DNA Polymerase Sequences of New World Monkey Cytomegaloviruses: Another Molecular Marker with Which To Infer Platyrrhini Systematics

Samantha James, Damien Donato, Jean-Francois Pouliquen, Manuel Ruiz-García, Anne Lavergne, Vincent Lacoste

HAL Id: pasteur-02197592
https://riip.hal.science/pasteur-02197592

Submitted on 3 Apr 2020

1   *DNA polymerase* **sequences of New World monkey cytomegaloviruses: another molecular**

2   **marker to infer Platyrrhini systematics**

3

4   *Samantha James,[a,b] Damien Donato,[a] Jean-François Pouliquen,[a] Manuel Ruiz-García,[c] Anne*

5   *Lavergne,[a,b] Vincent Lacoste[a,b,d,#]*

6

7   [a]Laboratoire des Interactions Virus-Hôtes, Institut Pasteur de la Guyane, Cayenne, French Guiana

8   [b]Université de la Guyane, Ecole doctorale 587 « Diversités, Santé et Développement en

9   Amazonie », Cayenne, French Guiana

10   [c]Laboratorio de Genética de Poblaciones - Biología Evolutiva, Unidad de Genética,

11   Departamento de Biología, Facultad de Ciencias, Pontificia Universidad Javeriana, Bogotá, DC,

12   Colombia

13   [d]Département de Virologie, Institut Pasteur, Paris, France

14

15   **Running head:** New World monkey cytomegaloviruses

16

17   # Address correspondence to Vincent Lacoste, vincent.lacoste@pasteur.fr

18

19   *Keywords: Cytomegalovirus*, CMV, New World monkeys, evolution, phylogeny.

20

21   **Word counts:** Abstract **245 words**   Importance **123 words**   Main text **4036 words**

22

23   Figures: **3**   Tables: **4**

ABSTRACT

Over the past few decades, a large number of studies have identified herpesvirus sequences from many mammalian species around the world. Among the different non-human primate species tested so far for cytomegaloviruses, only a few were from the New World. Seeking to identify cytomegalovirus homologues in New World monkeys (NWMs), we carried out molecular screening of 244 blood DNA samples from 20 NWM species from Central and South America. Our aim was to reach a better understanding of their evolutionary processes within the Platyrrhini parvorder. Using polymerase chain reaction amplification with degenerate consensus primers targeting highly conserved amino acid motifs of the herpesvirus *DNA polymerase* gene, we characterized novel viral sequences from 12 species belonging to seven genera representative of the three NWM families. BLAST searches, pairwise nucleotide and amino acid sequence comparisons, as well as phylogenetic analyses confirmed that they all belonged to the *Cytomegalovirus* genus. Previously determined host taxa allowed us to demonstrate a good correlation between the distinct monophyletic clades of viruses with those of the infected primates at the genus level. In addition, the evolutionary branching points that separate NWM CMVs were congruent with the divergence dates of their hosts at the genus level. These results significantly expand our knowledge of the host range of this viral genus and strongly support the co-speciation between these viruses and their hosts. In this respect, we propose that NWM CMV *DNA polymerase* sequences could serve as a reliable molecular marker to infer Platyrrhini phylogenetics.

IMPORTANCE

Investigating evolutionary processes between viruses and non-human primates has led to the discovery of a high number of herpesviruses. No study published so far on primate cytomegaloviruses has extensively studied New World monkeys (NWMs) at the subspecies, species, genus and family levels. The present study sought to identify cytomegalovirus homologues in NWMs and decipher their evolutionary relationships. This led us to characterize novel viruses in 12 of the 20 primate species tested representative of the three NWM families. The identification of distinct viruses in these primates not only significantly expands our knowledge of the host range of this viral genus, but has also shed light on their evolutionary history. Phylogenetic analyses and molecular dating of the sequences obtained support a virus–host coevolution.

INTRODUCTION

New World monkeys of tropical forests from Central to South America belong to the Platyrrhini parvorder (1). They first appeared in the Neotropics in the late Eocene or early Oligocene and have subsequently evolved into a broad diversity of families, subfamilies and genera (**Figure 1**) (2). To shed light on their phylogeny and evolution, NWMs have been extensively studied through morphological, bio-geographical, behavioral and molecular data (2-16). Over the last few decades, contrasting hypotheses have been proposed, presumably due to different markers and the presence of polymorphisms in the features considered. Agreement on the main clades of NWMs has been reached using different approaches, revealing a unique phylogenetic arrangement of Platyrrhini with three monophyletic families: Pitheciidae, Atelidae and Cebidae (**Table 1**, **Figure 1**) (3, 4, 10, 11, 13-15). Nevertheless, the relationships between them continue to be debated. Through the analysis of intergeneric and intrageneric relationships, intrafamily relationships have also been studied in depth. By incorporating all the available data, major advances have been made and many taxonomic controversies have been clarified (5). Therefore, the Pitheciidae family is composed of *Callicebus*, *Pithecia*, *Chiropotes* and *Cacajao*, the Atelidae family of *Alouatta*, *Ateles*, *Brachyteles* and *Lagothrix* while the Cebidae family is divided into *Cebuella*, *Mico*, *Callithrix*, *Callimico*, *Saguinus*, *Leontopithecus*, *Samiri*, *Cebus*, *Sapajus* and *Aotus* genera. However, relationships between or within some subfamilies and/or genera remain under discussion. Among Cebidae, the phylogenetic position of the Aotinae subfamily remains unclear (14). Indeed, molecular data did not allow determining if Aotinae is a sister clade of Callitrichinae or if, alternatively, Aotinae, Saimiriinae and Cebinae are sisters to Callitrichinae (3, 11, 13-15). Moreover, the number of platyrrhine genera is also still under discussion, such as the division of *Cebus* into the *Sapajus* (tufted capuchins) and *Cebus* (untufted capuchins) genera (3, 16-18). As a result, neither the diversity nor the taxonomy of NWMs are fully known. To

79 appreciate the details of Platyrrhini's evolution, much work still needs to be done at various
80 taxonomic levels.

81

82   Viruses of the genus *Cytomegalovirus* belong to the *Betaherpesvirinae* subfamily within
83 the *Herpesviridae* family, order *Herpesvirales* (19). Eight cytomegaloviruses are recognized as
84 species by the International Committee for Taxonomy of Viruses (ICTV) according to the latest
85 master species list (MSL# 32) released on March 12, 2018
86 (https://talk.ictvonline.org/files/master-species-lists/m/msl/7185). *Human betaherpesvirus 5*
87 (HHV5), commonly referred to in the literature as human cytomegalovirus (HCMV), is the
88 cytomegalovirus (CMV) type species. So far, cytomegaloviruses have been characterized only
89 from primates. Since the initial description of cytomegalovirus in African green monkeys in
90 1957, whose current species name is *Cercopithecine betaherpesvirus 5* (CeHV5), natural
91 infections by such viruses have been described in several Old World monkey (OWM) species,
92 including baboons, macaques, colobuses, chimpanzees, gorillas and others (20-31). In contrast,
93 cytomegaloviruses of New World monkeys (NWMs) are represented by only three viral entities
94 from *Aotus trivirgatus* (northern owl monkey), *Saimiri sciureus* (common squirrel monkey) and
95 *Cebus* spp. (capuchin) animals, despite the wide diversity of platyrrhines (32-34). It is presumed
96 that all primate species harbor CMVs following a co-speciation process, but data supporting this
97 assumption are scarce. The most extensive analyses of primate CMVs conducted to date are those
98 of Leendertz *et al.* (26) and Anoh *et al.* (30). These studies have demonstrated, through
99 phylogenetic analyses, a species-specific distribution of these viruses. This species specificity
100 indicates a long-term coevolution of CMVs with their natural hosts. The identification of two
101 clades, each composed of chimpanzee and gorilla CMVs, suggests that they have coevolved

102 following a horizontal transmission event between these great apes millions of years ago (26).

103 Nevertheless, interspecies transmissions in the wild are rare events (26, 28, 30, 31).

104

105   With the exception of the three above-mentioned CMVs of NWMs, there has been little

106 prior organized effort to discover cytomegaloviruses in neotropical primates. The number of

107 NWM species tested to date therefore accounts for only a tiny part of their diversity. We thought

108 that additional investigations on a larger number of species were required. We therefore

109 addressed the possible presence of CMVs in different NWM species from which we previously

110 partially characterized Epstein-Barr-like viruses (35, 36). The purpose was to gain greater insight

111 into the distribution and diversity of CMVs infecting the Platyrrhine primates. Furthermore,

112 based on the coevolution observed between OWMs and their specific CMVs, we wished to

113 determine whether NWM CMV sequences could help decipher evolutionary relationships of their

114 host species (26, 30). Given that multiple molecular markers of mitochondrial and nuclear DNAs

115 are available, host species can be characterized along with their viruses, allowing progress to be

116 made on their respective pattern of diversification. Here, we report finding sequences of

117 cytomegaloviruses in different NWM species and achieve a better understanding of the

118 evolutionary processes between these viruses and their Platyrrhini hosts.

119

120 **RESULTS**

121 To look for the presence of CMV-like viruses in our collection of NWMs, we attempted to

122 amplify a fragment of the highly conserved herpesvirus *DNA polymerase* gene from the PBMC

123 DNA of each wild-caught primate using the PCR conditions as described previously (27, 35). A

124 total of 244 samples from 20 different species of the three NWM families were tested (**Table 1**).

125 DNA samples from 12 species scored positive after the nested PCRs (nPCRs) (**Table 1**). No

126   primate belonging to the *Saguinus* and *Lagothrix* genera scored positive. Indeed, no amplification

127   was observed in any samples from the three tamarin species (*Saguinus midas*, *S. labiatus* and *S.*

128   *oedipus*) and the four woolly monkey subspecies (*Lagothrix lagotricha cana*, *L. l. lagotricha*, *L.*

129   *l. lugens* and *L. l. poeppigii*). We then used different pairs of consensus-degenerate and specific

130   PCR primers to obtain longer sequences of the *DNA polymerase* gene from each positive animal

131   (**Table 2, Figure 2**). The concatenated nucleotide sequences generated are between 448 and

132   2,026 bp in size depending on the viral strain (**Table 1**).

