Cimodo virus belongs to a novel lineage of reoviruses isolated from African mosquitoes

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A novel reovirus, designated Cimodo virus (CMDV), was isolated from mosquitoes collected in a rainforest region in Côte d’Ivoire. The entire genome comprised 24 835 bp divided into 12 segments ranging from 585 to 4080 bp. The icosahedral non-enveloped virions were 80 nm in diameter. Eight major viral proteins of about 150, 135, 120, 80, 66, 59, 42 and 30 kDa were identified and seven proteins were mapped to the corresponding genome segments by liquid chromatography mass spectrometry. Predicted protein genes diverged by > 77% encoded amino acids from their closest reovirus relatives. The deep phylogenetic branching suggests that CMDV defines an as-yet-unidentified genus within the subfamily Spinareovirinae.

The family Reoviridae comprises two subfamilies that are distinguished by their turreted (Spinareovirinae) or smooth (Sedoreovirinae) virions (Attoui et al., 2012). Members of the subfamily Spinareovirinae contain usually two layers of capsid proteins. Virions in the Sedoreovirinae subfamily have a three-layered core. The non-enveloped virions are 60–80 nm in diameter and contain 9–12 segments of linear dsRNA depending on the genus. The family is divided into 15 genera and viruses have been isolated from a wide variety of vertebrate and invertebrate hosts, fungi and plants (Attoui et al., 2012). Distantly related reoviruses tentatively defining novel species or genera have recently been isolated from non-blood-feeding insects (Chen et al., 2011; Deng et al., 2012; Huang et al., 2012; Quito-Avila et al., 2011).

During studies on mosquito-associated viruses in and around the Tai National Park, Côte d’Ivoire, unknown viruses were isolated in C6/36 cells that were inoculated with pools of homogenized mosquitoes (Junglen et al., 2009b). Five of those infected C6/36 cell cultures (C70/CI/2004, C75/CI/2004, C76/CI/2004, C89/CI/2004, C92/CI/2004) (Fig. 1a) and were further investigated during this study. Supernatants of the five cultures were purified by gradient ultracentrifugation and icosahedral, non-enveloped particles of 80 nm in diameter with morphology similar to reoviruses were identified by electron microscopy in all cultures (Fig. 1b). RNA was extracted from putative virions purified by gradient ultracentrifugation on a continuous sucrose gradient and separated by PAGE, confirming the presence of 12 genome segments (Fig. 1c). According to the phylogenetic relationship (see below), the genome segments were assumed to consist of dsRNA, like other reoviruses.

Viral stocks were generated in C6/36 cells and the fourth passage of isolates C74/CI/2004 and C95/CI/2004 were completely sequenced by adaptor-based random amplification methods or by deep sequencing on the 454 Junior platform as described previously (Junglen et al., 2009a; Marklewitz et al., 2013; Stang et al., 2005). The 5’ and 3’ genome termini were only determined for isolate C74/CI/2004 using 5’ RACE kits (Invitrogen and Roche). The sequences were deposited in GenBank under the accession numbers KF880748–KF880771. The length of the entire genome was 24 835 bp divided into 12 segments (S1–S12) ranging from 585 to 4080 bp (Table 1). Nucleotide and amino acid pairwise sequence identities for the ORFs of all segments ranged between 86.2–98.9% and 98.6–100%, respectively, suggesting that both isolates are strains of the same species, tentatively named Cimodo virus (CMDV, after Côte d’Ivoire, mosquito and dodeca).

All genome segments shared the conserved terminal nucleotides 5’-GAUAAA and UGAUC-3’ (Table 1). The 5’ terminus was identical to that found in genome segments of rice ragged stunt virus (RRSV) (genus Oryzavirus) (Yan et al., 1992) and piscine orthoreovirus (unclassified) (Markussen et al., 2013). The 3’ terminus was identical to that of aquatic and orthoreoviruses (Attoui et al., 2012; Markussen et al., 2013). All segments were monocistronic except S11 which contained two putative ORFs. Analyses of nucleotide sequences using BLASTN or BLASTX yielded no similarity to any other viral or cellular proteins on the GenBank database. Subsequent searches of translated sequences were deposited in GenBank under the accession numbers KF880748–KF880788. The ORFs of the other isolates of CMDV are KF880748–KF880771 and for the RdRp gene sequences of the other isolates of CMDV are KF880772–KF880788.

