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ANNOTATED SEQUENCE RECORD

Nanopore sequencing of a novel bipartite New World begomovirus infecting cowpea

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Abstract

A new bipartite begomovirus (family *Geminiviridae*) was detected on cowpea (*Vigna unguiculata*) plants exhibiting bright golden mosaic symptoms on leaves under field conditions in Brazil. Complete consensus sequences of DNA-A and DNA-B components of an isolate of the virus (PE–088) were obtained by nanopore sequencing and confirmed by Sanger sequencing. The genome components presented the typical genomic organization of New World (NW) begomoviruses. Pairwise sequence comparisons revealed low levels of identity with other begomovirus species previously reported infecting cowpea around the world. Phylogenetic analysis using complete sequences of DNA-A components revealed that the closest relatives of PE–088 (85-87% nucleotide sequence identities) were three legume-infecting begomoviruses from Brazil: bean golden mosaic virus, macroptilium common mosaic virus and macroptilium yellow vein virus. According to the current classification criteria, PE–088 represents a new species in the genus *Begomovirus*, tentatively named as cowpea bright yellow mosaic virus (CoBYMV).

Whitefly-transmitted geminiviruses (genus *Begomovirus*, family *Geminiviridae*) are of great economic relevance in many tropical and subtropical regions [1, 2]. In terms of legumes and pulses, begomovirus diseases cause more often economic problems in common bean (*Phaseolus vulgaris*) in the New World (NW) and in mung bean (*Vigna radiata*) in the Old World (OW). However, to date, begomovirus-induced diseases have not been a major problem in cowpea (*Vigna unguiculata*) crops in the OW.

Although at least five begomovirus species have been characterized to various degrees in association with cowpea (cowpea golden mosaic virus, CGMV; mungbean yellow mosaic virus, MYMV; mungbean yellow mosaic India virus, MYMIV; horsegram yellow mosaic virus, HgYMV and dolichos yellow mosaic virus, DoYMV), there is a need for further studies on the potential importance of begomovirus diseases in this crop and to better understand their diversity across distinct geographic areas. The need for further characterization is evidenced by the fact that the same name – *Cowpea golden mosaic virus* (CGMV) has been given to unrelated viruses from India (GenBank AY618902), Nigeria (AF029217), and Brazil (AF188708). Actually, the isolate from India is mungbean yellow mosaic India virus (MYMIV), based on having 98% sequence identity to isolates of this virus.

Cowpea is the most important legume crop in the Brazilian semi-arid region [3]. Cowpea yields in Brazil can be severely affected by a "golden leaf mosaic disease" associated with a begomovirus that has not been fully characterized. Here, we describe a novel bipartite begomovirus infecting cowpea in Brazil.

Cowpea leaf samples without and with conspicuous begomovirus-like symptoms (bright yellow/golden mosaic) were collected in commercial fields in Petrolina in Pernambuco State, Brazil (Latitude: 09º 23' 55''S/Longitude: 40º 30'03'' W and 376 meters above sea level) in 2011. The disease

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prevalence in this area was about 30%. Total DNA was extracted from single representative leaf samples (1 g each) using 2X CTAB buffer followed by rolling circle amplification (RCA) [4]. These DNA samples were used as templates in PCR assays with the degenerate begomovirus primer pairs designed to direct the amplification of genomic segments of the DNA-A and DNA-B components [5]. Amplicons of expected-size of DNA fragments of 1.1 kb (DNA-A) and 0.5 kb (DNA-B) were observed in agarose gel electrophoresis only in samples exhibiting bright yellow (golden) mosaic symptoms (Fig. 1).

The complete genome of the PE–088 isolate was obtained by nanopore sequencing with the Ligation Sequencing Kit 1D (Oxford Nanopore Technologies) following manufacturer's instructions. Briefly, the RCA product was mixed with the NEBNext Ultra II End Repair/dA-Tailing Module (New England Biolabs) and incubated for 5 min at 20 °C for end-repair reaction, and then 5 min at 65 °C for dA-tailing reaction, followed by purification with AMPure XP beads (Beckman Coulter) according to manufacturer's instructions. Blunt/TA Ligase Master Mix (New England Biolabs) was used to ligate native barcode adapters (NB01) to end prepared DNA for 10 min at room temperature, and then purified using AMPure XP beads. The barcoded samples were pooled and the adapter ligation and tethering were then carried out with Adapter Mix (Oxford Nanopore Technologies) and NEB Blunt/TA ligation Master Mix (New England Biolabs). The adapter-ligated DNA library was then purified with AMPure beads, followed by the addition of Adapter Bead binding buffer (Oxford Nanopore Technologies), and finally eluted in Elution Buffer. Each R9 flow cell was primed with 1000 µL of a mixture containing Running Buffer with Fuel (RBF) and nuclease-free water. Then 12 µL of the library was diluted in 35 µL RBF, 25.5 µL of Library Loading Beads, and 2.5 µL nuclease-free water and loaded onto a Spot-on flowcell (FLO-MIN106) (Oxford Nanopore Technologies) on a MinION Mk1B (Oxford Nanopore Technologies). The raw FAST5 reads were uploaded to the online server for base calling through the Metrichor EPI2ME platform. The basecalled MinION reads were converted to FASTQ and assembled with Canu v1.7 [6]. The resulting assembly was polished using Nanopolish v0.9.0 [7], to improve the accuracy of the consensus sequences. The resulting contigs were compared to the viral protein RefSeq database with Blastx [8] implemented in Geneious version 9.1.2 [9]. The assembled contigs of the putative complete DNA-A and DNA-B components (corresponding to an isolate of a putative new species present in our cowpea samples) were manually annotated and deposited in GenBank (accessions MH469731 and MH469732). The complete DNA-A and DNA-B sequences was confirmed by PCR and Sanger sequencing using primers described in Supplementary Table 1. MinION derived consensus sequences showed 100% nucleotide identity with the corresponding Sanger sequences, except for one nucleotide in a T-rich region of DNA B.

