_isolate_LZ55602	HBOV2_LZ55602	Human
KM624027.1	Human_bocavirus_strain_LZFB086	HBOV_LZFB086	Human
KC461233.1	Human_bocavirus_strain_CMH-S011-11	HBOV_CMH-S011-11	Human
GQ200737.1	Human bocavirus 2 isolate KU1	HBOV2_KU1	Human
FJ170278.1	Human bocavirus 2c PK isolate PK-5510	HBOV2c_PK5510	Human
FJ973561.2	Human bocavirus 4 NI strain NI-385	HBOV4_NI385	Human
KJ649741.1	Human bocavirus isolate RUS_NSC_11-N2655	HBOV_RUS_NSC_11_N2655	Human
KJ649742.1	Human bocavirus isolate RUS_NSC_11-N2657	HBOV_RUS_NSC_11_N2657	Human
FJ973562.1	Human bocavirus 3 strain 3B-TU-A-210-0		Human
EU918736.1	Human bocavirus 3 strain W471	HBOV3_W471	Human
KM624026.1	Human bocavirus strain LZFB199	HBOV_LZFB199	Human
GU048665.1	Human bocavirus 3 strain CU2139UK	HBOV3_CU2139UK	Human
FJ948861.1	Human bocavirus 3 strain W855	HBOV3_W855	Human
HM132056.1	Human bocavirus 3 isolate 46-BJ07	HBOV3_46-BJ07	Human
GQ867667.1	Human bocavirus 3 strain IM10	HBOV3_IM10	Human
GQ867666.1	Human bocavirus 3 strain MC8	HBOV3_MC8	Human
JN086998.1	Human bocavirus strain HBOV3	HBOV3	Human
EU984242.1	Human bocavirus isolate TW141_07	HBOVT_W141_07	Human
JN794566.1	Human bocavirus isolate GZ9081	HBOV_GZ9081	Human
EU984245.1	Human bocavirus isolate TW925_07	HBOV_TW925_07	Human
JX887481.1	Human bocavirus strain ZJ68	HBOV_ZJ68	Human
KJ684073.1	Human bocavirus isolate 2012GZ2332	HBOV_2012GZ2332	Human
JF327788.1	Human bocavirus strain TUN4134	HBOV_TUN4134	Human
EF450740.1	Human bocavirus isolate HK24	HBOV_HK24	Human
AB481074.1	Human bocavirus strain: JPOC06-089	HBOV_JPOC06-089	Human

AB481073.1	Human bocavirus strain: JPOC06-077	HBOV_JPOC06-077	Human
JX434060.1	Human bocavirus isolate CQ90	HBOV_CQ90	Human
DQ988934.2	Human bocavirus strain BJ3722	HBOV_BJ3722	Human
EU984232.1	Human bocavirus isolate TW2715_06	HBOV_TW2715_06	Human

*These viruses are named in this study. **Captive: hosted in a zoo.

Table 2.4 List of models used for the Maximum Likelihood test

Region	Model	Figure
2-4872	HKY+G	Figure 4
16-1938	HKY+G	Suppl. Figure 2
2802-4797	GTR+G	Suppl. Figure 3
2168-2815	GTR+G	Suppl. Figure 4
890-1344	T92+G	Figure 5A
4077-4406	TN93+G	Figure 5B
1-133	T92+G	Figure 6B
134-864	T92+G	Figure 6C
865-1499	T92+I	Figure 6D
1500-2336	GTR+G	Figure 6E
2337-2640	T92+I	Figure 6F
2641-3068	T92+I	Figure 6G
3069-3374	TN93+I	Figure 6H
3375-3556	HKY+I	Figure 6I
3557-4034	TN93+I	Figure 6J
4259-4443	TN93+G	Figure 5K

Table 2.5. Pairwise amino acid percentage identities of NS1 of GBOV2 and other primate bocaparvovirus reference strains.