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The 3' terminus was identical to that of aqua- and piscine orthoreovirus (unclassified) (Markussen et al., 2013). The 3’ terminus was identical to that of aquatic and orthoreoviruses (Attoui et al., 2012; Markussen et al., 2013). All segments were monocistronic except S11 which contained two putative ORFs. Analyses of nucleotide sequences using BLASTN or BLASTX yielded no similarity to any other viral or cellular proteins on the GenBank database. Subsequent searches of translated sequences were deposited in GenBank under the accession numbers KF880748–KF880788. The ORFs of the other isolates of CMDV are KF880748–KF880771 and for the RdRp gene sequences of the other isolates of CMDV are KF880772–KF880788.

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Fig. 1. Cimodo virus (CMDV) morphology, growth and genome characteristics. (a) Cytopathic effect of C6/36 cells 5 days after infection with CMDV-C70/CI/2004. Uninfected C6/36 cells are shown in the smaller box. (b) Negative staining electron microscopy of purified CMDV-C70/CI/2004 particles. Bar, 100 nm. (c) RNA extracted from purified virions of CMDV-C74/CI/2004 was used. M, Molecular marker. (d) SDS-PAGE analysis of the protein content of purified virions of CMDV-C74/CI/2004. Proteins were stained with Coomassie blue R-250 and identified by LC-MS. In lane 2 a 1 : 3 dilution of the protein amount of lane 1 was used. M, Molecular marker. (e) C6/36 cells were infected with CMDV-C89/CI/2004 and CMDV-C92/CI/2004, respectively, at an m.o.i. of 0.001 and numbers of genome copies per millilitre were measured by specific real-time RT-PCR for 9 days.

ORFs using BLASTp and PSI-BLAST yielded low (<28%) levels of identity with reoviral proteins for the predicted proteins VP1, VP2, VP3, VP6 and VP12 (Table 1).

The protein putatively encoded by S1 (VP1 protein) contained the conserved RNA dependent RNA polymerase (RdRp) motifs between amino acids 551-DRD and KMLK (Nakashima et al., 1996; Poch et al., 1989, 1990). A significant rate of amino acid identity (256/1222 aa, 23%; BLAST e-value 7 x 10^-41) was found to the RdRp of Operophtera brumata reovirus (OpbuRV), an unclassified reovirus with 10 segments that is proposed to belong to the genus Idnorovirus (Attoui et al., 2012; Graham et al., 2008). For the putative protein encoded on S2 (VP2), low but significant identity (55/271 aa, 20%, e-value 0.013) to an uncharacterized protein (VP2) of OpbuRV was detected. CMDV VP3 showed some identity to VP4 of Fiji disease virus, genus Fijivirus (53/195 aa, 27%, e-value 1 x 10^-2) and to VP4 of Heliothis armigera cypovirus 5, genus Cypovirus (135/568 aa, 24%, e-value 2 x 10^-6) (Li et al., 2006), both uncharacterized proteins. Nucleoside triphosphate (NTP)-binding motifs conserved within members of the subfamily Spinareovirinae (Nibert & Kim, 2004; Spear et al., 2012) were identified for CMDV VP6 between S1 and S11. CMDV VP6 showed some identity to the NTP-binding protein P-S8 of Mal de Rio Cuarto virus (MRCV), genus Fijivirus (44/128 aa, 28%, e-value 0.028) and to VP6 of Aedes pseudoscutellaris reovirus (APRV), genus Dinovernavirus (50/201 aa, 25%, e-value 0.038). For CMDV VP12, a less significant rate of identity (11/33 aa, 33%, e-value 4.7) to the nucleic-acid-binding protein P6 of RRSV, genus Oryzavirus, a movement and RNA silencing suppressor protein (Shao et al., 2004; Wu et al., 2010a, b), was only identified using a database restricted to reoviruses. No significant similarities to other viral or cellular proteins were identified for the predicted proteins VP4, VP5, VP7, VP8, VP9, VP10 and VP11. All generated pools of homogenized mosquitoes (Junglen et al., 2009b) were measured for virus replication by specific real-time reverse transcription (RT)-PCR. CMDV was isolated from 19 pools of either Aedes, Anopholes, Culex or unclassified mosquito species in C6/36 cells. A 426 bp fragment of the RdRp gene of all isolates was analysed showing that the isolates formed two major subgroups with pairwise nucleotide identities >86.6% (Genbank accession numbers KF880772–KF880788). Phylogenetic analyses of CMDV and representative members of the family Reoviridae based on the RdRp genes showed that CMDV clustered within the subfamily Spinareovirinae and branched from a deep node in basal relationship to coltiviruses and mycoreoviruses (Fig. 2). This phylogenetic relationship was verified by phylogenetic analyses including only CMDV, coltiviruses,
### Table 1. Genome organization of CMDV

