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A new bunya-like virus associated with mass mortality of white-clawed crayfish in the wild

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ABSTRACT

We report the discovery of a new enveloped, spherical virus belonging to the Phenuiviridae family of negative ssRNA viruses associated with a massive outbreak in a French population of the endangered white-clawed crayfish *Austropotamobius pallipes*. We call this virus Bunya-like Brown Spot Virus (BBSV) and characterize it using transmission electronic microscopy, genome sequencing and clinical signs. Infected specimens show discolored brown spots on the cuticle. Using RNA-seq data we assembled a partial sequence for the L, M and S genome segments of BBSV. Phylogenetic analyses using all three segments show this virus is closely related to the Wenling crustacean virus 7 (China) and to a bunya-like virus found in feces of the European otter. Our survey of the mass mortality event indicates the virus is less virulent than the crayfish plague caused by *Aphanomyces astaci*. Overall, the discovery of BBSV provides a new important asset to monitor *A. pallipes* populations.

1. Introduction

The white-clawed crayfish *Austropotamobius pallipes* Lereboullet, 1858, is a species particularly sensitive to water and habitat quality, native to Western Europe (Souty-Grosset et al., 2006; Trouilhe et al. 2007, 2012; Grandjean et al., 2011; Kouba et al., 2014). Over the last decades, important population declines have been recorded, with a recent 50–80% collapse over the species' global range (Bramard et al., 2006; Souty-Grosset et al., 2006; Füreder et al., 2010). As a result, *A. pallipes* has been included in the IUCN Red List of endangered species in 2010 (Füreder et al., 2010) and listed as a species requiring special conservation measures in the European Union under the Species and Habitats Directive, Annex 2. Among the various factors responsible for its regression, the crayfish plague, a disease caused by the parasite *Aphanomyces astaci* (Oomycota), is considered to be the main threat. This disease has been introduced in European freshwater ecosystems by its natural hosts, North American freshwater crayfish species which are acting as safe carriers (Bohman et al., 2006). For example, in a screening of the invasive signal crayfish populations, *Pacifastacus leniusculus* in France, Filipova et al. (2013) and Grandjean et al. (2017) reported that the pathogen infects more than 50% of the populations, with in some cases an infection rate within populations reaching up to 80%. Then, it is not surprising that this pathogen has been found to be involved in a large series of outbreaks throughout Europe and is now considered as one of the major threat for the preservation of all European crayfish species (Souty-Grosset et al., 2006; Kozubikova et al., 2011; Grandjean et al., 2014; Maguire et al., 2016; Kaldre et al., 2017). Recently, this disease has decimated entirely one of the widest French population of white-clawed crayfish in East of France, which was estimated at 150,000 individuals spread over 12 km of the river (Collas et al., 2016). Due to its devastating impact, *A. astaci* has been included among the 100 worst invasive alien species (Lowe et al., 2000).

In this paper, we identify the likely cause of a mass mortality event in a population of *A. pallipes* showing symptoms characteristic of the crayfish plague, but in which *A. astaci* could not be identified using well-established qPCR tests. Instead, using an approach combining

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electron microscopy, RNA-seq, PCR screenings and phylogenetic analyses, we show that the mortality event was most likely due to a new negative single-stranded RNA virus belonging to the Phenuiviridae family (Order Bunyavirales). We report the nearly complete genome sequence of the virus and develop a rapid and reliable PCR test allowing accurate diagnosis of crayfish infections for future epidemiological screening of natural populations. This new virus, which we call Bunya-like Brown Spot Virus (BBSV), is the first disease-causing RNA virus reported in the endangered *A. pallipes*.

2. Materials and methods

2.1. Samples

A total of 14 live individuals of the white-clawed crayfish *A. pallipes* were sampled at night by hand in 2017 (n = 4) and 2018 (n = 10) on the Bonneille brook (Department of Doubs, Eastern France), where the mass mortality event was observed. All these specimens were transported alive in a cold box to the laboratory where they were placed individually in aquariums of 20 L each. In addition to these live individuals, 20 additional dead individuals were sampled in the Bonneille mortality front in 2017 (n = 9) and in 2018 (n = 11). These dead individuals were stored in 96% ethanol. Another 8 specimens per site were collected in two localities (Navarre and Rieumançon, department of Dordogne, West of France), where no sign of mortality were observed. We sampled 300 µl of hemolymph from these 16 specimens before releasing them back into the water. Hemolymph was homogenized in 300 µl of RNA shield (Zymo Research, Irvine, CA).

2.2. Diagnosis of plague

The cuticle of live specimens sampled in the Bonneille was examined carefully for the presence of distinctive clinical signs such as discolored brown spots or shell lesions. All live individuals from the Bonneille were then tested for the presence of the crayfish plague (*A. astaci*) according to the protocol developed by Vralstad et al. (2009). We used DNeasy blood and tissue kit (Qiagen) to isolate DNA from the cuticle samples immersed in 360 µl of Buffer ATL and crushed by stainless steel beads (1.6 mm diameter) in a BBX24B Bullet Blender (Next Advance) for 10 min. DNA extractions were done using the DNeasy blood and tissue kit but the volume of the reagents was doubled. The presence of *A. astaci* was tested by the TaqMan MGB real-time PCR assay of Vralstad et al. (2009) in 25 µl reaction volumes, using a LightCycler 480 Instrument (Roche).