133

134   BLAST searches demonstrated that all sequences identified belonged to the

135   *Cytomegalovirus* genus and revealed the presence of 12 distinct sequences. Four sequences were

136   identified twice in the *Saimiri boliviensis boliviensis*, *Aotus nancymaae*, *Pithecia pithecia* and

137   *Alouatta seniculus* species while the sequence in the *Alouatta macconnelli* was identified in five

138   individuals (**Table 1**). Virus names and abbreviations were given to the 12 distinct viruses (**Table**

139   **1**): the viruses were named after the host species (for *Saimiri*, after the subspecies) followed by

140   three upper case letters corresponding to the viral genus (CMV for *Cytomegalovirus*) to which

141   they were then assigned the Arabic numeral 1, as has already been done by us and others (26, 35).

142

143   To obtain a full vision of the genetic diversity of these new CMV sequences, pairwise

144   sequence comparisons were made on the 447-bp/149-aa fragments of the *DNA polymerase* gene

145   common to all primate CMVs. All 12 sequences obtained differed from each other at the

146   nucleotide level. Sequences that were identified in different specimens of the same primate

147   species, e.g., AnanCMV1, AsenCMV1, AmacCMV1, SbolCMV1 and PpitCMV1, were 100%

148   identical with the exception of the two PpitCMV1 sequences showing 99.6% nucleotide identity

149   (**Table 3**). For clarity, comparison of the percentage of identity between the different newly

7

150 identified NWM CMVs have been reported by grouping viral sequences at the host genus level

151 (**Table 3**). Overall, the new sequences exhibited among themselves and the other available NWM

152 CMV sequences from 71.1% (AnanCMV1 *vs.* ApanCMV1) to 99.6% (CalbCMV1 *vs.* CebHV1

153 or *Cebus* sp. herpesvirus) nucleotide identity and from 79.6% (CcapCMV1 *vs.* ApanCMV1 and

154 AnanCMV1 *vs.* ApalCMV1) to 100% (AmacCMV1 *vs.* AsenCMV1 as well as SbolCMV1 *vs.*

155 SalbCMV1) amino acid identity (**Table 3**). Viruses infecting NWMs of the same genus presented

156 more than 92% of nucleotide and amino acid identities (**Table 3**). Comparison between CMVs of

157 different NWM genera ranged from 71.1% (*Aotus* CMVs *vs. Ateles* CMVs) to 88.7% (*Sapajus*

158 CMVs *vs. Cebus* CMVs) at the nucleotide level and from 79.6% between *Aotus* and *Alouatta*

159 CMVs and *Cebus* and *Ateles* CMVs to 99.3% between *Sapajus* and *Cebus* CMVs at the amino

160 acid level. NWM CMV sequences exhibited 59.8% (*Cebus* CMVs *vs. Macaca* CMVs) to 72.7%

161 (*Aotus* CMVs *vs. Colobus* CMVs) nucleotide sequence identities and 61.9 to 68.7% amino acid

162 sequence identities with OWM CMVs. The level of nucleotide and amino acid sequence identity

163 with CMVs of Hominidae, except HHV5, ranged from 61 to 72% and from 66 to 72.8%,

164 respectively. Identities with HHV5 ranged from 61.6% (*Alouatta* CMVs) to 70.9% (*Aotus*

165 CMVs) at the nucleotide level and from 66% (*Alouatta* CMVs) to 72.8% (*Aotus* CMVs) at the

166 amino acid level.

167

168   All phylogenetic analyses, performed on nucleotide or amino acid sequences between the

169 newly characterized CMV sequences and those of other primate CMVs available in the databases

170 clearly placed the new sequences in a monophyletic lineage of NWM viruses in the

171 *Cytomegalovirus* genus. The phylogenetic analysis presented in **Figure 3** is based on amino acid

172 sequences. The NWM CMV lineage diverged from the OWM CMV lineage with a posterior

173 probability value of 1. Remarkably, considering the OWM CMV lineage, the phylogenetic tree

8

174 formed two major monophyletic groups consisting of Hominoidea and Cercopithecoidea viruses,

175 respectively. Within the Cercopithecoidea, the *Colobus* CMV sequences are the basal taxa with

176 the formation of additional clades, comprising Asian *Macaca*, *Cercopithecus*/*Chlorocebus* and

177 *Papio/Mandrillus/Cercocebus* taxa.

178

179 Considering NWM CMVs, analyses demonstrated the existence of five distinct lineages

180 supported by high posterior probability values. The phylogenetic relationships between the

181 different NWM CMVs were correlated with the families and genera to which the infected

182 primates belong. The only exception was the hierarchical branching order of the

183 *Aotus*/*Saimiri*/*Cebus* genera within the Cebidae, which was not supported. Thus, viruses of *Aotus*

184 (AoHV1, AvocCMV1 and AnanCMV1) all grouped together in a monophyletic clade as did

185 those of *Saimiri* (SaHV4, SscHV, SalbCMV1 and SbolCMV1), *Cebus/Sapajus* (CebHV1,

186 CebusHV, CalbCMV1, CcapCMV1 and SapeCMV1), *Alouatta* (ApalCMV1, AmacCMV1 and

187 AsenCMV1) and *Pithecia* (PpitCMV1). Furthermore, viruses from *Alouatta* species were related

188 to ApanCMV1 from *Ateles* in a monophyletic clade of viruses infecting Atelidae monkeys with a

189 posterior probability of 1, while those of *Saimiri*, *Aotus* and *Cebus/Sapajus* belonged to a

190 monophyletic clade of Cebidae, which was supported by a posterior probability of 0.91.

191

192 To explore the cospeciation hypothesis, a time calibration analysis was performed on our

193 data set. Cytomegaloviruses identified in NWMs diverged from those of OWMs at about 32.45

194 MYA (95% HPD: 17.76–52.33) (**Table 4**). In the New World clade, three major groups were

195 identified corresponding to viruses hosted by (i) Pitheciidae, (ii) Atelidae and (iii) Cebidae. The

196 Pitheciidae viruses diverged from those of the two other groups 22.33 MYA (95% HPD: 16.25–

197 28.21) and intraspecific divergence of PpitCMV1 occurred 1.15 MYA. The divergence between

198   Atelidae and Cebidae viruses is estimated at 17.16 MYA (95% HPD: 10.29–24.25). Within the

199   group of Atelidae viruses, the divergence between ApanCMV1 identified in *Ateles paniscus* with

200   those identified in *Alouatta* spp. (ApalCMV1, AmacCMV1 and AsenCMV1) is estimated at 8.29

201   MYA (95% HPD: 2.57–14.82). Viruses identified in the three different *Alouatta* species

202   (*palliata*, *macconnelli* and *seniculus*) diverged at 3.11 MYA (95% HPD: 0.47–6.82) (ApalCMV1

203   *vs.* AmacCMV1 /AsenCMV1) while AmacCMV1 diverged from AsenCMV1 1.33 MYA (95%

204   HPD: 0.02–3.45). Within the Cebidae group, the divergence between viruses of Aotinae, Cebinae

205   and Saimiriinae occurred around 11.54 MYA (95% HPD: 5.82–17.89). Within the group of

206   cytomegaloviruses hosted by the different *Aotus* species, AoHV1 identified in *A. trivirgatus*

207   diverged from the others about 3.98 MYA (95% HPD: 0.8–8.25) while AnanCMV1 diverged

208   from AvocCMV1 2.15 MYA (95% HPD: 0.2–4.88). Within the group of viruses identified in the

209   Cebinae, CcapCMV1 from *C. capucinus* diverged from the others 3.26 MYA (95% HPD: 0.59–

210   6.94). SapCMV1 identified in *Sapajus apella* diverged from the other viruses detected in Cebus

211   (*albifrons* and *Cebus* sp.) 2.09 MYA (0.32–4.68). Finally, within the group of viruses identified

212   in the *Saimiri* genus, SsciCMV1/SaHV4 detected in *S. sciureus* diverged from those hosted by *S.*

213   *boliviensis* and *S. albigena*, SbolCMV1 and SalbCMV1, 2.68 MYA (95% HPD: 0.28–6.14).

214

215   **DISCUSSION**

216   This study is the largest conducted to date to molecularly characterize CMVs in NWMs in terms

217   of species diversity. It has partially characterized 12 cytomegaloviruses from 12 distinct species

218   belonging to seven genera and three NWM families. BLAST searches of the *Cytomegalovirus*

219   sequences identified further revealed that all but one were new viral sequences close to, but

220   distinct from, already published CMV sequences from *Aotus trivirgatus*, *Saimiri sciureus* and

221   *Cebus* sp. The only exception was the viral sequence from *C. albifrons* showing 99.6% identity at

222   the nucleotide level to CebusHV (AF292067) and CebHV1 (JQ264772), both identified from

223   unspecified *Cebus* spp. These three viral sequences were therefore considered to correspond to

224   the same viral species. In addition, the newly identified viral sequences are completely host-

225   specific, with no identification of cross-species transmission in our sample. Observations on

226   sequence comparisons, phylogenetic analysis and host-specificity of the sequences reported in

227   this paper are close to species demarcation criteria outlined in the 9th ICTV report for formal

228   recognition of new herpesvirus species (https://talk.ictvonline.org/ictv-reports/ictv_9th_report/)

229   (37).

230

231   By refining the degeneracy of the PCR primers used to screen the sample collection, we

232   were able to specifically target and identify CMV sequences, even though some of the primates

233   tested were coinfected with lymphocryptoviruses (35). Indeed, we formerly identified 17 EBV-

234   related viruses from 15 NWM species belonging to seven genera and three families from the

235   same collection of samples (35, 36). These new combinations of screening primers are therefore

236   good molecular tools to be used for future studies. Nevertheless, on the 20 NWM species tested,

237   we did not characterize any CMV sequence from our collection of *Saguinus* spp. and *Lagothrix*

238   spp. samples. Considering the relatively small sampling size of most species belonging to these

239   two genera, with the exception of *Saguinus midas*, it is conceivable that we missed a CMV-like

240   virus from them. Nevertheless, for the other primate species tested, the sampling size was

241   equivalent or even smaller and we identified CMV sequences for almost all of them. More

242   strikingly, despite the large sample size of *Saguinus midas* screened (54 individuals) and the

243   different PCR approaches used (different combinations of primers with different levels of

244   degeneracy and PCR cycling conditions), no PCR product was identified. The negativity of the

245   *Saguinus* and *Lagothrix* genera for CMV-related viruses can be explained by a lack of primer

11

246 matching or by a loss of CMV viruses during evolution within these genera. Likewise, in our

247 former studies of EBV-related sequences, we were unsuccessful in amplifying EBV sequences

248 from individuals of the *Aotus* and *Alouatta* genera (35, 36). Taken together, these results

249 highlight the need for more in-depth analyses of a representative sample from these and other

250 species of these genera to clarify this point. Moreover, for part of the positive samples, we were

251 unsuccessful in generating longer sequences of the *DNA polymerase* gene. Whether this is due to

252 the low quality/low amount of the remaining DNA, a low viral load, or reflects technical

253 difficulties, *i.e.* an inadequate level of degeneracy of the primers designed for some of these

254 viruses, is not clear. Nonetheless, sequence data generated here were sufficient to gain insight on

255 their genetic relationships.