Accession number	Strain	GBOV2	GBOV1	CPzh2	HBOV1	HBOV2	HBOV3
KY581737	GBOV2						
HM145750.1	GBOV1	**76.1**					
KT223502.1	CPzh2	**75.6**	**91.5**				
JQ923422.1	HBOV1	**77.2**	**92**	**90.5**			
GU048662.1	HBOV2	**92.8**	**76.4**	**75.7**	**77.9**		
EU918736.1	HBOV3	**76.7**	**91.1**	**93**	**91.4**	**77.2**	
FJ973561.2	HBOV4	**87.8**	**75**	**74.6**	**77.5**	**91.1**	**75.4**

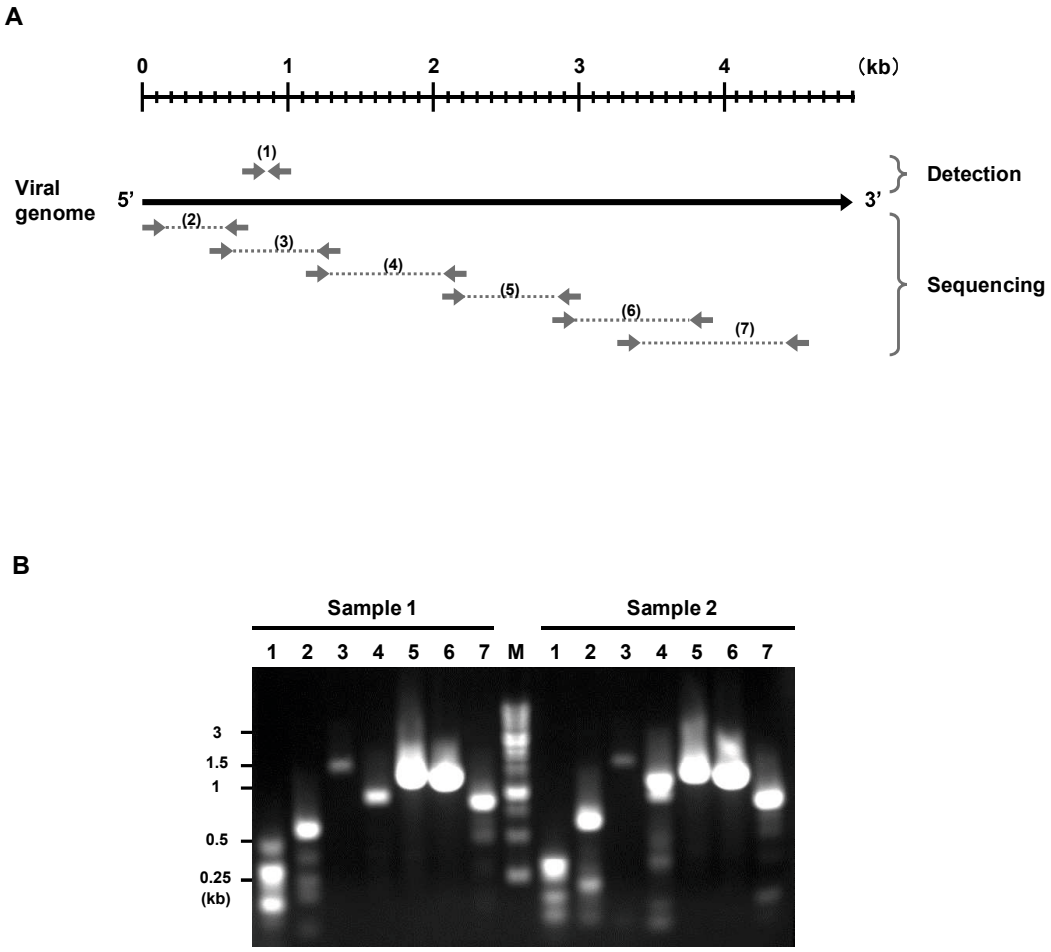

Figure 2.1. **(A)** Schematic diagram of the PCR strategy **(B)** Gel electrophoresis results showing the amplicon sizes of both positive samples.