The PE–088 DNA-A component is 2,632 nt and exhibited all major characteristics of NW begomoviruses, including the virion-sense gene (AV1) and the five complementary sense (AC1, AC2, AC3, AC4, and AC5) genes (Fig. S1). The DNA-B genome is 2,593 nt and harbored genes homologous to BV1 and BC1 (Fig. S1). The common region (CR) was identified by comparing the DNA-A and DNA-B sequences and it was 241 nt in size. The CR possessed its functional elements, including the conserved hairpin containing stem-loop, the conserved TAATATTAC nonanucleotide as well as repeated sequences ('iterons") (Fig. S1). Interestingly, the common region includes 93 nt of Rep coding region (Fig. S1), which in DNA-B forms a small ORF (31 aa) encoding a Rep-like peptide with conserved motif I of Rep proteins (Fig. S1). Short Rep homologous sequences (sRepHS) were described in some begomoviruses and could be involved in the posttranscriptional regulation of the cognate viral Rep gene [10].

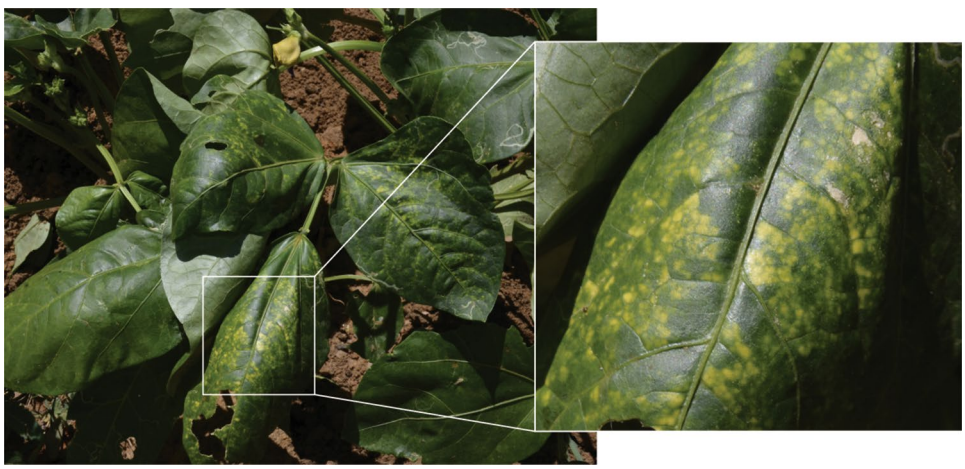

Fig. 1 Symptoms of bright yellow mosaic in cowpea (*Vigna unguiculata*) plants collected in a commercial field in Petrolina-PE, Brazil

The BLASTn analyses of the complete DNA-A sequence revealed 86% nt identity of the isolate PE–088 with macroptilium common mosaic virus (MacCMV) from North-East Brazil. Moreover, a partial genomic sequence (1,365 bp encompassing the Rep associated protein and CP genes) of a cowpea-infecting begomovirus isolate from Brazil (AF188708), which was tentatively named CGMV, was 97% identical with that of PE–088 DNA-A. Furthermore, these sequences of cowpea-infecting begomovirus from Brazil were genetically distinct (57% nt sequence identity) from two other cowpea-infecting begomoviruses from the OW: CGMV (AF029217) and MYMIV (AY618902). Overall, these results suggest that both Brazilian isolates (PE-088 and AF188708) represent variants of a novel viral species, which is distinct from CGMV and MYMIV.