256

257 Pairwise nucleotide and amino acid sequence comparisons demonstrated that the viral

258 sequences analyzed present different levels of genetic diversity among them (**Table 3**); the

259 smallest divergences were detected when viral sequences from primates belonging to the same

260 genus were analyzed. Phylogenetic analyses showed that CMV sequences are grouped according

261 to the primate genus from which they were detected. Thereafter, the phylogenetic clustering and

262 diversification follow those proposed for NWM species, corroborating the hypothesis of joint

263 evolution of the viruses with the speciation of their hosts (3, 5). Comparatively, analyses of

264 NWM EBV sequences have fallen short of achieving a completely resolved phylogeny (35).

265 While a clear co-speciation can be seen in the terminal branchings within major lineages

266 according to the primate subfamilies, the phylogenetic relationships between them are not

267 concordant with the current interpretations of their hosts' pattern of diversification at the family

268 level. In addition, for OWMs, there is a similar incongruence between the *Lymphocryptovirus*

269 phylogeny and that of the corresponding host lineages (38, 39). One can therefore argue that,

12

270 within the *Herpesviridae* family, *DNA polymerase* sequences from viruses of the

271 *Cytomegalovirus* genus are a better molecular marker than those of the *Lymphocryptovirus* genus

272 to test hypotheses of herpesvirus/primate co-evolution. On the basis of the available data, our

273 analysis nevertheless has two limitations regarding viruses of Cebidae that do not perfectly reflect

274 current interpretations of their hosts' diversification pattern. While viral sequences of Cebidae

275 segregate into three well-supported clades, each corresponding to the host genus from which they

276 have been identified, *i.e. Cebus*/*Sapajus*, *Saimiri* and *Aotus*, the relationships between the three

277 clades are not phylogenetically supported (**Figure 3**). The second limitation concerns

278 SapeCMV1, identified from *Sapajus apella*, which, phylogenetically, falls within the group of

279 *Cebus* viruses (**Figure 3**). Nevertheless, pairwise sequence comparison of SapeCMV1 with the

280 viral sequences identified from *Cebus* spp. shows that the nucleotide divergence of SapeCMV1 is

281 over the maximum 8% observed for viral sequences identified from NWM species of the same

282 genus (**Table 3**). These combined results (on SapeCMV1 and the other *Cebus* viruses) do not, for

283 the moment, make it possible to confidently separate the *Cebus* genus into two genera as

284 observed on analyses of *Alu* elements and on phylogenomics (3, 17). However, our virus results

285 agree quite well with the mitogenomics findings obtained in recent studies, where *Sapajus* is a

286 taxon within *Cebus* (18). These limits should be resolved by screening an extensive taxon

287 sampling of the different *Sapajus* spp. as well as of Callithrichinae for the presence of

288 cytomegaloviruses.

289

290   Finally, these data support virus–host coevolution in terms of branching order as well as

291 divergence time. Indeed, for each NWM genus tested, the estimated timing of diversification of

292 viruses is in agreement with host sequence divergence date estimates from previously published

293 studies (**Table 4**) (3, 10, 15). Nevertheless, dates obtained at superior taxonomic levels are more

13

294   recent than those based on different types of data sets or models. These discrepancies can be

295   attributed to the fact that a majority of NWM taxa remain to be tested and that no CMV sequence

296   is available for numerous genera. This therefore limits the significance of our estimates for the

297   major primate lineages for the moment and emphasizes the need for further studies.

298

299   Here, we conclusively expand our knowledge of the viral diversity, distribution and

300   evolutionary relationships of NWM CMVs. Even if the evolutionary history of these viruses is

301   not fully resolved, and in spite of the limitations mentioned above, these results strongly support

302   the co-evolution hypothesis of these new viruses with their hosts. In light of these data, we

303   propose that CMV *DNA polymerase* sequences could serve as a genetic marker to define the

304   evolutionary links of their host species. Indeed, despite the number of studies conducted over the

305   past few decades and the fast-growing number of host DNA sequence data sets, an unifying

306   consensus of the evolutionary hierarchy of NWMs has not been fully reached, partly because not

307   all phylogenies from these data sets agree but also due to a large proportion of missing data for

308   some taxa (2-15). The search for and identification of CMV *DNA polymerase* sequences

309   therefore seems to be an alternative to help solve this issue. Given the high prevalence rate of

310   CMVs in wild primates, their spread through close contact with infectious bodily fluids and their

311   persistence for the lifelong of the host, cytomegalovirus sequences, if present, should be easily

312   obtained through our PCR approach (20, 21, 40-42). Considering the number of all presently

313   known NWM species, we only tested here a fraction of their diversity. This suggests that a great

314   number of cytomegaloviruses remains to be identified in this important group of primates. These

315   results argue for a wider and more systematic sampling and exploration of NWMs to evaluate the

316   presence of CMVs and to confirm the usefulness of those sequences as a new molecular tool to

317   infer the systematics of Platyrrhini.

14

318

MATERIALS AND METHODS

Sample collection. The collection of blood DNA samples has been described in detail elsewhere

(**Table 1**) (35, 36, 43-45). In brief, we tested a total of 244 DNA samples from 20 NWM species

(26 subspecies) belonging to the three families and to six of the seven subfamilies according to

Schneider and Sampaio (5). All samples were previously genetically identified by phylogenetic

analysis of mtDNA genes, including cytochrome *c* oxydase subunit I (*COX1*) and/or cytochrome

b (*CytB*) (35).

Initial screening of samples. Molecular screening was done by semi-nested polymerase

chain reaction amplification with degenerate consensus primers targeting highly conserved amino

acid (aa) motifs of the herpesvirus *DNA polymerase* gene (**Table 2**). To maximize the chances of

amplifying CMV-like sequences, the primers of Rose *et al.* were refined from the alignment of all

primate cytomegalovirus DNA polymerase sequences available in the databases (**Table 2**) (46).

CMV3F1, CMV3F2 and CMV3R1 primers were designed and used in place of QAHNA, VYGA

and GDTD1B, respectively, while the sense primer DFASA was kept as it was. Two different

combinations of primers (DFASA/ CMV3R1 or CMV3F1/ CMV3R1) were used on each DNA

sample in separate reactions for the first-round PCR (**Figure 2**). In the second-round PCR,

CMV3F2/CMV3R1 primers were used. PCR analyses were performed at an annealing

temperature of 60°C, with an elongation time of 30 s, for 35 cycles. All amplicons of

approximately the expected size were purified, cloned by TA cloning and sent for sequencing to

Genewiz®, Takeley, UK (https://www.genewiz.com/).

341   **Partial *DNA polymerase* gene amplification.** To obtain the nucleotide sequence upstream

342   of the CMV3F2 motif, a degenerate primer (CMV3R2) was derived from the complementary

343   sequences of the small fragments and used in an nPCR amplification with the DFASA or

344   CMV3F1 primer pools using the initial PCR products as templates (**Table 2, Figure 2**). Then, to

345   generate longer segments of the *DNA polymerase* gene for each newly identified virus, we tried

346   to obtain upstream and downstream sequences using different sets of consensus degenerate and

347   species-specific primers designed using the primate CMV DNA polymerase sequence alignment

348   (**Table 2, Figure 2**). Overlapping amplicons were generated, cloned and sequenced as described

349   above. Each sequence corresponds to at least three independent clones sequenced on both strands.

350   Contig sequences were then assembled using MEGA 5.05 software (47).

351

352   **Phylogenetic analysis.** Raw sequences were analyzed and edited in MEGA 5.05 (47).

353   Sequences were confirmed as CMV by homology analysis using the NCBI BLAST search tool

354   (48). Then multiple sequence alignments were constructed using ClustalW with all other

355   previously published primate CMV sequences and alignments were checked manually.

356   Sequences were translated into amino acids and both nucleotide and amino acid sequences were

357   checked for irregularities. Hypervariable regions were removed before performing analyses.

358   Sequence identity was calculated using uncorrected p-distances. Phylogenetic trees were inferred

359   from the aligned amino acid sequences. The JTT + G model was selected as the best-fitted model

360   of amino acid evolution under corrected Akaike information criteria (AICc) using MEGA 5.05

361   and used for the Bayesian approach (49), which was performed with Mr. Bayes 3.2.2 to infer

362   phylogenetic relationships (50). Markov Chain Monte Carlo (MCMC) simulations were run for

363   10,000,000 generations, with four simultaneous chains, using a sample frequency of 500 and a

364   burn-in of 25,000. Majority rule consensus trees were obtained from the output. Validation of the

365   inference was assessed based on the standard deviation of split frequencies, which was less than

366   the expected threshold value of 0.01 (calculated value of 0.002).

367

368   **Time calibration.** Divergence times between clades were calculated using a relaxed

369   Bayesian molecular clock model with an uncorrelated lognormal rate of distribution, as

370   implemented in BEAST version 1.7.4 (51). A monophyletic constraint was imposed for the nodes

371   used to calibrate evolutionary rates. Two calibration points were applied as normal prior to

372   constraining the age of the Platyrrhini and Homo-Pan clades, the time of the most recent common

373   ancestor (tMRCA) of Platyrrhini to 23.5 MYA (stdev = 3.0) and for Homo-Pan to 6.5 MYA

374   (stdev = 0.8) (52-54). These calibration points are based on the fossil date (55). The amino acid

375   substitution model was the same as the one described above. A Yule process of speciation was

376   used as the tree prior. Results were obtained for 10,000,000 generations with the first 2,500,000

377   discarded as burn-in and parameter values sampled every 100 generations. The effective sample

378   size for parameter estimates and convergence were checked using Tracer version 1.5.0 software

379   (56). The final tree with divergence estimates and their 95% highest posterior densities was

380   computed in TreeAnnotator v1.4.5 (51).