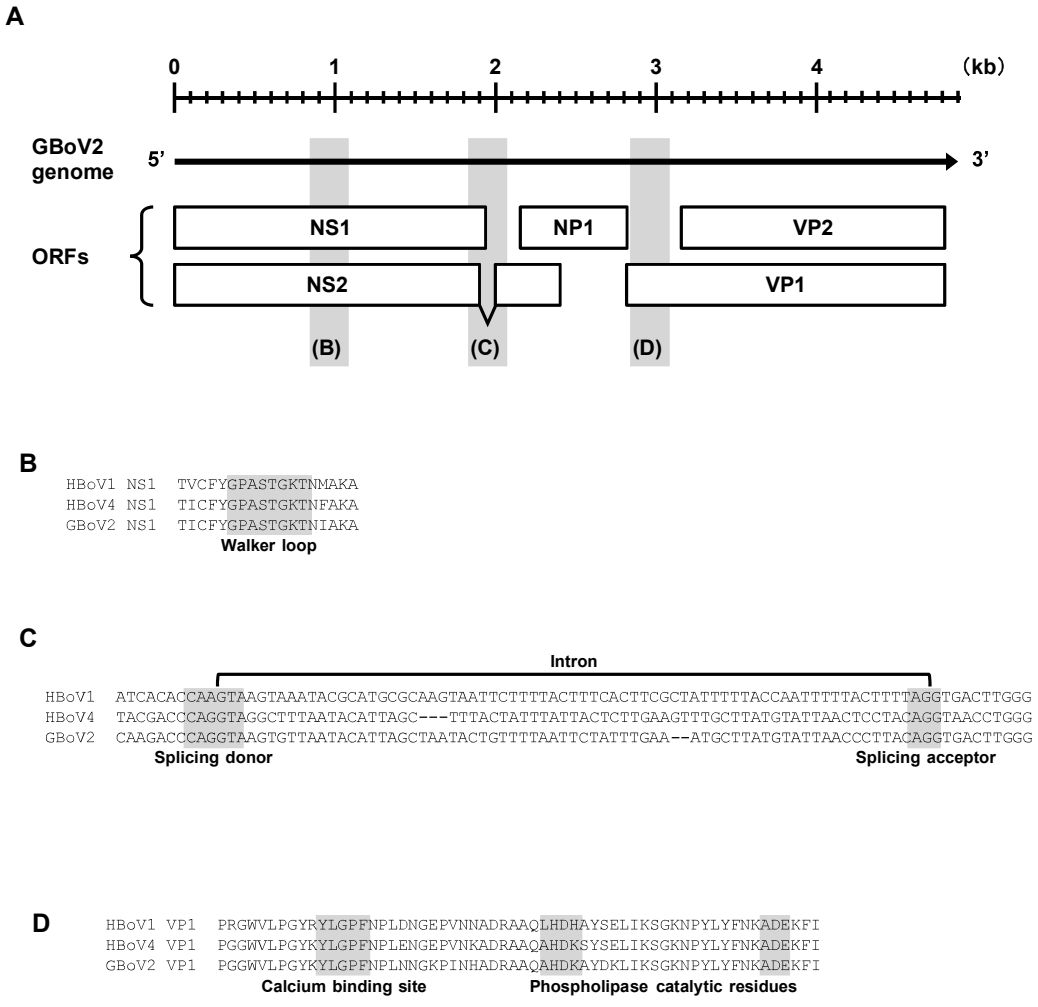

Figure 2.2. Genome organization of the gorilla bocaparvovirus 2.