<table>
<thead>
<tr>
<th>S</th>
<th>Size (bp)</th>
<th>5’-NCR (bp)</th>
<th>3’-NCR (bp)</th>
<th>Conserved terminal nucleotides</th>
<th>ORF (kDa)</th>
<th>Protein</th>
<th>Amino acid identities (%; BLAST e-value) to other reovirus proteins/conserved motifs</th>
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<tr>
<td>1</td>
<td>4080</td>
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<td>34</td>
<td>5'-GAUAAAUC...UAAUGAUC-3'</td>
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<td>256/1222 (23 %; 7 × 10^-41) RdRp of OpbuRV/RdRp motifs 553IDR–KML288 (Nakashima et al., 1996; Poch et al., 1989, 1990)</td>
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<td>2</td>
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<td>43</td>
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<td>55/271 (20 %; 0.013) VP2 (uncharacterized protein) of OpbuRV</td>
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<td>1055</td>
<td>121</td>
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<td>66</td>
<td>44/128 (28 %; 0.028) NTP-binding protein P-S8 of MRCV; 50/201 (25 %; 0.038) VP6 of APRV/NTP motifs 371VVA–KWI405 (Nibert &amp; Kim, 2004; Spear et al., 2012)</td>
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<td>96</td>
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<td>16</td>
<td>11/33 (33 %; 4.7) P6 of RRSV, a nucleic-acid-binding, movement and RNA silencing suppressor protein (Shao et al., 2004; Wu et al., 2010a, b)*</td>
</tr>
</tbody>
</table>


*, Database restricted to reoviruses.
mycoreoviruses and idnoreoviruses. The phylogenetic positioning corresponded well with the low protein sequence similarity observed for CMDV.

To identify the major proteins present in mature CMDV particles, virions were purified by gradient ultracentrifugation and viral proteins were separated by SDS-PAGE as described previously (Marklewitz et al., 2013; Zirkel et al., 2013). Eight proteins of 150, 135, 120, 80, 66, 59, 42 and 30 kDa were detected and seven proteins (VP1–4, VP6–8) were mapped to the corresponding genome segments by liquid chromatography mass spectrometry (LC-MS) (Fig. 1d).

Virus growth was studied on insect cells using isolates from each subgroup. Both isolates induced cytopathic effects 3–4 days p.i. Maximum amounts of genome copies were reached 7 days p.i. on C6/36 cells (Fig. 1e). TCID<sub>50</sub> titration of cell culture supernatants yielded virus titres of up to 8 × 10<sup>6</sup> ml<sup>–1</sup>. Twelve different vertebrate cell lines from monkeys (Vero E6), rodents [BHK-21, MEF-MDA5<sup>−/−</sup>, Crocsu-Lu (Corman et al., 2014)], humans (Huh-7), swine (PSEK), bats (RoNis/7, RhiLu/1.1; Hoffmann et al., 2013), goats (ZN-R), frogs (ICR-2A) and snakes (VH-2) were inoculated with an m.o.i. of 1 (based on TCID<sub>50</sub> titration on C6/36 cells) and cultivated for 21 days including two passages of infectious cell culture supernatant on fresh cells (Marklewitz et al., 2013; Zirkel et al., 2013). No replication was detected by specific real-time RT-PCR in any of the tested vertebrate cell lines.

In summary, we have identified and characterized a new reovirus that differs from all previously known reoviruses. According to the International Committee on Taxonomy of Viruses genus demarcation criteria, viruses from different genera share <26% amino acid identity within their RdRp genes (Attoui et al., 2012) suggesting that CMDV defines a novel genus. Additionally, with regard to other genetic features, similarity to reoviruses of seven different genera was identified, for example genome termini (5’ genome termini similar to oryzaviruses, 3’ termini similar to aqua- and orthoreoviruses), encoded genes (highest identity to idnoreoviruses and fijiviruses) and number of genome segments (similar to myco- and coltiviruses). Based on the phylogenetic relationship of the RdRp gene, CMDV was most closely related to myco- and coltiviruses. Further phylogenetic analyses based on other segments cannot be investigated as reoviruses encode homologous proteins on different segments (Attoui et al., 2012) and no significant similarity for those segments was identified. The in vitro infection experiments suggest that CMDV may be restricted to growth in insect cells. The related coltiviruses infect different mammals and are transmitted predominantly by ticks. Colorado tick fever virus grows well in mammalian cells, also in those tested in this study (Attoui et al., 2005). However, Eyach virus does not replicate in mammalian cells and can only be grown in suckling mice (Charrel et al., 2004; Chastel et al., 1984). CMDV is clearly separated from mycoreoviruses by host restriction as mycoreoviruses only infect fungi (Hillman & Suzuki, 2004; Suzuki et al., 2004). Taken together, CMDV putatively defines a novel genus within the subfamily of Spinareovirinae.

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References