The complete sequence of the PE–088 DNA-A component was aligned to other related sequences available in GenBank with MAFFT algorithm [11] implemented in Geneious 9.1.2 [9]. A maximum likelihood phylogenetic tree was inferred with FastTree [12] and a pairwise nucleotide comparison was performed using SDT [13]. The pairwise comparison heatmap were plotted with Evolview v2 [14]. As shown in Fig 2, PE–088 grouped together with legume-infecting begomoviruses from Brazil: MacCMV and bean golden mosaic virus (BGMV) with nucleotide sequence identities ranging from 85 to 87% (Figure S2). Bipartite tomato-infecting begomoviruses from the NW formed a distinct group with a maximum identity of 81% with PE–088 (Figure S2).

DNA-A genome pairwise identities of 91% and 94% are currently accepted as the demarcation thresholds for distinct species and strains, respectively for members of the genus *Begomovirus* [1]. Therefore, PE–088 is a new bipartite species for which the name *Cowpea bright yellow mosaic virus* (CoBYMV) is proposed. More nationwide surveys are needed in order to establish CoBYMV as a major economic problem for the cowpea crop in Brazil as well as to guide resistance breeding programs. For that will be necessary to fulfill Koch's postulates with the development of infectious clones and an agroinoculation method for screening cowpea germplasm collection in search for natural sources of genetic resistance to CoBYMV. Our work also demonstrated that a portable nanopore sequencing device is a rapid and accurate alternative tool for the characterization of plant viral genomes, as recently published [15].

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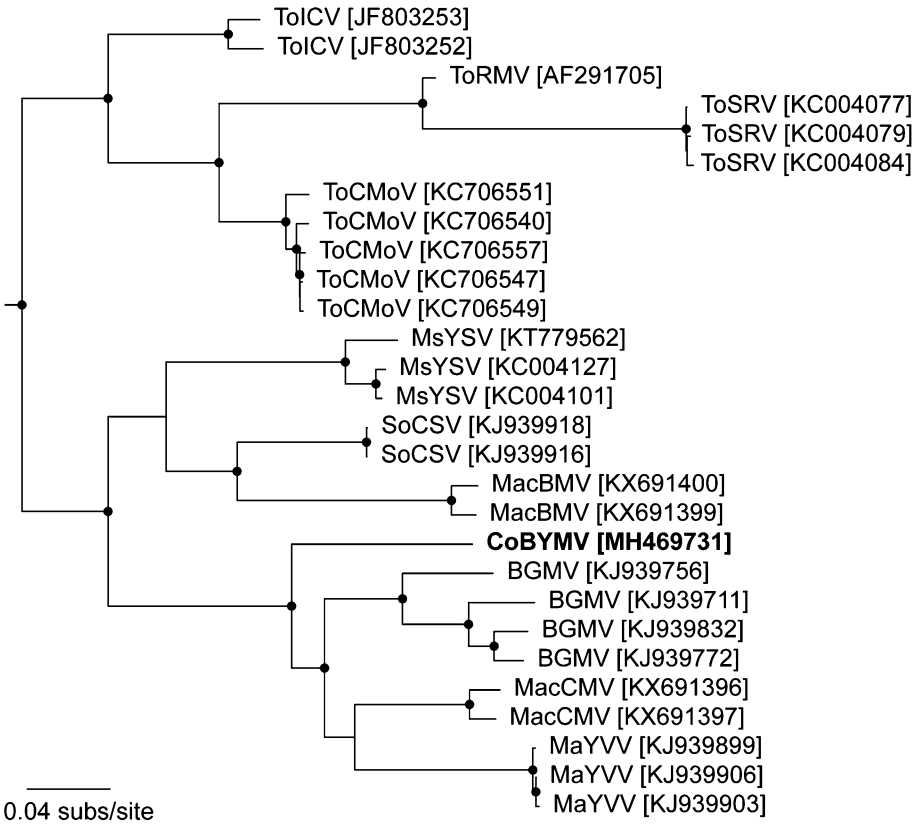

Fig. 2 Maximum-likelihood tree of cowpea bright yellow mosaic virus (CoBYMV, in bold) and related begomoviruses, inferred using complete DNA-A component sequences. The GenBank accession number for each sequence is given in bracket next to the abbreviated virus name. ToICV, tomato interveinal chlorosis virus; ToRMV, tomato rugose mosaic virus; ToSRV, tomato severe rugose virus; ToCMoV, tomato chlorotic mottle virus; MsYMSV, macroptilium yellow spot virus; SoCSV, soybean chlorotic spot virus; MacBMV, macroptilium bright mosaic virus; BGMV, bean golden mosaic virus; MacCMV, macroptilium common mosaic virus; MaYVV, macroptilium yellow vein virus. The tree is midpoint rooted for clarity. The branch support was assessed by a Shimodaira-Hasegawa-like test and values > 90 are indicated by black dots

Compliance with ethical standards

Conflict of interest The authors declare that no conflict of interest exists.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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