381

382   **Accession numbers.** The sequences reported in this paper have been deposited in the

383   GenBank database under accession numbers **KU963225** to **KU963240**.

384

17

COMPETING INTERESTS

The authors declare that they have no competing financial interests.

AUTHORS' CONTRIBUTION

A.L., M.R-G. and V.L. contributed to the study design. M.R-G. collected some of the samples. S.J., D.D., J.-F.P., A.L. and V.L. performed the molecular and genetic analyses. S.J., A.L. and V.L. analyzed the data and wrote the article. All authors participated in its final writing and editing.

ETHICAL APPROVAL

Study based on samples collected several years earlier. Biological material from French Guiana was collected in 1994–1995 along the Sinnamary River, Petit Saut Hydroelectric dam, under the supervision of veterinarians of "Faune Sauvage" team led by Jean-Christophe Vié (57). Blood

18

409   sampling from live animals was carried out in accordance with French animal care regulations

410   and the laws of France. The other samples were collected directly from animals killed, in the

411   field, by indigenous hunters for their own purposes, with the full consent of the hunters and in

412   accordance with the laws of Brazil, Colombia, Mexico, Peru, Guatemala and Argentina.

REFERENCES

1. Groves CP. 2005. Order Primates, p 111-184. *In* Wilson DE, Reeder DM (ed), Mammal species of the World: a taxonomic and geographic reference, Third ed, vol 2. The Johns Hopkins University Press, Baltimore, MD.

2. Rylands AB, Mittermeier RA. 2009. The diversity of the New World Primates (Platyrrhini): an annotated taxonomy., p 23 - 54. *In* Garber PA, Estrada A, Bicca-Marques JC, Heymann EW, Stier KB (ed), South American Primates - Comparative Perspectives in the Study of Behavior, Ecology, and Conservation doi:10.1007/978-0-387-78705-3. Springer-Verlag New York, New York.

3. Perelman P, Johnson WE, Roos C, Seuanez HN, Horvath JE, Moreira MA, Kessing B, Pontius J, Roelke M, Rumpler Y, Schneider MP, Silva A, O'Brien SJ, Pecon-Slattery J. 2011. A molecular phylogeny of living primates. PLoS Genet 7:e1001342.

4. Schneider H, Canavez FC, Sampaio I, Moreira MA, Tagliaro CH, Seuanez HN. 2001. Can molecular data place each neotropical monkey in its own branch? Chromosoma 109:515-23.

5. Schneider H, Sampaio I. 2015. The systematics and evolution of New World primates - A review. Mol Phylogenet Evol 82 Pt B:348-57.

6. Schneider H, Sampaio I, Harada ML, Barroso CM, Schneider MP, Czelusniak J, Goodman M. 1996. Molecular phylogeny of the New World monkeys (Platyrrhini, primates) based on two unlinked nuclear genes: IRBP intron 1 and epsilon-globin sequences. Am J Phys Anthropol 100:153-79.

7. Schneider H, Schneider MP, Sampaio I, Harada ML, Stanhope M, Czelusniak J, Goodman M. 1993. Molecular phylogeny of the New World monkeys (Platyrrhini, primates). Mol Phylogenet Evol 2:225-42.

437   8.   Rylands AB, Mittermeier RA, Silva Jr. JS. 2012. Neotropical primates: taxonomy and

438   recently described species and subspecies. Int Zoo Yearb 46:11-24.

439   9.   Rylands AB, Schneider H, Langguth A, Mittermeier RA, Groves CP, Rodrigues-Luna E.

440   2000. An assessment of the diversity of new World primates. Neotrop Primates 8:61-93.

441   10.   Opazo JC, Wildman DE, Prychitko T, Johnson RM, Goodman M. 2006. Phylogenetic

442   relationships and divergence times among New World monkeys (Platyrrhini, Primates).

443   Mol Phylogenet Evol 40:274-80.

444   11.   Wildman DE, Jameson NM, Opazo JC, Yi SV. 2009. A fully resolved genus level

445   phylogeny of neotropical primates (Platyrrhini). Mol Phylogenet Evol 53:694-702.

446   12.   Singer SS, Schmitz J, Schwiegk C, Zischler H. 2003. Molecular cladistic markers in New

447   World monkey phylogeny (Platyrrhini, Primates). Mol Phylogenet Evol 26:490-501.

448   13.   Ray DA, Xing J, Hedges DJ, Hall MA, Laborde ME, Anders BA, White BR, Stoilova N,

449   Fowlkes JD, Landry KE, Chemnick LG, Ryder OA, Batzer MA. 2005. Alu insertion loci

450   and platyrrhine primate phylogeny. Mol Phylogenet Evol 35:117-26.

451   14.   Osterholz M, Walter L, Roos C. 2009. Retropositional events consolidate the branching

452   order among New World monkey genera. Mol Phylogenet Evol 50:507-13.

453   15.   Jameson Kiesling NM, Yi SV, Xu K, Gianluca Sperone F, Wildman DE. 2015. The tempo

454   and mode of New World monkey evolution and biogeography in the context of

455   phylogenomic analysis. Mol Phylogenet Evol 82 Pt B:386-99.

456   16.   Alfaro JW, Silva JD, Jr., Rylands AB. 2012. How different are robust and gracile capuchin

457   monkeys? An argument for the use of sapajus and cebus. Am J Primatol 74:273-86.

458   17.   Martins AM, Jr., Amorim N, Carneiro JC, de Mello Affonso PR, Sampaio I, Schneider H.

459   2015. Alu elements and the phylogeny of capuchin (Cebus and Sapajus) monkeys. Am J

460   Primatol 77:368-75.

461   18.   Ruiz-Garcia M, Castillo MI, Luengas-Villamil K. 2016. It is misleading to use Sapajus (robust capuchins) as a genus? : A review of the evolution of the capuchins and suggestions on their systematics, p 209-268. *In* Ruiz-Garcia M, Shostell JM (ed), Molecular population genetics, evolutionary biology and biological conservation of the Neotropical Prinates. Nova Science Publisher, Inc., New York, USA.

466   19.   Davison AJ, Eberle R, Ehlers B, Hayward GS, McGeoch DJ, Minson AC, Pellett PE, Roizman B, Studdert MJ, Thiry E. 2009. The order Herpesvirales. Arch Virol 154:171-7.

468   20.   Blewett EL, White G, Saliki JT, Eberle R. 2001. Isolation and characterization of an endogenous cytomegalovirus (BaCMV) from baboons. Arch Virol 146:1723-38.

470   21.   Blewett EL, Lewis J, Gadsby EL, Neubauer SR, Eberle R. 2003. Isolation of cytomegalovirus and foamy virus from the drill monkey (Mandrillus leucophaeus) and prevalence of antibodies to these viruses amongst wild-born and captive-bred individuals. Arch Virol 148:423-33.

474   22.   Asher DM, Gibbs CJ, Jr., Lang DJ, Gajdusek DC, Chanock RM. 1974. Persistent shedding of cytomegalovirus in the urine of healthy Rhesus monkeys. Proc Soc Exp Biol Med 145:794-801.

477   23.   Malherbe H, Harwin R. 1957. Seven viruses isolated from the vervet monkey. Br J Exp Pathol 38:539-41.

479   24.   Black PH, Hartley JW, Rowe WP. 1963. Isolation of a cytomegalovirus from African green monkey. Proc Soc Exp Biol Med 112:601-5.

481   25.   Wroblewska Z, Gilden D, Devlin M, Huang ES, Rorke LB, Hamada T, Furukawa T, Cummins L, Kalter S, Koprowski H. 1979. Cytomegalovirus isolation from a chimpanzee with acute demyelinating disease after inoculation of multiple sclerosis brain cells. Infect Immun 25:1008-15.

485   26.   Leendertz FH, Deckers M, Schempp W, Lankester F, Boesch C, Mugisha L, Dolan A,

486   Gatherer D, McGeoch DJ, Ehlers B. 2009. Novel cytomegaloviruses in free-ranging and

487   captive great apes: phylogenetic evidence for bidirectional horizontal transmission. J Gen

488   Virol 90:2386-94.

489   27.   Lacoste V, Mauclere P, Dubreuil G, Lewis J, Georges-Courbot MC, Rigoulet J, Petit T,

490   Gessain A. 2000. Simian homologues of human gamma-2 and betaherpesviruses in

491   mandrill and drill monkeys. J Virol 74:11993-9.

492   28.   Murthy S, Couacy-Hymann E, Metzger S, Nowak K, De Nys H, Boesch C, Wittig R, Jarvis

493   MA, Leendertz FH, Ehlers B. 2013. Absence of frequent herpesvirus transmission in a

494   nonhuman primate predator-prey system in the wild. J Virol 87:10651-9.

495   29.   Prepens S, Kreuzer KA, Leendertz F, Nitsche A, Ehlers B. 2007. Discovery of

496   herpesviruses in multi-infected primates using locked nucleic acids (LNA) and a bigenic

497   PCR approach. Virol J 4:84.

498   30.   Anoh AE, Murthy S, Akoua-Koffi C, Couacy-Hymann E, Leendertz FH, Calvignac-

499   Spencer S, Ehlers B. 2017. Cytomegaloviruses in a Community of Wild Nonhuman

500   Primates in Tai National Park, Cote D'Ivoire. Viruses 10.

501   31.   Seimon TA, Olson SH, Lee KJ, Rosen G, Ondzie A, Cameron K, Reed P, Anthony SJ, Joly

502   DO, Karesh WB, McAloose D, Lipkin WI. 2015. Adenovirus and herpesvirus diversity in

503   free-ranging great apes in the Sangha region of the Republic Of Congo. PLoS One

504   10:e0118543.

505   32.   Daniel MD, Melendez LV, King NW, Barahona HH, Fraser CE, Garcia FG, Silva D. 1973.

506   Isolation and characterization of a new virus from owl monkeys: herpesvirus aotus type 3.

507   Am J Phys Anthropol 38:497-500.

508   33.   Daniel MD, Melendez LV, King NW, Fraser CE, Barahona HH, Hunt RD, Garcia FG,
509   Trum BF. 1971. Herpes virus aotus: a latent herpesvirus from owl monkeys (Aotus
510   trivirgatus) isolation and characterization. Proc Soc Exp Biol Med 138:835-45.