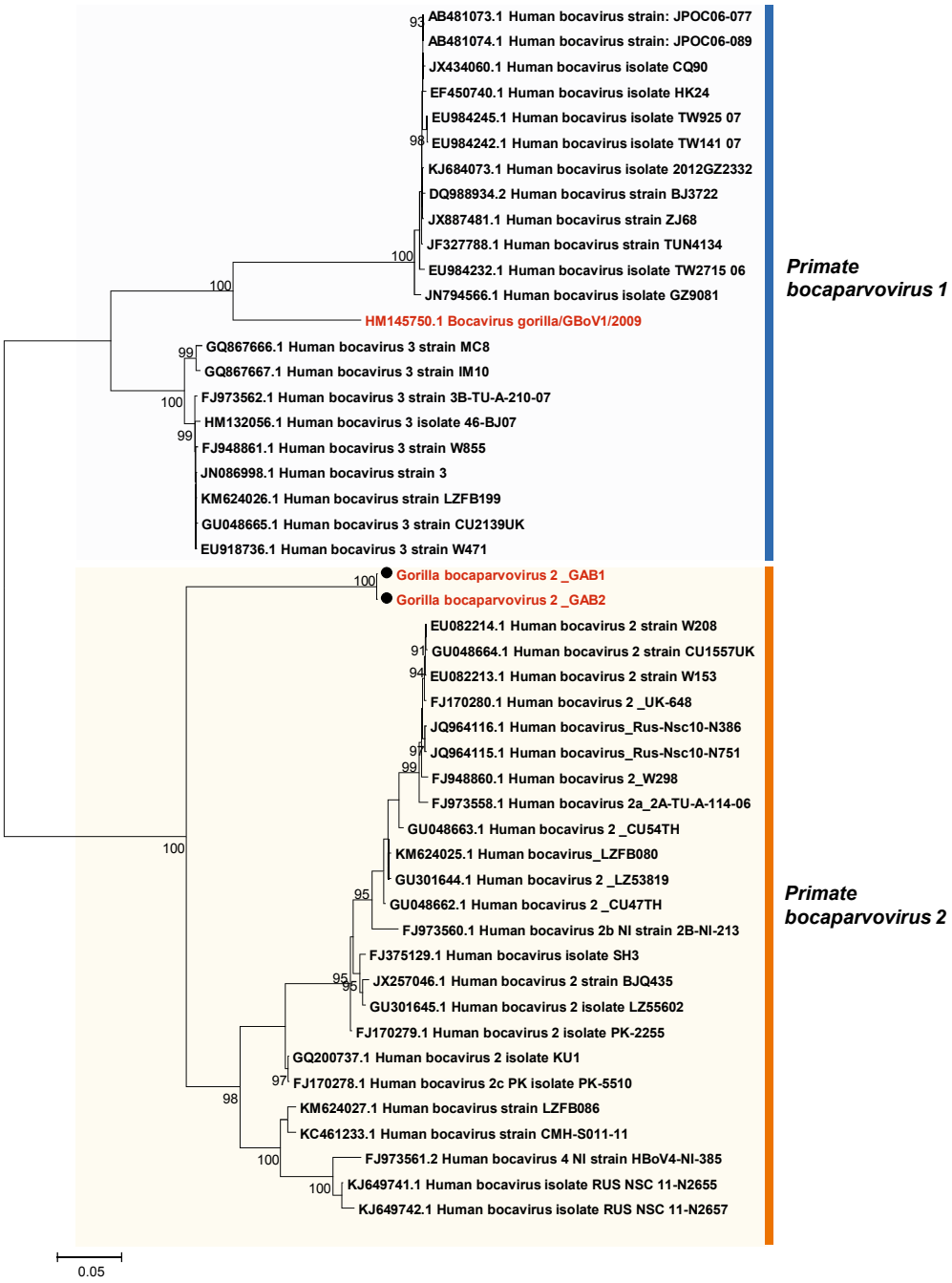

Figure 2.3. Phylogenetic tree based on the complete coding sequences of primate bocaparvoviruses