2.3. Sample preparation for RNA-Seq

We performed RNA-seq on the 4 live specimens collected in 2017 in Bonneille, 2 of which were caught in the mortality front while the 2 others were trapped 800 m upstream of the front. 600 µl of hemolymph were sampled for each individual and homogenized in 600 µl of RNA shield (Zymo Research, Irvine, CA). Total RNA extraction was performed on the homogenate using Quick-RNA MicroPrep (Zymo Research, Irvine, CA) and stored at −80 °C. The RNA-seq was not initially performed to detect potential infectious agents (including viruses) responsible for the mass mortality. mRNA enrichment and library preparation were done using NEBNext Poly(A) mRNA Magnetic Isolation Module and NEBNext Ultra RNA Library Prep Kit for Illumina. The concentration of the libraries was measured using Qubit 2.0 fluorometer and the fragment sizes of the libraries were determined using TapeStation 2200 (Agilent). Sequencing was performed on a MiSeq Illumina platform using a paired-end 2 × 75bp running configuration.

2.4. Bioinformatics analyses

The raw RNA-seq reads were quality checked using fastqc (Simon, 2010) and filtered and trimmed with trimmomatic with default parameters (Bolger et al., 2014). Remaining adaptors were removed using cutadapt (Martin, 2011). The resulting reads from the four individuals were pooled and assembled using the Trinity software, with default parameters (Grabherr et al., 2011). Transcriptome annotation was performed using blastx (Camacho et al., 2009) on the non-redundant (nr) protein database available in Genbank with an e-value cut-off of 0.0001. Transcripts that could not be annotated this way were used as queries to perform megablast searches (Morgulis et al., 2008) against the Genbank nucleotide (nt) database to detect possible rRNA, bacterial contaminants and other sequences. To identify possible viral transcripts, we inspected the annotation by keyword searching for virus hits, and by refining blast searches against custom virus databases. Viral transcripts were extracted from the assembly and were submitted to an additional assembly step using CAP3 (Huang and Madan, 1999) with default parameters. The order of the resulting contigs was inferred from blastx alignments on a closely related virus (Wenling crustacean virus 7 (Shi et al., 2016)) and contig extension was performed using SSPACE (Boetzer et al., 2011). To assess which of the four individuals were infected by the virus, we mapped the reads back to the viral contigs using bowtie2 (Langmead and Salzberg, 2012).

2.5. RNA extraction and cDNA template preparation for polymerase chain reaction

Total RNA was extracted from pieces of muscle tissue, green glands, nerve chain, hepatopancreas and gills for each of the 4 specimens collected in the Bonneille brook in 2017 using total RNA Purification Kit (NorgenBiotek®, CA) according to the manufacturer's instructions. Total RNA extraction was also performed from 300 µl of hemolymph for the 10 live specimens collected in 2018 in Bonneille and for the 16 specimens caught in Rieumançon and Navarre. In addition, we attempted to extract RNA from the 20 dead individuals stored in ethanol sampled in the mortality front in the Bonneille in 2017 and 2018. Ethanol-stored tissues were washed in sterile water to drain off the ethanol before RNA extraction. cDNA were produced using Tetro cDNA Synthesis Kit (Bioline, UK) following the manufacturer's protocol and used as a template for RT-PCR.

2.6. Virus detection by RT-PCR

We have designed specific primer pairs (forward primer: CTAATG TCTTATATTGAATGCCCCTATTAATC and, reverse primer AACTACTG ATCAAACTGTGTCTATAATGGAAT targeting the viral RNA polymerase gene to check for the presence of the virus using Primer3 (Untergasser et al., 2012). Amplicons were generated with Taq polymerase Master Mix in a 25 µl reaction volume with 100 nM of forward and reverse primers and 1 µl of cDNA template. The cycling conditions were as follows: initial denaturation at 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s, followed by one step of final extension at 72 °C for 5 min. PCR products (3 µl) were separated on a 1,5% agarose gel containing ethidium bromide in TAE buffer. Visualization was performed using a UV transilluminator and the images were captured with a Polaroid camera and video documentation system (Bioprofil-Vilber Lourmat, France). The expected length of the amplified fragment was 485 pb. The specificity of the PCR was confirmed by Sanger-sequencing PCR products. The cDNA samples from crayfish free of the virus identified by RNA-seq and from two other French populations (Rieumançon and Navarre) were used as negative controls and those infected by the virus as positive controls.

2.7. Transmission electron microscopy

Several tissues (nerve chain, hepatopancreas and muscle) from the 4 specimens used for RNA-seq were fixed for 2 h in ice-cold 2,5%

glutaraldehyde in 0.1 M sodium chloride, 0.1 M sodium cacodylate buffer pH 7.4. After washing with cacodylate buffer for 2 h, the samples were postfixed with 1.3% OsO_4 in 0.1 M cacodylate buffer for 1 h at room temperature. Nerve chains, hepatopancreas and muscle tissues were then dehydrated in a graded acetone series and finally embedded in Epon resin. Ultrathin sections (70 nm) were contrasted with 2% uranyl acetate and lead citrate and analyzed using a Jeol JEM 1010 electron microscope (EM). Additionally, nerve chains and muscle tissues from 2 specimens sampled in 2018 showing clinical signs of infection and positive for virus presence by PCR, have been also fixed and observed according to the same protocol. Mean size of the virus was estimated by measuring 20 viral particles in EM.

2.8. Phylogenetic analyses

In order to assess the phylogenetic position of the new RNA virus discovered in this study, we aligned the largest of the RdRP contig as well as the nucleocapsid and glycoprotein contigs with the corresponding proteins from a diversity of viruses belonging to the Bunyavirales order, including tick and sandfly-borne phleboviruses, phasiviruses, tenuiviruses, goukoviruses and a number of closely related unclassified viruses. Our sampling of bunya-like viruses to construct these alignments was inspired from (Xin et al., 2017; Matsuno et al., 2018; Harvey et al., 2019). Alignments at the amino-acid level and removal of ambiguous regions were performed by hand. Alignments devoid of ambiguous regions are provided in Supplementary Material 1–3. The amino acid evolutionary model best fitting each alignment (LG + I + G + F for all three alignments) was selected using the Akaike Information Criterion implemented in ProtTest 3 (Darriba et al., 2011). Maximum likelihood analyses were then performed using PhyML 3.1 (Guindon and Gascuel, 2003). Node support was evaluated with a bootstrap analysis (100 replicates).