511   34.   Rangan SR, Chaiban J. 1980. Isolation and characterization of a cytomegalovirus from the
512   salivary gland of a squirrel monkey (Saimiri sciureus). Lab Anim Sci 30:532-40.

513   35.   Lavergne A, de Thoisy B, Pouliquen JF, Ruiz-Garcia M, Lacoste V. 2011. Partial
514   molecular characterisation of New World non-human primate lymphocryptoviruses. Infect
515   Genet Evol 11:1782-9.

516   36.   de Thoisy B, Pouliquen JF, Lacoste V, Gessain A, Kazanji M. 2003. Novel gamma-1
517   herpesviruses identified in free-ranging new world monkeys (golden-handed tamarin
518   [Saguinus midas], squirrel monkey [Saimiri sciureus], and white-faced saki [Pithecia
519   pithecia]) in French Guiana. J Virol 77:9099-105.

520   37.   Pellett PE, Davison AJ, Eberle R, B. E, Hayward GS, Lacoste V, Minson AC, Nicholas J,
521   Roizman B, Studdert MJ, Wang F. 2011. Order *Herpesvirales*, p 99-107. *In* King AMQ,
522   Adams MJ, Carstens EB, Lefkowitz EJ (ed), Virus Taxonomy: Ninth report of the
523   International Committee on Taxonomy of Viruses. Elsevier Inc., Oxford, United Kingdom.

524   38.   Ehlers B, Ochs A, Leendertz F, Goltz M, Boesch C, Matz-Rensing K. 2003. Novel simian
525   homologues of Epstein-Barr virus. J Virol 77:10695-9.

526   39.   Ehlers B, Spiess K, Leendertz F, Peeters M, Boesch C, Gatherer D, McGeoch DJ. 2010.
527   Lymphocryptovirus phylogeny and the origins of Epstein-Barr virus. J Gen Virol 91:630-
528   42.

529   40.   Mocarski ES, Shenk T, Griffiths PD, Pass RF. 2013. Cytomegaloviruses, p 1960 - 2014. *In*
530   Knipe DM, Howley PM (ed), Field's Virology, vol 2. Lippincott Williams & Wilkins,
531   Philadelphia.

532   41.   Cannon MJ, Hyde TB, Schmid DS. 2011. Review of cytomegalovirus shedding in bodily

533   fluids and relevance to congenital cytomegalovirus infection. Rev Med Virol 21:240-55.

534   42.   Reeves M, Sinclair J. 2008. Aspects of human cytomegalovirus latency and reactivation.

535   Curr Top Microbiol Immunol 325:297-313.

536   43.   de Thoisy B, Vogel I, Reynes JM, Pouliquen JF, Carme B, Kazanji M, Vie JC. 2001.

537   Health evaluation of translocated free-ranging primates in French Guiana. Am J Primatol

538   54:1-16.

539   44.   Ruiz-Garcia M, Castillo MI, Vasquez C, Rodriguez K, Pinedo-Castro M, Shostell J,

540   Leguizamon N. 2010. Molecular phylogenetics and phylogeography of the white-fronted

541   capuchin (Cebus albifrons; Cebidae, Primates) by means of mtCOII gene sequences. Mol

542   Phylogenet Evol 57:1049-61.

543   45.   Ruiz-Garcia M, Pinedo-Castro MO. 2010. Molecular systematics and phylogeography of

544   the genus Lagothrix (Atelidae, Primates) by means of the mitochondrial COII gene. Folia

545   Primatol (Basel) 81:109-28.

546   46.   Rose TM, Strand KB, Schultz ER, Schaefer G, Rankin GW, Jr., Thouless ME, Tsai CC,

547   Bosch ML. 1997. Identification of two homologs of the Kaposi's sarcoma-associated

548   herpesvirus (human herpesvirus 8) in retroperitoneal fibromatosis of different macaque

549   species. J Virol 71:4138-44.

550   47.   Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular

551   evolutionary genetics analysis using maximum likelihood, evolutionary distance, and

552   maximum parsimony methods. Mol Biol Evol 28:2731-9.

553   48.   Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden TL. 2008. NCBI

554   BLAST: a better web interface. Nucleic Acids Res 36:W5-9.

555   49.   Darriba D, Taboada GL, Doallo R, Posada D. 2011. ProtTest 3: fast selection of best-fit

556   models of protein evolution. Bioinformatics 27:1164-5.

557   50.   Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Hohna S, Larget B, Liu L,

558   Suchard MA, Huelsenbeck JP. 2012. MrBayes 3.2: efficient Bayesian phylogenetic

559   inference and model choice across a large model space. Syst Biol 61:539-42.

560   51.   Drummond AJ, Suchard MA, Xie D, Rambaut A. 2012. Bayesian phylogenetics with

561   BEAUti and the BEAST 1.7. Mol Biol Evol 29:1969-73.

562   52.   Poux C, Douzery EJ. 2004. Primate phylogeny, evolutionary rate variations, and divergence

563   times: a contribution from the nuclear gene IRBP. Am J Phys Anthropol 124:1-16.

564   53.   Kay RF, Fleagle JG, Mitchell TR, Colbert M, Bown T, Powers DW. 2008. The anatomy of

565   Dolichocebus gaimanensis, a stem platyrrhine monkey from Argentina. J Hum Evol

566   54:323-82.

567   54.   Vignaud P, Duringer P, Mackaye HT, Likius A, Blondel C, Boisserie JR, De Bonis L,

568   Eisenmann V, Etienne ME, Geraads D, Guy F, Lehmann T, Lihoreau F, Lopez-Martinez N,

569   Mourer-Chauvire C, Otero O, Rage JC, Schuster M, Viriot L, Zazzo A, Brunet M. 2002.

570   Geology and palaeontology of the Upper Miocene Toros-Menalla hominid locality, Chad.

571   Nature 418:152-5.

572   55.   Tavare S, Marshall CR, Will O, Soligo C, Martin RD. 2002. Using the fossil record to

573   estimate the age of the last common ancestor of extant primates. Nature 416:726-9.

574   56.   Rambaut A, Drummond AJ. 2009. Tracer. Version 1.5.0.

575   57.   Vie JC. 1999. Wildlife rescues - The case of the Petit Saut hydroelectric dam in French

576   Guiana. Oryx 33:115-126.

577 58. Blewett EL, Sherrod CJ, Texier JR, Conrad TM, Dittmer DP. 2015. Complete Genome

578   Sequences of Mandrillus leucophaeus and Papio ursinus Cytomegaloviruses. Genome

579   Announc 3.

580 59. Johnson G, Dick D, Ayers M, Petric M, Tellier R. 2003. Detection and species-level

581   identification of primate herpesviruses with a comprehensive PCR test for human

582   herpesviruses. J Clin Microbiol 41:1256-8.

583 60. Marsh AK, Willer DO, Ambagala AP, Dzamba M, Chan JK, Pilon R, Fournier J,

584   Sandstrom P, Brudno M, MacDonald KS. 2011. Genomic sequencing and characterization

585   of cynomolgus macaque cytomegalovirus. J Virol 85:12995-3009.

586 61. Russell JN, Marsh AK, Willer DO, Ambagala AP, Dzamba M, Chan JK, Pilon R, Fournier

587   J, Brudno M, Antony JM, Sandstrom P, Evans BJ, MacDonald KS. 2016. A novel strain of

588   cynomolgus macaque cytomegalovirus: implications for host-virus co-evolution. BMC

589   Genomics 17:277.

590 62. Swanson R, Bergquam E, Wong SW. 1998. Characterization of rhesus cytomegalovirus

591   genes associated with anti-viral susceptibility. Virology 240:338-48.

592 63. Rivailler P, Kaur A, Johnson RP, Wang F. 2006. Genomic sequence of rhesus

593   cytomegalovirus 180.92: insights into the coding potential of rhesus cytomegalovirus. J

594   Virol 80:4179-82.

595 64. Kouzarides T, Bankier AT, Satchwell SC, Weston K, Tomlinson P, Barrell BG. 1987.

596   Sequence and transcription analysis of the human cytomegalovirus DNA polymerase gene.

597   J Virol 61:125-33.

598 65. Davison AJ, Dolan A, Akter P, Addison C, Dargan DJ, Alcendor DJ, McGeoch DJ,

599   Hayward GS. 2003. The human cytomegalovirus genome revisited: comparison with the

600   chimpanzee cytomegalovirus genome. J Gen Virol 84:17-28.

601   66.   Dunn W, Chou C, Li H, Hai R, Patterson D, Stolc V, Zhu H, Liu F. 2003. Functional

602   profiling of a human cytomegalovirus genome. Proc Natl Acad Sci U S A 100:14223-8.

603   67.   Murphy E, Yu D, Grimwood J, Schmutz J, Dickson M, Jarvis MA, Hahn G, Nelson JA,

604   Myers RM, Shenk TE. 2003. Coding potential of laboratory and clinical strains of human

605   cytomegalovirus. Proc Natl Acad Sci U S A 100:14976-81.

606

607 **FIGURE LEGEND**

608

609 **FIG.1. Diagram representation of Plathyrrhini taxa in descending order down to genus**

610 **level.** Adapted from Perelman et al. (3). Black and gray boxes represent NWM genera tested for

611 CMVs. Black boxes correspond to NWM genera from which CMV sequences have been

612 characterized while gray boxes represent NWM genera from which no CMV sequence has been

613 obtained in the present study.

614

615 **FIG. 2. Relative position and orientation of the PCR primers used.** The different

616 combinations of primers used in nested or semi-nested PCRs are represented above the

617 Herpesviral *DNA polymerase* gene sequence in different grey boxes. Primers XxxR1 and XxxR2,

618 represented by dotted arrows, correspond to the different antisense specific primers used in a

619 degenerate (CMV2F1 or CMV2F2) - nondegenerate nPCR assay (see **Table 2**). Bars below the

620 sequence represent the different nested PCR products expected. The pairs of primers on the left-

621 hand side of the bars indicate those used for the first-round PCR, while those on the right-hand

622 side correspond to the one used in the nested PCR reactions. The sequences of the

623 oligonucleotide primers are given in **Table 2**.