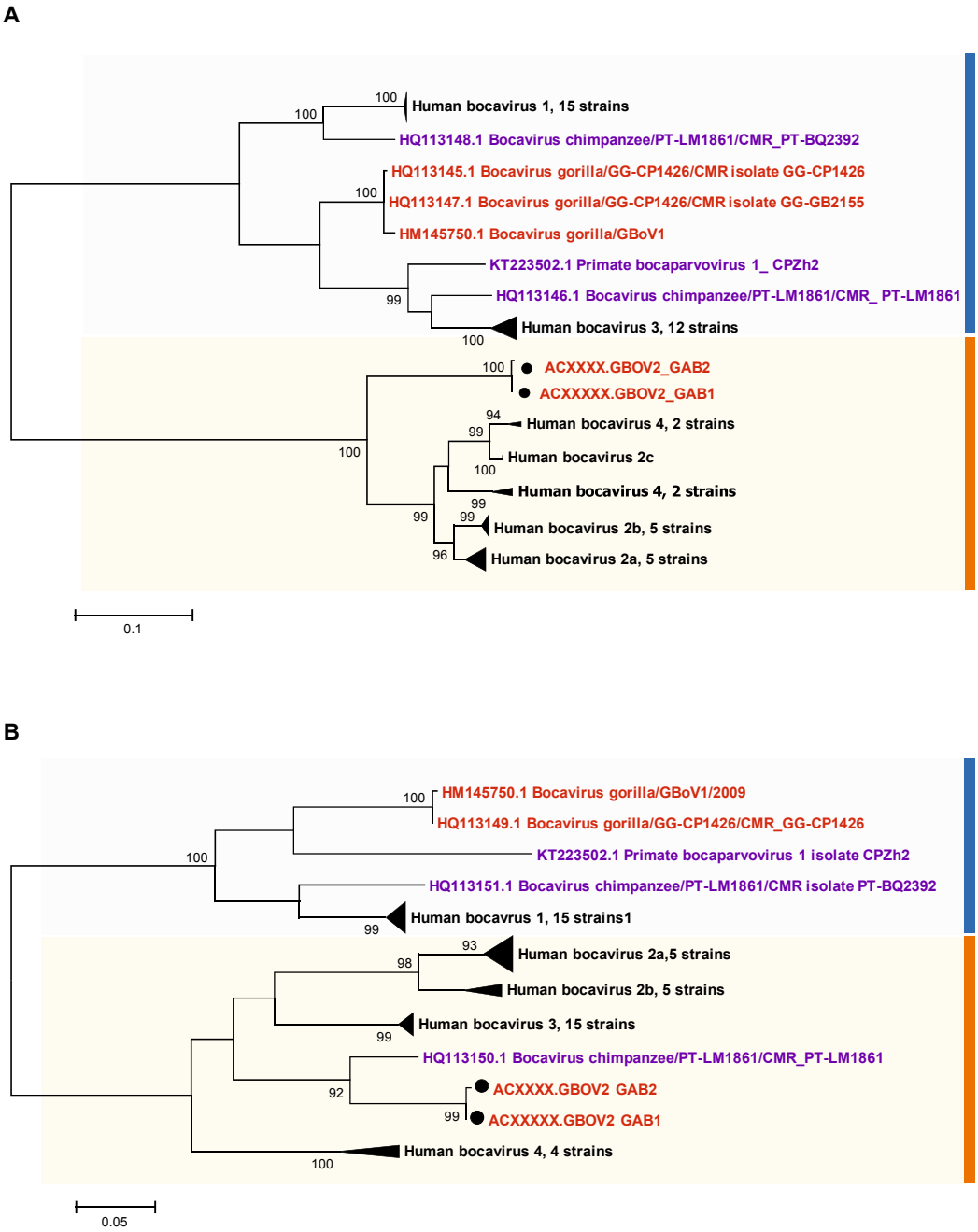

Figure 2. 4. Phylogenetic tree based on the partial NS and VP2 genes of primate bocaparvoviruses.