3. Results

3.1. A mass mortality event in French Austropotamobius pallipes populations not due to the crayfish plague

The mass mortality event from which the *A. pallipes* individuals studied here were sampled was initially reported on the 24th of July 2017 in the Bonneille brook by French Agency of Biodiversity (AFB, unpublished). The Bonneille brook is a tributary of the Loue hydrographic basin in the French department of Doubs (Eastern France). The *A. pallipes* populations affected by the mortality event showed characteristics that are generally considered as diagnostic of plague outbreaks. These include the observation of moribund crayfish during the day around dead ones, and spread of the disease upstream from the initial outbreak. On the 27th of September 2017, two living individuals of *A. pallipes* were collected in the mortality front and two other individuals were sampled approximately 2 km upstream, in an area suspected to be free of the disease, where none of the signs associated to crayfish plague could be observed. An additional sampling of 10 individuals was performed on the 10th of October 2018 in the mortality front, which had moved approximately 1,5 km upstream since the initial report of the mortality event (September 27th, 2017). Surprisingly, our search for the presence of *A. astaci* in all 14 sampled individuals using the test developed by Vralstad et al. (2009) revealed no sign of the crayfish plague, with level of infection of A0.

3.2. A new virus identified in infected individuals using RNA-seq and transmission electron microscopy

While our test for the presence of the crayfish plague in *A. pallipes* sampled in a population affected by mass mortality were negative, the spread of the mortality upstream of the initial outbreak over two successive summers (2017–2018) was highly suggestive of an infectious cause. Careful examination of the two individuals sampled in 2017 upstream of the area affected by the disease revealed no distinctive clinical signs. Interestingly, the two other individuals caught in 2017 in the mortality front showed shell lesions on the abdomen and discolored brown spots on the cuticle, the diameter of which varied from 1 to about 20 mm (Fig. 1). Among the 10 crayfish collected in 2018 in the mortality front, 6 of them showed brown spots on the cuticle. No clinical sign was observed on the cuticle of 8 specimens sampled in two other locations unaffected by the disease (Rieumançon and Navarre populations) in 2018.

Fig. 1. Discolored brown spots on the cuticle of two males of *A. pallipes* from Bonneille population.

To identify infectious agents other than the crayfish plague that would be potentially responsible for the *A. pallipes* mass mortality event in Bonneille, we performed RNA-seq on hemolymph samples extracted from the four individuals caught in 2017. All statistics about the read counts obtained through the different steps of preprocessing are given in Supplementary Table 1. Our assembly yielded 1251 Trinity genes, with 1514 transcripts measuring 385 bp on average. Among the 1514 assembled transcripts, 15 transcripts had virus blastx hits (Supplementary Table 2). CAP3 extending procedures assembled them into 13 transcripts and SSPACE extending procedures further reduced this number to 7 scaffolds. Blastx searches on the Genbank non-redundant protein database revealed that these scaffolds were most similar to phlebovirus-like viruses, which are segmented negative ssRNA viruses belonging to the Bunyavirales order. Using PCR with primers designed on the extremities of the five fragments aligning on the L segment, we were able to close the gaps in this segment by assembling a 5939-bp long L Scaffold. This Scaffold covers 94% of the L segment encoding the RNA-dependent RNA-polymerase of the most closely related virus, the Wenling crustacean virus 7, an unclassified bunya-like virus discovered as part of a large-scale metagenomics study (Shi et al., 2016). Amino acid identity between the two sequences varies from 30

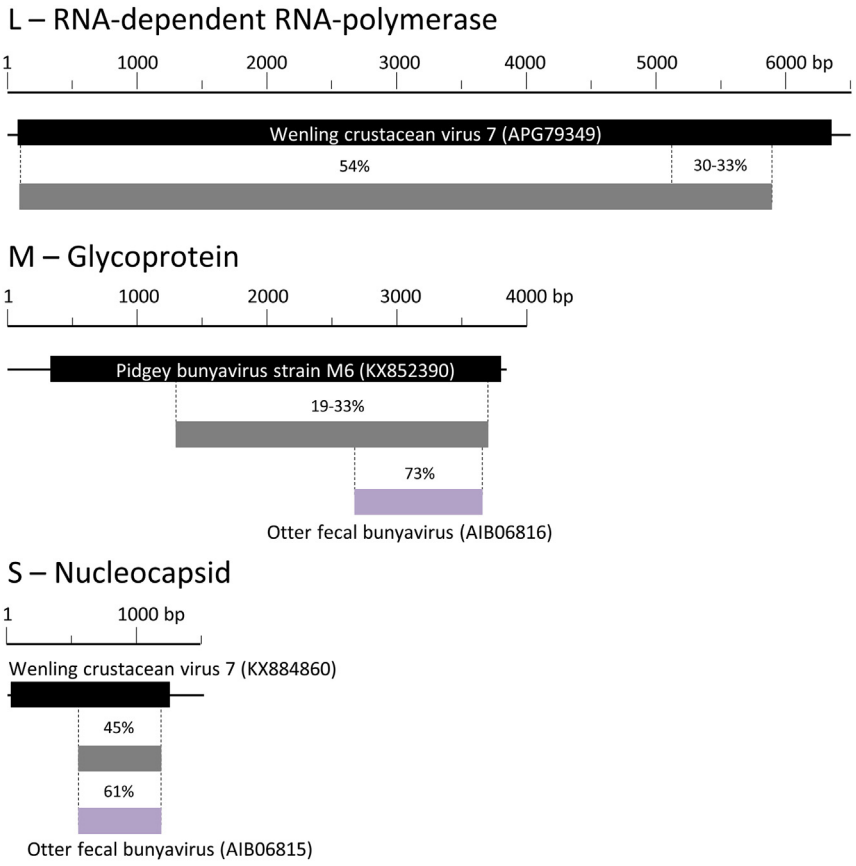

Fig. 2. Schematic alignment of the 3 partial BBSV segments with their closest complete viral gene retrieved using BLASTX on the non-redundant Genbank protein database. Percentage of identity are provided for each segment. For segments M and S, a schematic alignment is also shown with the partial sequences from the Otter fecal bunyavirus, which are more similar to BBSV than any available complete bunyaviral genome sequence.