624

625 **FIG. 3. Phylogenetic analysis of primate cytomegalovirus sequences.** The phylogenetic tree

626 was derived from the partial amino acid sequences of the *DNA polymerase* gene (149 aa) of 50

627 representatives of primate cytomegaloviruses using the Bayesian method with the JTT + G model

628 of amino acid evolution. Sequences generated in this study are in boldface. Posterior probabilities

629 of the Bayesian analysis (>0.9) are shown next to each node. The scale bar indicates amino acid

630 substitutions per site. The major clades representing Old World and New World primate families,

631   super-families (for OWM) and parvorders are labeled on the right-hand side of the figure. The

632   virus names are associated with their accession numbers. Viruses of Cercopithecidae comprise

633   those of the chacma (BaCMV NC_027016 (20, 58)) and olive (BaCMV AF387664 (21))

634   baboons, the moustached guenon (CceCMV1 AY728178), the agile mangabey (CagCMV1

635   AY608713), the drill (MndCMV AF282941 and MndCMV AF387665 strain OCOM6-2 (21,

636   27)), the mandrill (MndCMV AY129399), the African green monkey (CeHV5 AY117754,

637   CeHV5 FJ483969 strain Colburn, CeHV5 FJ483969 strain 2715, CaeCMV AF292066,

638   VervetCMV AY049066 strain CSG and CeHV3 AY049065 (21, 59)), the cynomolgus

639   (MfasCMV1 JN227533 strain Ottawa, MfasCMV1 AY728171 and MfasCMV KP796148 strain

640   Mauritius (60, 61)) and rhesus (McHV3 AF033184 and McHV3 DQ120516 isolate CMV 180.92

641   (62, 63)) macaques and the mantled guereza (CgueCMV1.1 AY129397 and CgueCMV1.2

642   EU118147 (29)). Viruses of Hominidae comprise those of the Bornean orangutan (PpygCMV1.1

643   AY129396), the human (HHV5 M14709 strain AD169, HHV5 NC_006273 strain Merlin, HHV5

644   AY315197 strain Towne and HHV5 AC146905 isolate Toledo (64-67)), the Western gorilla

645   (GgorCMV2.1 FJ538490 (26)) and the common chimpanzee (PnHV2 AF480884 strain

646   Heberling, PtroCMV1.1 FJ538485 and PtroCMV AF292063 (26, 65)). Regarding viruses of New

647   World monkeys, in addition to the ones describe in the present study, viruses of Cebidae

648   comprise those of the capuchin monkey (CebHV1 JQ264772 and CebusHV AF292067 from

649   *Cebus* sp.), the common squirrel monkey (SaHV4 FJ483967 and SscHV AF292065) and the

650   three-striped night monkey (AoHV1 FJ483970).

Parvorder	Platyrrhini	
Family	Pitheciidae Atelidae Cebidae	
Subfamily	Callicebinae Pitheciinae Alouattinae Atelinae Callitrichinae Cebinae Saimirinae Aotinae	
Genus	*Callicebus* **Pithecia** *Chiropotes* *Cacajao* **Alouatta** **Ateles** **Lagothrix** *Brachyteles* **Saguinus** *Callithrix* *Leontopithecus* *Callimico* *Cebuella* *Mico* **Cebus** **Sapajus** **Saimiri** **Aotus**	
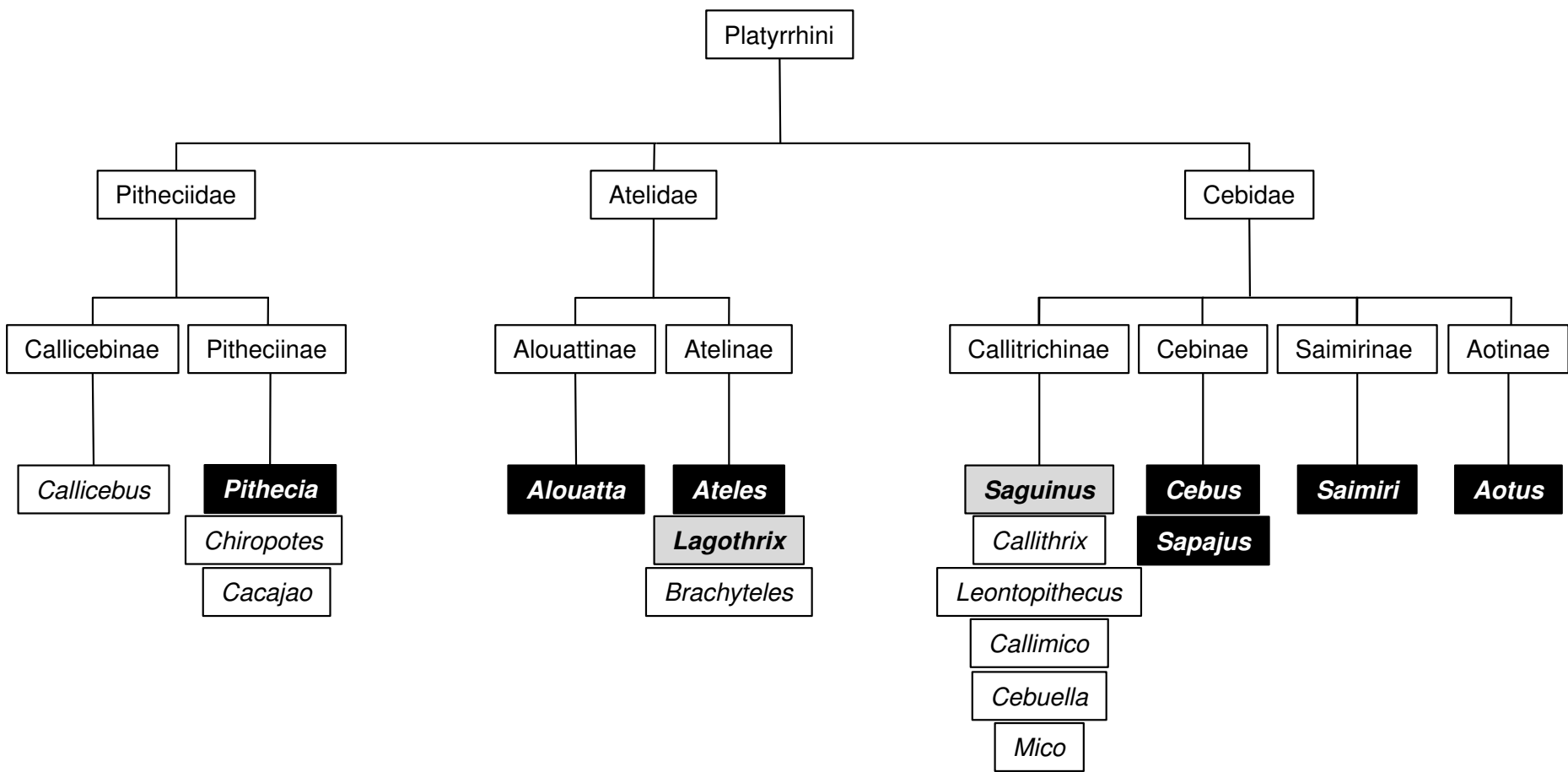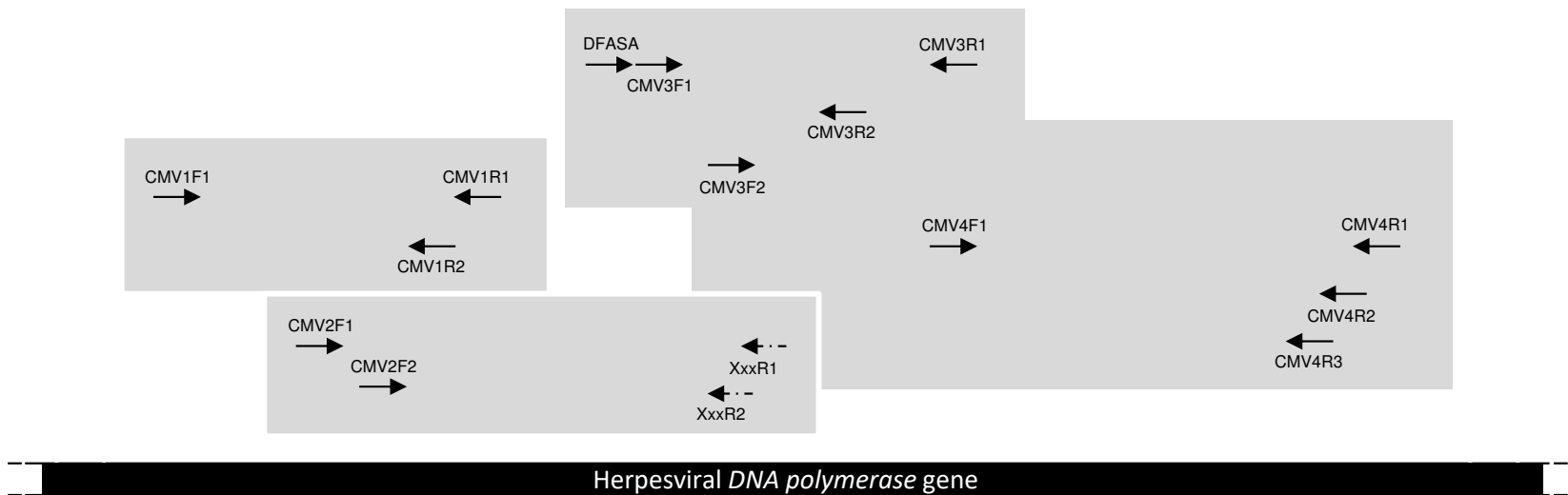
TABLE 1. New World non-human primates tested for cytomegaloviruses using molecular methods and survey results