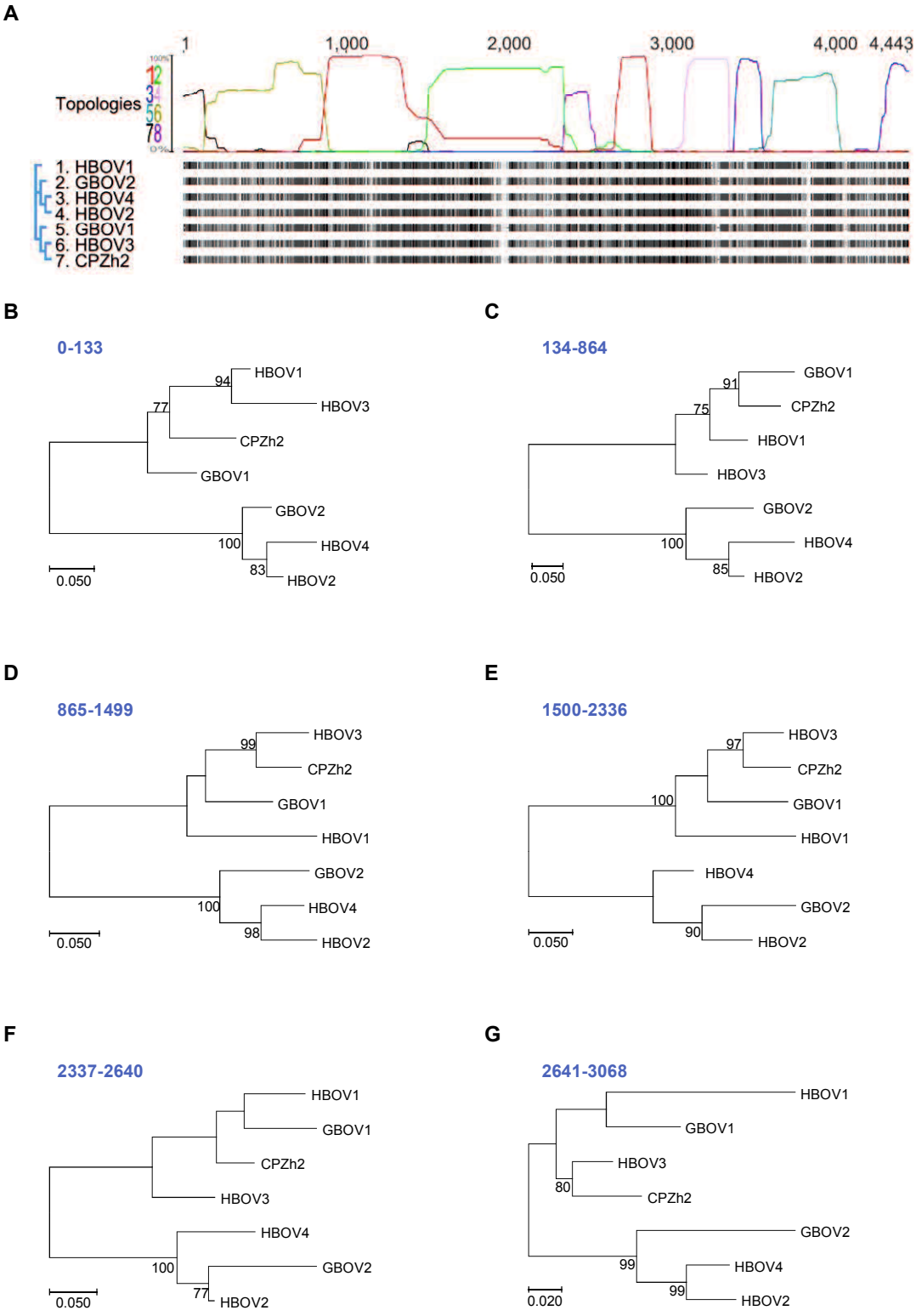

Figure 2.5. Recombination analysis.