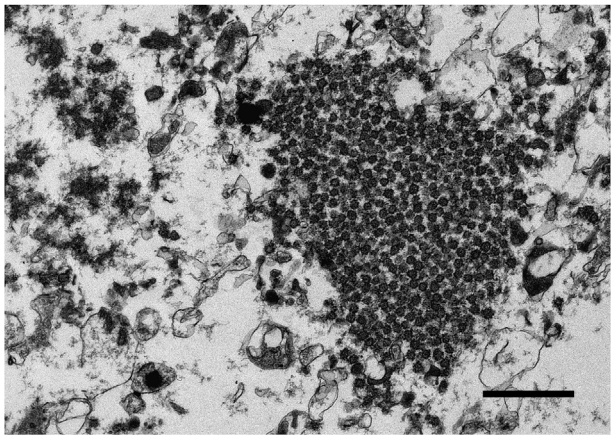

Fig. 3. Aggregates of BBSV particles from nerve chain observed in transmission electron microscopy. scale bar = 1 µm. Other pictures are provided in Supplementary Fig. 1.

to 54% along the L gene (Fig. 2). The Scaffold corresponding to the M segment of the viral genome (glycoprotein precursor) covers 66% of and is 19–33% identical to the M gene of the Pidgey bunyavirus strain M6. This virus was identified using a metagenomics approach performed on a moth (*Choristoneura rosaceana*) caught in Seattle (USA) (Makhsous et al., 2017). The M segment also shows 73% similarity to the partial M segment of a virus detected as part of a metagenomics study of otter feces (Bodewes et al., 2014). Finally, the Scaffold corresponding to the S segment of the viral genome (nucleocapsid) covers 52% of and is 45% similar to the Wenling crustacean virus 7. It is also 61% similar to the Otter fecal bunyavirus sequenced by Bodewes et al. (2014). It is noteworthy that the M segment of the Wenling crustacean virus 7 was not sequenced by Shi et al. (2016) and that the L segment of the Otter fecal bunyavirus was not sequenced by Bodewes et al. (2014), which explains why we do not report similarity between these sequences and the ones we are characterizing in this study. Individual mapping of the reads obtained from the four crayfish collected in Bonneille in 2017 revealed the presence of the virus only in the two individuals sampled on the mortality front that showed brown spots on their cuticle. The partial sequence of the three viral genome segments produced in this study have been deposited to Genbank under accession number (L Segment: MK881590, M Segment: MK881591, S Segment: MK881592).

Based on the blastx results indicating that the virus newly discovered in the white-clawed crayfish is most similar to phlebo-like viruses from the Bunyavirales order, we call it Bunya-like Brown Spot Virus (BBSV). Particles of viruses closely related to phleboviruses within the Bunyavirales order are typically spherical, with diameters generally ranging from 80 to 120 nm (Plyusnina et al., 2012; Adams et al., 2017). In agreement with this description, transmission electron microscopy (TEM) performed on muscle, nerve chain and hepatopancreas tissues from the two infected individuals collected in 2017 revealed aggregates of spherical particles, with diameters ranging from 72 nm to 97 nm (Fig. 3; Supplementary Figs. 1a and 1b). Such particles were not observed in the two other, non-infected specimens on which we performed TEM.

3.3. Phylogenetic position of the Bunya-like Brown Spot Virus

The phylogenetic analysis of the L segment places the BBSV discovered in the white-clawed crayfish as sister to the Wenling crustacean virus 7 (bootstrap = 100) (Fig. 4). Together with two other unclassified viruses discovered in Chinese invertebrates by Shi et al. (2016) through metagenomics, these viruses fall in an unresolved position within the Phenuiviridae family, which among others, also contains viruses belonging to the Phasivirus, Tenuivirus and Goukovirus genera (Adams