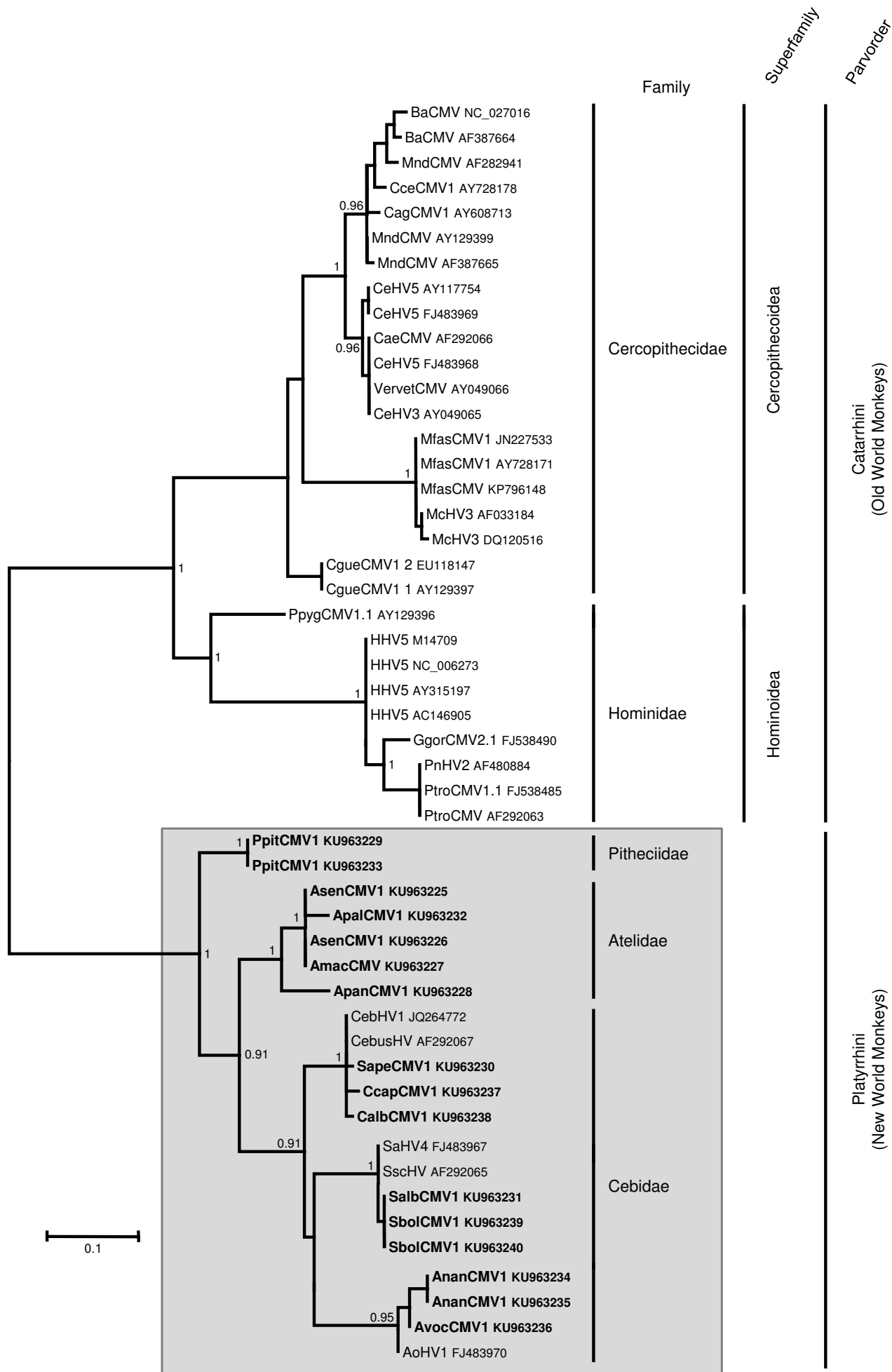| O	sO	iO	pO	Family[a]	Subfamily	Genus	species	subspecies	Common Name	Origin	Number[b]	Size[c]	Name	Acronym	Accession numbers
													Informal names		
Primates	Haplorrhini	Simiiformes	Platyrrhini		Callitrichinae	*Saguinus*	*midas*		Red-handed tamarin	French Guiana	0/54				
							labiatus		White-lipped tamarin	Peru	0/2				
							oedipus		Cottontop tamarin	Colombia	0/2				
				Cebidae	Cebinae	*Sapajus*[d]	*apella*		Tufted capuchin	French Guiana	0/5				
							apella		Tufted capuchin	Colombia	1/10	910	*S. apella* CMV1	**SapeCMV1**	KU963230
						Cebus	*albifrons*		White-fronted capuchin	Colombia	1/10	448	*C. albifrons* CMV1	**CalbCMV1**	KU963238
							capucinus		White-headed capuchin	Colombia	1/10	448	*C. capucinus* CMV1	**CcapCMV1**	KU963237
					Saimirinae	*Saimiri*	*boliviensis*	*boliviensis*	Black-capped squirrel monkey	Colombia	2/3	448	*S. b. boliviensis* CMV1	**SbolCMV1**	KU963239, KU963240
							sciureus	*sciureus*	Common squirrel monkey	French Guiana	0/4				
							sciureus	*albigena*	Colombian common squirrel monkey	Colombia	1/1	910	*S. s. albigena* CMV1	**SalbCMV1**	KU963231
							sciureus	*macrodon*	Ecuadorian common squirrel monkey	Colombia	0/1				
					Aotinae	*Aotus*	*vociferans*		Spix's night monkey	Peru	1/5	451	*A. vociferans* CMV1	**AvocCMV1**	KU963236
							nancymaae		Nancy Ma's night monkey	Peru	2/6	964	*A. nancymaae* CMV1	**AnanCMV1**	KU963234, KU963235
				Pitheciidae	Pitheciinae	*Pithecia*	*pithecia*		White-faced saki	French Guiana	2/4	916	*P. pithecia* CMV1	**PpitCMV1**	KU963229, KU963233
				Atelidae	Alouattinae	*Alouatta*	*macconnelli*		Guyanan red howler	French Guiana	5/94	1724	*A. macconnelli* CMV1	**AmacCMV1**	KU963227
							seniculus		Venezuelan red howler	Colombia	2/8	1724	*A. seniculus* CMV1	**AsenCMV1**	KU963225, KU963226
							caraya		Black howler	Argentina	0/1				
							palliata		Mantled howler	Mexico	1/2	988	*A. palliata* CMV1	**ApalCMV1**	KU963232
					Atelinae	*Ateles*	*paniscus*		Red-faced spider monkey	French Guiana	1/5	2026	*A. paniscus* CMV1	**ApanCMV1**	KU963228
							belzebuth		White-fronted spider monkey	Colombia	0/3				
							fusciceps	*robustus*	Black-headed spider monkey	Colombia	0/4				
							geoffroyi		Geoffroy's spider monkey	Guatemala	0/3				
						Lagothrix	*lagotricha*	*cana*	Gray woolly monkey	Brazil	0/1				
							lagotricha	*lagotricha*	Brown woolly monkey	Colombia	0/2				
							lagotricha	*lugens*	Colombian woolly monkey	Colombia	0/2				
							lagotricha	*poeppigii*	Silvery woolly monkey	Peru	0/2				

Abbreviations: O, order; sO, suborder; iO, infraorder; pO, parvorder; CMV, Cytomegalovirus.

[a] According to Perelman *et al.* (3).

[b] Number: number of CMV-positive animals (by PCR, cloning and sequencing)/number of tested animals.

[c] Size: size of the *DNA polymerase* gene fragments obtained, in bp.

[d] According to Alfaro *et al.* (16).

TABLE 2. Oligonucletide primers used for Cytomegalovirus *DNA polymerase* consensus and specific PCRs

Oligonucleotide	Orientation[a]	Location[b]	5' -> 3' sequence[c]	CMV sequence amplified
DNA pol degenerate primers				
CMV1F1	+	721 - 749	GAC AAG AAG TTG ACN ACN TTY GGN TGG TG	AmacCMV1,AsenCMV1, ApanCMV1
CMV1R1	-	1534 - 1559	ACG CCG GCY TCR TAR TGR AAR TTD AT	AmacCMV1,AsenCMV1, ApanCMV1
CMV1R2	-	1480 - 1504	CGT CCT GHA CRC ART AYT TNC CNA C	AmacCMV1,AsenCMV1, ApanCMV1
CMV2F1	+	1330 - 1352	GAY ATG TAY CCN GTS TGY ATG GC	AmacCMV1, AsenCMV1, ApanCMV1, PpitCMV1, SapeCMV1, SalbCMV1
CMV2F2	+	1372 - 1394	TAC AAR YTV AAY ACB ATG GCS GA	AmacCMV1, AsenCMV1, ApanCMV1, PpitCMV1, SapeCMV1, SalbCMV1
DFASA[d]	+	1768 - 1793	GTG TTC GAC TTY GCN AGY YTN TAY CC	All
CMV3F1	+	1795 - 1817	TCH ATY ATY ATG GCN CAY AAY CT	All
CMV3F2	+	2062 - 2090	ACG TGC AAT TCT TTY TAY GGB TTY ACN GG	All
CMV3R1	-	2269 - 2303	CGA TAG CAC ACA AAC ACR CTR TCN GTR TCN CCR TA	All
CMV3R2	-	2125 - 2147	CCG ATD CGN GTR ATR CTR GCC GC	All
CMV4F1	+	2266 - 2291	ATC TAY GGK GAC ACS GAY AGY GTS TT	AmacCMV1, AsenCMV1, ApalCMV1, AnanCMV1, ApanCMV1
CMV4R1	-	2782 - 2801	GCC GCY ARN CGY TTD ATG AC	ApalCMV1, AnanCMV1, ApanCMV1
CMV4R2	-	2477 - 2498	CGC ACC ARR TCR ACN CCY TTC A	AmacCMV1, AsenCMV1, ApalCMV1, AnanCMV1, ApanCMV1
CMV4R3	-	2419 - 2444	ATA TAC CGY TTY TTR CAG ATC ATC AT	AmacCMV1, AsenCMV1, ApalCMV1, AnanCMV1, ApanCMV1
DNA pol antisense specific primers (in combination with CMV2F1 or CMV2F2) [e]				
PitR1	-	1958 - 1978	TGC GCT GAG CAA CCC ATT TAG	PpitCMV1
PitR2	-	1912 - 1932	ACG CAC CTC CGA CTT CAC AAA	PpitCMV1
AotR1	-	2019 - 2039	TTG TCG AGC AGC GTC CTC TTG	AnanCMV1, AvocCMV1
AotR2	-	1884 - 1904	ACC GTA CCG TTT TCG AAG TTA	AnanCMV1, AvocCMV1
SapR1	-	2106 - 2126	GCG ACT GGC AAA CAC GGT AAC	SapeCMV1
CapR2	-	1994 - 2016	GGG ATC TGT GCA ATC TTT CAT GG	CalbCMV1, CcapCMV1
CapR3	-	1939 - 1961	CGG GTC AAC AAT TCA GAA AGC AC	SapeCMV1, CalbCMV1, CcapCMV1
AteR1	-	1994 - 2016	CGG ATC TCT GCA ATT TTT CAT GG	ApanCMV1
AteR2	-	1935 - 1957	TCA GCA GTT CGG ACA ACA CTG AA	ApanCMV1
SaiR1	-	1948 - 1969	CCA CCC ACT TCG TTA GCA GCT C	SbolCMV1, SalbCMV1
SaiR2	-	2155 - 2175	ACG CGC GGT GTC TTG TAA CAT	SbolCMV1, SalbCMV1
AloR1	-	1959 - 1979	TTC CGT TGA GCC ACC CAT TTA	AmacCMV1, AsenCMV1, ApalCMV1
AloR2	-	1932 - 1954	GCA ATT CCG ATA GCA CTG AGG AA	AmacCMV1, AsenCMV1, ApalCMV1

[a] +, sense; -, antisense.