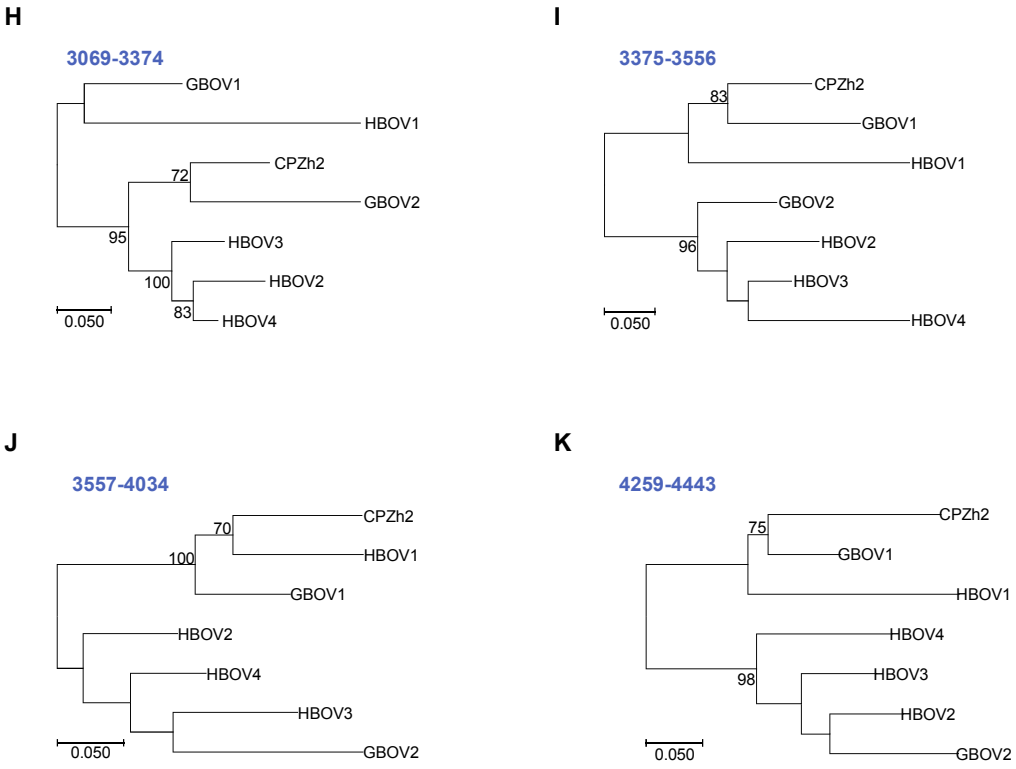

Figure 2.5. Recombination analysis (continued)

Chapter IV. General discussion

The 21st century has been marked by the emergence in the human population of various diseases originating from animals (wildlife, domestic animals, etc.) (Taylor et al., 2001) and causing significant morbidity and mortality (Muyembe-Tamfum et al., 2012; Suzuki and Nei, 2000). In the year 2000's the World Health Organization (WHO) has brought up a new concept known as "One health" which supports a multidisciplinary collaboration of health practitioners for the benefit of human, animal and environment health. Molecular epidemiological studies of viruses infecting wild apes provide data in the "One Health approach" (Gibbs, 2014).

The survey of adenoviruses infecting wild western lowland gorillas has revealed the presence of 3 species namely Human adenovirus B (HAdV-B), HAdV-C and HAdV-E. The species HAdV-C was found in the local people living the vicinity of the park, but both gorilla and HAdVs of this study were genetically distinct. In addition, the description of HAdV-E closely related to chimpanzee strains highlights the possible ape-to-ape cross transmission of mastadenoviruses in the wild.

The survey of bocaparvoviruses revealed the presence of novel primate bocaparvovirus members of the species *Primate bocaparvovirus 2.* To date, all the non-human primate bocaparvoviruses including gorillas and chimpanzee strains belong to the species *Primate bocaparvovirus 1*. The description of a new genotype of primate bocaparvovirus 2 in gorillas highlights the possible existence of more non-human primate bocaparvoviruses strains members of that species in the wild.

IV.1. Genetic diversity and evolution of viruses

The study of the genetic diversity and evolution of viruses in natural host populations provides a better understanding of the origin of viral diseases and their distribution among several hosts.

Viruses infecting wild apes display a high genetic diversity such as mastadenoviruses (Hoppe, 2015; Wevers et al., 2011) but generally each primate is infected with a species-specific virus. Therefore, the close phylogenetic relationship between apes and humans (>98% homology) (Scally et al., 2012), coupled with the exponential expansion of human populations and human activities within apes' habitats (mining, agriculture, eco-tourism, etc.), has led to an exceptionally high potential for

pathogen exchange (Calvignac-Spencer et al., 2012; Gillespie et al., 2008; Woodford et al., 2002). Ape-to-human (and *vice versa*) as well as ape-to-ape cross-species transmissions have been documented (Calvignac-Spencer et al., 2012; Hoppe et al., 2015; Mouinga-Ondeme et al., 2012).

More than 10 virus families have been reported so far in wild African apes (Calvignac-Spencer et al., 2012). The viruses infecting gorillas belong to 11 families including the *Adenoviridae* and the *Parvoviridae* (Table 4.1). The knowledge of the diversity and evolution of infectious agents in wild apes has contributed answering the question of origin of some of the human pathogens (Liu et al., 2010; Sharp and Hahn, 2010; Switzer et al., 2006) such as the HIV and HTLV (Gao et al., 1999; Switzer et al., 2006).

Although most microorganisms identified in wild apes are enzootic without being associated with acute disease, several host switches may change the microbe's properties which can become more hazardous for the new host. However, it is difficult to study about the pathogenicity in the wild animals.