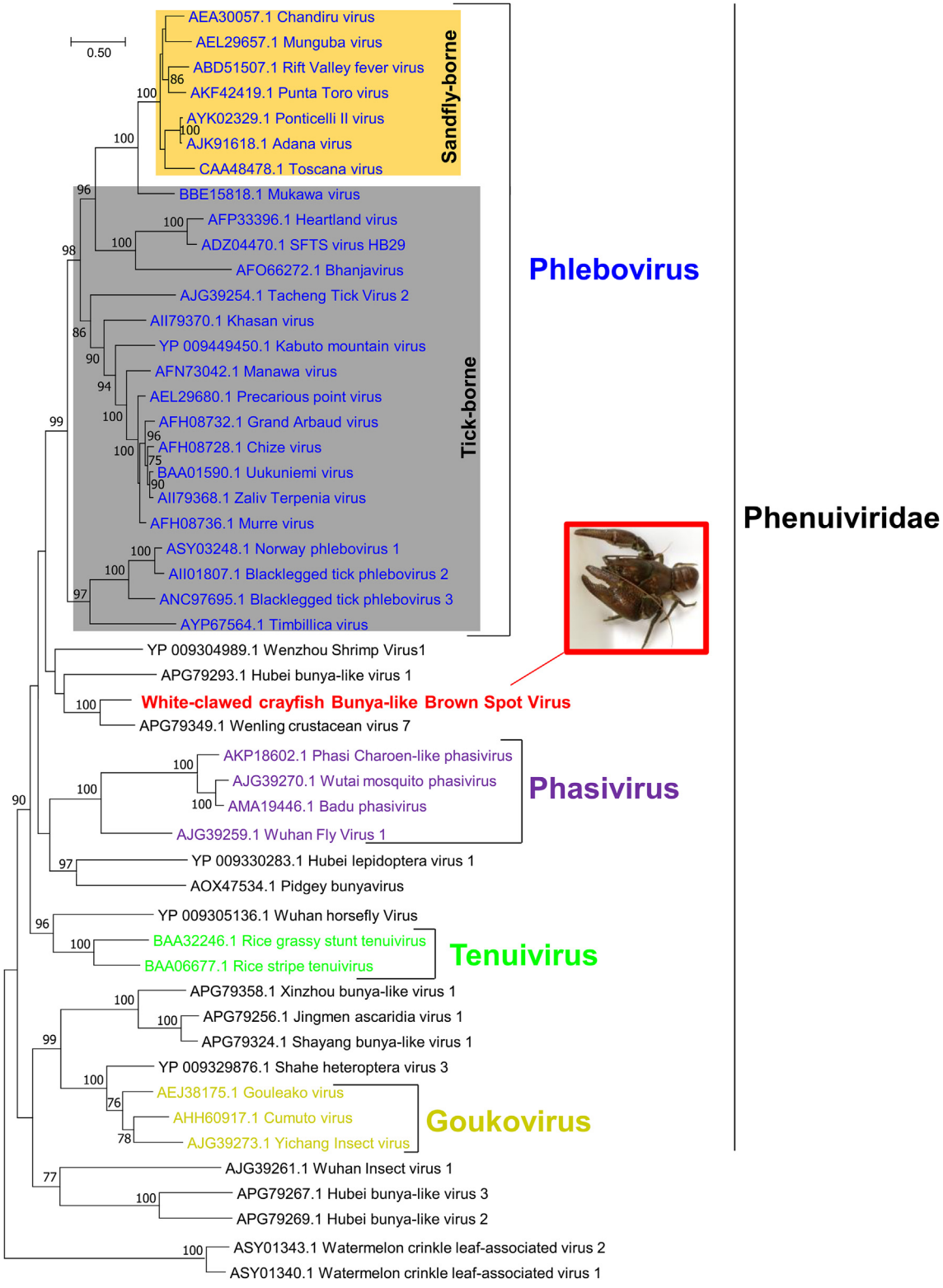

Fig. 4. Phylogenetic tree of the RNA-dependent RNA-polymerase from a selection of viruses belonging to the Bunyavirales order. The tree was reconstructed using a maximum likelihood approach on an alignment of 868 amino acids. The most likely tree is presented with bootstrap values obtained after 100 replicates. Classified viruses are colored following the code used by Xin et al. (2017).

et al., 2017; Xin et al., 2017). The phylogenies of both glycoprotein and nucleocapsid are in agreement with this position. In the glycoprotein tree, BBSV is sister to the Otter fecal bunyavirus (bootstrap = 100) and falls in a clade gathering the Wenzhou Shrimp virus 1 and the Pidgey bunyavirus, the position of which is unresolved among Phenuiviridae (Fig. 5). The analyses of the nucleocapsid, which is available for both the Wenling crustacean virus 7 and the Otter fecal bunyavirus, places BBSV with these two viruses in a strongly supported clade (bootstrap = 99), the position of which is also unresolved among Phenuiviridae (Fig. 6). It is noteworthy that our phylogenies recover the