[b] Positions relative to ATG of the *DNA polymerase* gene of AoHV1, accession number FJ483970.

[c] Positions of degeneracy follow the International Unit Base codes.

[d] Degenerate oligonucleotide primer described in Rose *et al.* (45).

[e] For clarity, all antisense specific primers are indicated as XxxR1 for R1 primers and XxxR2 for R2 primers in Figure 2.

TABLE 3. Nucleotide and amino acid identities between the novel Cytomegaloviruses and all other NHP Cytomegaloviruses and HCMV[a]

% identity[b] with

		Viruses of	Sapajus (SapeCMV1) Nucleotide	Sapajus Amino acid	Cebus (CalbCMV1, CcapCMV1) Nucleotide	Cebus Amino acid	Saimiri (SbolCMV1, SalbCMV1) Nucleotide	Saimiri Amino acid	Aotus (AvocCMV1, AnanCMV1) Nucleotide	Aotus Amino acid	Pithecia (PpitCMV1) Nucleotide	Pithecia Amino acid	Alouatta (AmacCMV1, AsenCMV1, ApalCMV1) Nucleotide	Alouatta Amino acid	Ateles (ApanCMV1) Nucleotide	Ateles Amino acid
Cd[c]	Cn	Sapajus			87.6 - 88.7	98.0 - 99.3	76.1 - 77.0	89.1	74.3 - 75.4	85.7	72.9 - 73.1	83.7	74.5 - 74.7	83.0 - 85.0	75.9	80.3
		Cebus[d]	87.6 - 88.7	98.0 - 99.3	95.7 - 99.6	98.0 - 99.3	72.2 - 77.2	87.1 - 88.4	74.0 - 75.4	85.0 - 87.1	72.0 - 72.5	83.0 - 84.4	74.3 - 75.4	81.6 - 83.7	74.5 - 74.7	79.6 - 81.0
	Sn	Saimiri[e]	76.1 - 77.4	89.1	75.2 - 77.7	87.1 - 87.8	97.1 - 97.7	99.3 - 100	74.3 - 74.9	85.71	74.7 - 75.6	82.31	74.7 - 76.1	85.0 - 85.7	75.2 - 75.6	83.67
	An	Aotus[f]	74.3 - 75.4	85.7 - 86.4	74.0 - 75.6	85.0 - 87.8	74.0 - 74.7	85.7 - 86.4	94.8 - 96.2	96.6 - 98.6	74.0 - 74.7	83.7 - 85.0	71.8 - 74.8	79.6 - 82.3	71.1 - 72.2	81.63
Pd	Pn	Pithecia	72.9 - 73.1	83.7	72.0 - 72.5	83.0 - 83.7	74.72	82.3 - 83.3	74.0 - 74.7	84.4 - 85.0	99.6	100	72.9 - 75.2	83.0 - 85.0	75.2 - 75.4	84.35
Ad	Al	Alouatta	74.5 - 74.7	82.3 - 83.0	74.3 - 75.4	81.6 - 83.0	74.9 - 76.1	85.0 - 85.7	71.8 - 73.1	79.6 - 81.6	72.9 - 75.2	83.0 - 85.0	92.1 - 98.9	97.3 - 100	82.2 - 83.5	91.2 - 92.5
	At	Ateles	75.9	80.3	74.5 - 74.7	79.6 - 80.3	75.4 - 75.6	83.67	71.1 - 71.6	81.63	75.2 - 75.4	84.35	82.2 - 83.5	91.2 - 92.5	-	-
Hd[g]	Hn	Homo	64.1 - 64.6	67.35	64.3 - 65.9	66.7 - 68.0	63.2 - 64.3	67.35	70.0 - 70.9	72.1 - 72.8	65.2 - 66.1	70.07	61.6 - 63.7	65.99	62.1 - 63.0	67.35
		Pan	61.85	66.67	63.2 - 63.7	66.0 - 66.7	63.2 - 63.4	66.67	71.6 - 72.0	71.4 - 72.1	66.1 - 66.6	70.07	61.2 - 62.7	66.67	62.08	68.03
		Gorilla	62.98	67.35	63.7 - 63.9	66.7 - 68.0	62.3 - 62.5	67.35	71.33	72.1 - 72.8	62.98	70.75	61.0 - 62.1	65.99	60.95	68.03
	Po	Pongo	64.11	69.39	63.4 - 64.1	69.4	65.5 - 65.7	68.03	70.4 - 70.7	71.43	66.14	69.39	62.5 - 63.4	68.0 - 68.7	62.53	68.71
Ce[g]	Cr	Macaca	62.1 - 63.4	63.3 - 64.0	59.8 - 61.6	61.9 - 63.3	62.1 - 63.2	64.6 - 65.3	65.9 - 66.6	65.3 - 67.4	63.4 - 63.9	64.6 - 65.3	60.3 - 62.1	64.6 - 66.7	62.5 - 63.0	64.6 - 65.3
		Papio	64.8 - 66.4	64.0 - 64.6	62.1 - 63.4	61.9 - 63.3	62.1 - 62.3	66.0 - 66.7	68.2 - 69.1	64.0 - 65.3	64.6 - 64.8	65.3 - 66.0	62.3 - 63.4	66.0 - 66.7	62.53	65.3
		Mandrillus	64.1 - 65.7	64.0 - 66.0	61.8 - 63.4	61.9 -64.6	61.4 - 63.0	66.7 - 68.0	67.5 - 68.8	64.6 - 67.4	64.3 - 65.9	66.0 - 68.0	60.9 - 64.6	66.0 - 68.0	61.8 - 62.7	65.3 - 67.4
		Cerco/Chloro	63.7- 65.5	64.6 - 66.0	61.6 - 68.8	63.3 - 65.3	60.9 - 63.7	66.7 - 68.0	67.5 - 69.3	65.3 - 68.0	63.7 - 65.5	66.7 - 68.7	61.8 - 65.0	66.7 - 68.7	60.5 - 63.2	66.0 - 68.0
	Co	Colobus	63.9 - 64.1	65.3	63.9	63.3 - 65.3	63.7 - 64.6	68.7	72.2 - 72.7	68,0	66.1 - 66.4	66.7	63.4 - 66.1	67.4 - 68.0	62.5 - 62.8	66.7

[a] Numbers refer to values obtained in comparison with the 447-bp fragment of the conserved *DNA polymerase* gene that is available for all viruses.

[b] Sequences identified from specimens from the same primate species showing 100% nucleotide identity *e.g.* SbolCMV1, AnanCMV1, AmacCMV1 and AsenCMV1 are not included.

[c] Abbrevations, Cd, Cebidae; Cn, Cebinae; Sn, Saimiriinae; An: Aotinae; Pd, Pitheciidae; Pn, Pitheciinae; Ad, Atelidae; Al, Alouattinae; At, Atelinae; Hd, Hominidae; Hn, Homininae; Po, Pongidae; Ce, Cercopithecidae; Cr; Cercopithecinae; Co, Colobinae.

[d] Nucleotide and amino acid identities of viruses of Cebus rely on the sequences generated in this study as well as on sequences of CebHV1 [JQ264772] and CebusHV [AF292067].

[e] Nucleotide and amino acid identities of viruses of Saimiri rely on the sequences generated in this study as well as on sequences of SaHV4 [FJ483967] and SscHV [AF292065].

[f] Nucleotide and amino acid identities of viruses of Aotus rely on the sequences generated in this study as well as on the sequence of AoHV1 [FJ483970].

[g] Hominidae and Cercopithecidae viral sequences used to calculate nucleotide and amino acid identities correspond to those shown in Figure 3. Their GenBank accession numbers and associated publications are all reported in the figure and its legend.

TABLE 4. Estimates of Platyrrhini divergence time from CMV *DNA polymerase* sequence data and comparison with other estimates

Node	This study	Perelman et al. (3)	Jameson Kiesling et al. (15)
Catarrhini / Platyrrhini	32.45[a] (17.76 - 52.33)[b]	43.47 (38.55 - 48.36)	37.72 (36.04 - 42.07)
Pitheciidae / Atelidae+Cebidae	22.33 (16.25 - 28.21)	24.82 (20.55 - 29.25)	25.51 (25.14 - 26.36)
Atelidae / Cebidae	17.16 (10.29 - 24.25)	22.76 (18.14 - 27.08)	24.04 (22.6 - 25.29)
Atelinae / Alouattinae	8.29 (2.57 - 14.82)	16.13 (10.52 - 21.35)	15.29 (13.29 - 17.99)
Within *Alouatta*	3.11 (0.47 - 6.82)	ND	5.14 (3.65 - 6.8)
Within Cebidae	11.54 (5.82 - 17.89)	19.95 (15.66 - 24.03)	20.86 (18.48 - 22.86)
Within *Aotus*	3.98 (0.8 - 8.25)	5.54 (3.20 - 7.85)	4.39 (3.12 - 5.75)
Within *Cebus*	3.26 (0.59 - 6.94)	6.00 (3.13 - 9.35)	5.19 (3.69 - 6.78)
Within *Saimiri*	2.68 (0.28 - 6.14)	2.24 (1.05 - 3.73)	0.97 (0.51 - 1.45)

[a] times in units of millions of years

[b] values given in the parentheses indicate 95% HPD in millions of years.