Evolution of viral pathogens may lead to altered virulence, enhanced transmission, altered tissue tropisms, and striking new disease manifestations (Robinson et al., 2013; Tyumentsev et al., 2014). Recombination and mutation are the central mechanisms driving such evolution (Farkov et al., 2017). For RNA viruses, for example, the concerted activities of mutation and recombination are key to virus spread and virulence in infected animals (Farkov et al., 2017). For single stranded DNA viruses such as bocaparvoviruses which exhibit a high mutation rate close to that of RNA viruses, recombination has been shown to contribute to their evolution (Nze-Nkogue et al., 2017). Also, double stranded DNA viruses with lower mutation rate, such as adenoviruses, are also prone to recombine (Robinson et al., 2013; Walsh et al., 2009).

Several factors may affect the rate of recombination events, including the genome structure, presence of sites sensitive to nucleases, and the mechanisms of virus replication (Tyumentsev et al., 2014). In particular, replication of viruses with single-stranded DNA genomes suggests the generation of concatemeric intermediates, and the breakpoints of concatemers may be recombination hotspots (Tyumentsev et al., 2014).

Homologous recombination is a critical mechanism for the maintenance of genome fitness and diversity (Eichler, 2009). Similarly, inter and intra-species recombination contribute to the virus diversity, and sometimes leading to the emergence of new variants or new viruses. The current study highlights the occurrence of recombination events in natural settings involving wild apes. The intra-species recombination among the members of primate bocaparvovirus 2 and the inter-species between both primate bocaparvovirus species (Nze-Nkogue et al., 2017) contribute to understand the mixed hosts origin of these viruses.

IV.2. Conclusions and recommendations

Epidemiological studies on microorganisms infecting wildlife have contributed to the understanding of the genetic diversity and the evolutionary history of the target agents. The knowledge of viral evolution is critical to predict and prevent future disease outbreaks.

The description of several adenoviruses and the identification of novel bocaparvoviruses in wild western gorillas contribute to a better understanding of the genetic diversity of these viruses as well as to clarify their evolutionary processes. Considering the genetic relatedness between gorillas and human, I would advise the

surveillance adenoviruses and bocaparvoviruses in gorillas as well as in humans (tourists, guides, local peoples, etc.) which potentially contact with these animals when eco-tourism is promoted. In addition, the continuation of the epidemiological study at a broader scale, i.e. the assessment of more viral families and in other ape populations in Gabon, is recommended.

The recent use of advanced sequencing technologies such as high throughput approaches for pathogen assessment in various animals including wildlife (bats: (Li et al., 2010) and domestic animals (pigs: (Amimo et al., 2016; Shan et al., 2011) has significantly increased the knowledge of microbial diversity in target animals which opens an important tool box for comprehending the natural history of various pathogens. The use of third generation sequencing such as MinION (Oxford Nanopore Technologies) which allows the screening of a broad range of microorganisms (RNA viruses for example) in short time could be considered for the study of gorilla's virome.

Table 4.1. Diversity of viruses described in gorillas

Viral family/genus	Closed human counterpart	Reference
Adenoviridae/Mastadenovirus	**HAdV-A-F**	(Hoppe et al., 2015; Nkogue et al., 2016; Seimon et al., 2015)
Filoviridae/Ebolavirus	**EBOV**	(Leroy et al., 2011)
Hepadnaviridae	**HBV**	(Njouom et al., 2010)
Herpesviridae/Lymphocryptovirus	**EBV**	(Ehlers et al., 2010)
Cytomegalovirus	**HCMV**	(Leendertz et al., 2009)
Paramyxoviridae/Metapneumovirus	**HMPV**	(Palacios et al., 2011)
Parvoviridae/Bocaparvovirus	**HBV**	(Kapoor et al., 2010a; Nze-Nkogue et al., 2017)
Picornaviridae/Enterovirus	**EV70/EV76**	(Harvala et al., 2011)
Polyomaviridae/Polyomavirus	**MCPV**	(Leendertz et al., 2011)
Retroviridae/Lentivirus	**HIV-I**	(Takehisa et al., 2009)
Deltaretrovirus	**HTLV**	(Dooren et al., 2007)
Spumavirus		(Betsem et al., 2011)

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