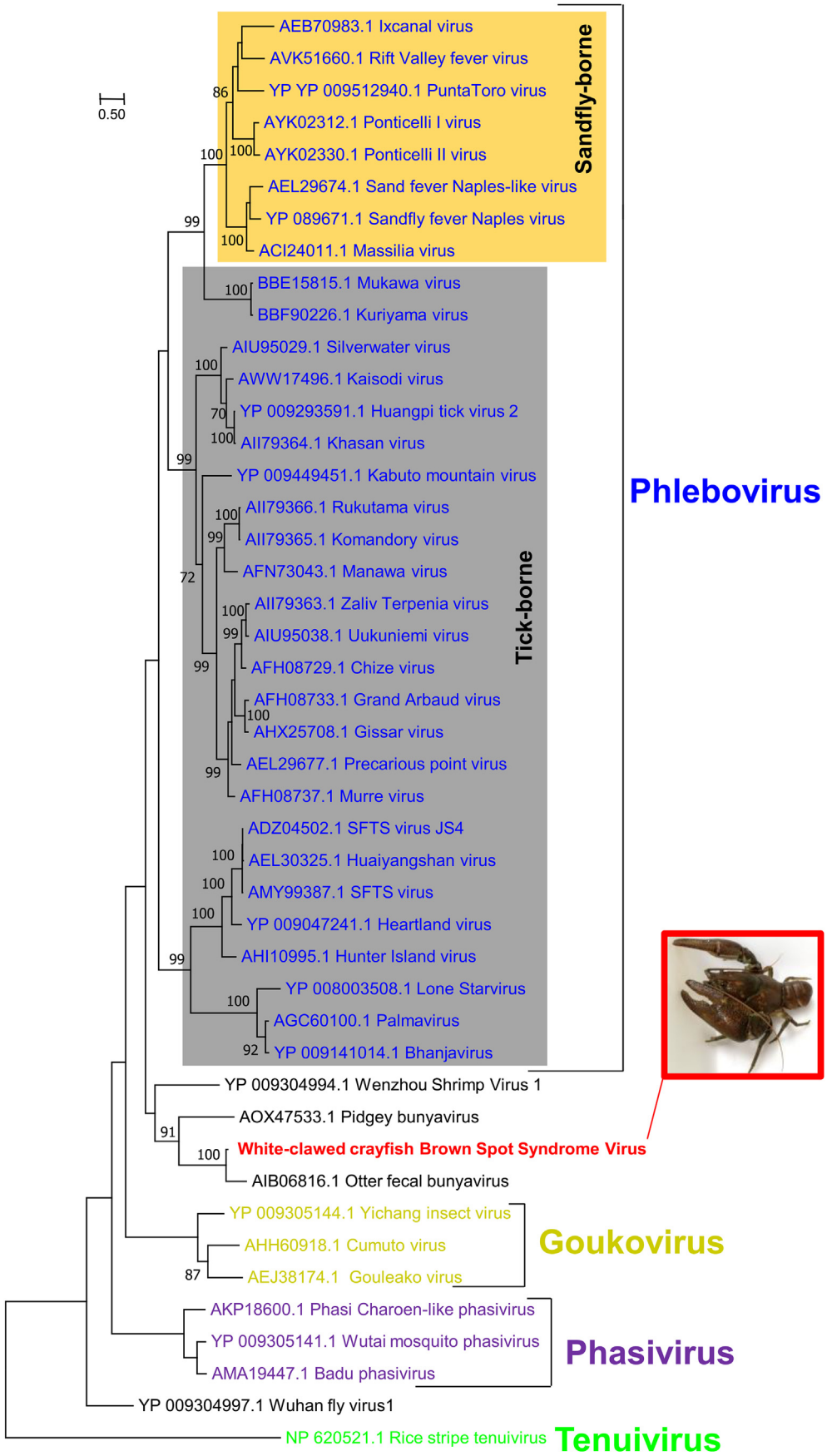

Fig. 5. Phylogenetic tree of the glycoprotein precursor from a selection of viruses belonging to the Bunyavirales order. The tree was reconstructed using a maximum likelihood approach on an alignment of 512 amino acids. The most likely tree is presented with bootstrap values obtained after 100 replicates. Classified viruses are colored following the code used by Xin et al. (2017).

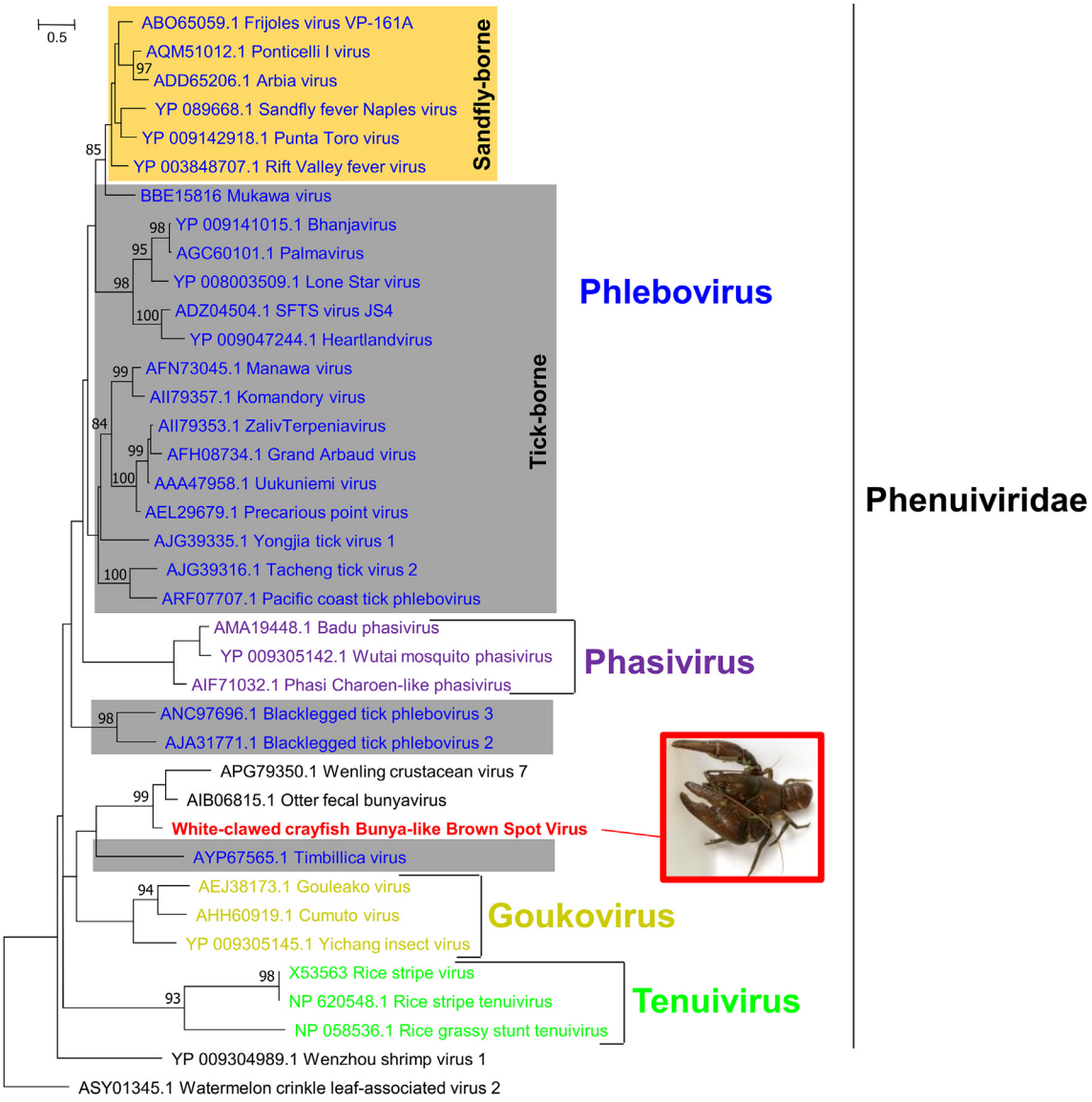

Fig. 6. Phylogenetic tree of the nucleocapsid from a selection of viruses belonging to the Bunyavirales order. The tree was reconstructed using a maximum likelihood approach on an alignment of 150 amino acids. The most likely tree is presented with bootstrap values obtained after 100 replicates. Classified viruses are colored following the code used by Xin et al. (2017).

paraphyly of tick-borne phleboviruses, with the tick-borne Mukawa virus being more closely related to sandfly-borne phleboviruses than to other tick-borne phleboviruses in all three trees (Figs. 4–6). This is all in agreement with the conclusion of (Matsuno et al., 2018) according to which the ancestor of sandfly-borne phleboviruses was tick-borne. Furthermore, in our RNA-dependent RNA-polymerase tree, this conclusion is further supported by the position of the Bhanjavirus group of tick-borne phleboviruses, which falls sister to the sandfly-borne + Mukawa phlebovirus clade (bootstrap = 96).

3.4. PCR screening for the Bunya-like Brown Spot Virus in white-clawed crayfish

The presence of the BBSV was evaluated by PCR on cDNA extracted from the hemolymph of all 10 live crayfish individuals collected in 2018 in the Bonneille. It was also checked in the cDNA samples extracted from several tissues for the four individuals sampled in 2017 and used for RNA-seq, as well as in all 20 dead individuals sampled in the Bonneille in 2017 and 2018. Virus amplicons of expected size (485 bp) were obtained in all tissues of the two infected crayfish individuals used for RNA-seq purposes, as well as in all 10 crayfish sampled in 2018 showing brown spots on their cuticle (Fig. 7). Our RNA extractions on dead ethanol-preserved individuals was successful and we obtained virus amplicons of the expected size in all these 20 individuals (Fig. 8). No amplification was obtained from the 16 specimens sampled in the two populations of Rieumançon and Navarre which did not show any external sign of infection. Sanger sequencing of the PCR products confirmed the presence of BBSV.

4. Discussion

In this study we have characterized a new enveloped, spherical virus belonging to the Phenuiviridae family of negative ssRNA viruses, called Bunya-like Brown Spot Virus (BBSV) in the white-clawed crayfish *A. pallipes*. Together with the Wenling crustacean virus 7 and the Otter fecal bunyavirus, which are viruses known only from their partial genome sequence obtained through metagenomics, it falls in an undetermined position within the Phenuiviridae. The phylogeny of the L and G segments place these viruses as sister to the Phlebovirus genus, but this position is not supported (bootstrap < 70). Furthermore, the

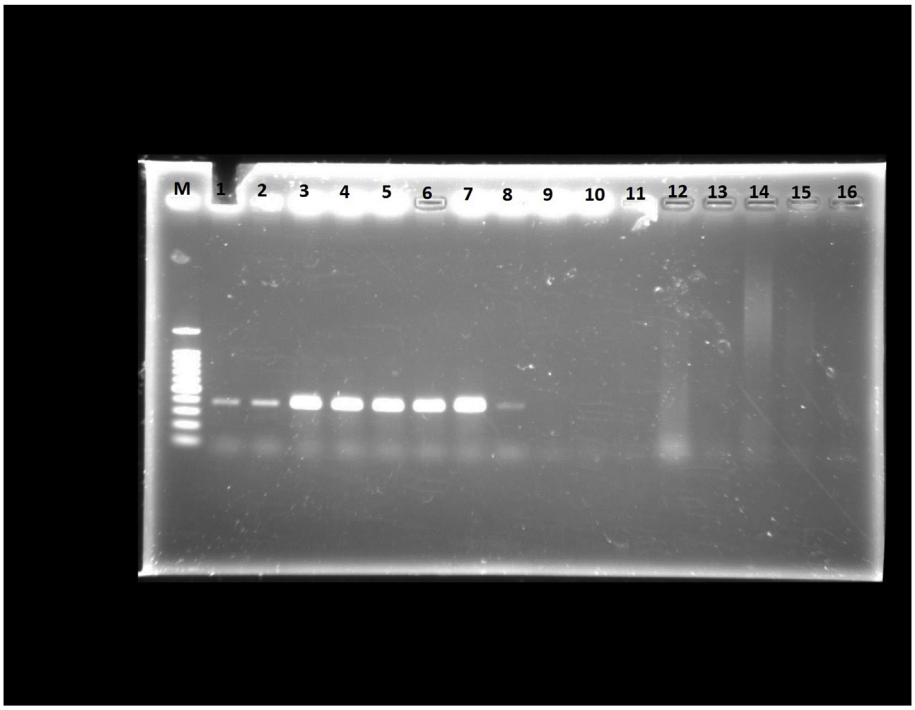

Fig. 7. Agarose gel electrophoresis of BBSV RT-PCR products. A single band of around 485bp was observed when using samples from different tissues for two infected specimens from Bonneille population (Lines 1 to 8) (1–2: hemolymph, 3–4: nerves chain, 4–5: gills, 6–7: hepathopancreas, 8: muscles). Lines 9 to 16: negative controls (9–10: on DNA extraction from hemolymph of infected specimens from Boneille, 11 to 14: RNA extraction of hemolymph and muscle samples from two non-infected specimens of Bonneille population sampled upstream of the mortality front in 2017, 15–16: RNA extraction from hemolymph of non-infected specimens sampled in Navarre population). MK 100 pb ladder.

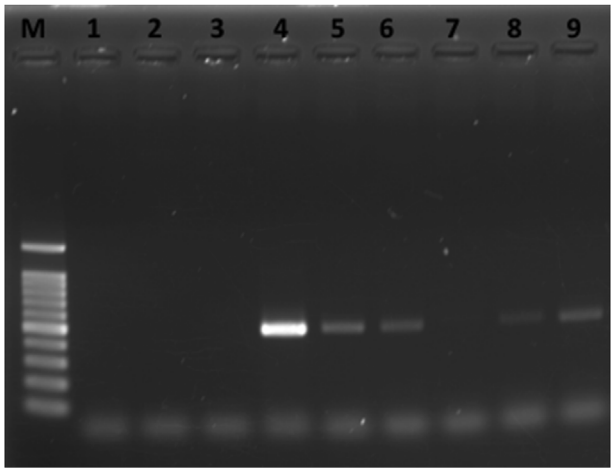

Fig. 8. Agarose gel electrophoresis of BBSV RT-PCR products. A single band of around 485 bp was observed for positive samples. Lines 1–3: negative controls: on DNA extraction from muscle samples of a dead specimen from Boneille sampled on the mortality front in 2017 and stored in 96% ethanol. Lines 4–6: RNA extraction from muscle samples of three dead specimens from Bonneille population stored in 96% ethanol and sampled in the mortality front in 2017. Line 7: RNA extraction from a muscle sample of a live specimen from Bonneille population stored in 96% ethanol and sampled upstream of the mortality front in 2017. Lines 8–9: RNA extractions from muscle samples of dead specimens from Bonneille stored in 96% ethanol and sampled in the mortality front in 2018.

branches separating these viruses from Phleboviruses are at least as long as those separating Phleboviruses from Phasiviruses. Finally, unlike the S genomic segment of Phleboviruses which encodes two relatively large ORF, that of BBSV and of the Wenling crustacean virus 7 encodes one large ORF only, i.e. it does not seem to be ambisense. Altogether, these observations suggest that BBSV, Wenling crustacean virus 7 and Otter fecal bunyavirus could be assigned to a new genus of Phenuiviridae that may be specific to crustacean species. The Wenling crustacean virus 7 has been sequenced from RNA extracted from a mix of various tissues sampled on 13 different crustacean species dwelling in the marine/coastal environment of Zhejiang (China) (Shi et al., 2016). The Otter fecal bunyavirus sequences were obtained from otter (*Lutra lutra*) feces collected in the north of Spain (Bodewes et al., 2014). To explain the presence of this bunya-like virus in the otter feces, Bodewes et al. (2014) proposed that the individual otter from which the sampled feces came from could have been systemically infected by the virus or that the virus could have infected a prey ingested by the otter. The later hypothesis is supported by the high identity between the BBSV found in crayfish and the Otter fecal bunyavirus (61–73%; Fig. 2) and the fact that crayfish are known to make up a substantial fraction of the European otter's diet (Britton et al., 2017).

A fairly large number of viruses have been described in crustaceans either based on morphological features only, on sequence data only, or both (Shi et al., 2016; Bateman and Stentiford, 2017). Many of these crustacean viruses were discovered because of the severe epidemiological outbreaks they cause in shrimp farming, which have important economic consequences (Walker and Mohan, 2009). Among crustacean viruses, relatively few were described in crayfish, and most crayfish viruses were characterized as nudi-like or parvo-like viruses only based on morphological features (Evans and Edgerton, 2002; Edgerton, 2004; Longshaw, 2011; Bateman and Stentiford, 2017). Perhaps the best known crayfish viruses are two RNA viruses recently characterized in Australian red claw crayfish (*Cherax quadricarinatus*) from farms (Sakuna et al. 2017, 2018). One is a positive ssRNA virus belonging to the Picornavirales order and the other is a bunya-like virus belonging to the Bunyavirales order, like BBSV. Unlike BBSV however, the *C. quadricarinatus* bunya-like virus belongs to the Peribunyaviridae family, which is distantly related to Phenuiviridae (Xin et al., 2017; Sakuna et al., 2018). Both viruses were characterized on the basis of morphological observations and whole genome sequencing. They are thought to cause 20–40% mortality after three weeks of transportation stress in crayfish farms (Sakuna et al. 2017, 2018). In the European Astacidae family of crayfish, Edgerton (1996) reported the existence of a bacilliform virus in *Astacus astacus* and *A. pallipes*. Originally thought to be responsible for mass mortality events in *A. pallipes* (Edgerton et al., 2002), it was later shown to be a common infection in various French populations (Edgerton, 2003; Bateman and Stentiford, 2017). Thus, our knowledge on crayfish viruses is still very limited, it is mainly drawn

from farmed species and no viruses infecting wild crayfish have been sequenced so far. In this context, our discovery of the BBSV in wild white-clawed crayfish is a significant contribution to our understanding of crayfish virus diversity and of crayfish disease-causing agents in the wild. We acknowledge that a direct and definitive link between BBSV and crayfish disease and mortality requires infection of naïve crayfish with purified viral particles or with hemolymph sampled from infected individuals. Such an experiment would entail sacrificing a substantial number of individuals, which is problematic because *A. pallipes* is listed on the IUCN Red List as an endangered species requiring special conservation measures in the European Union under the Species and Habitats Directive, Annex 2 (Füreder et al., 2010). However, the upstream spread of the mortality front observed over two successive summers can only be explained by the presence of an infectious agent. Furthermore, all 12 crayfish sampled live on the mortality front that had brown spots on their cuticles were positive for the virus while all those devoid of brown spots (sampled upstream of the mortality front or elsewhere) were negative (n = 18). Additionally, all 20 crayfish sampled on the mortality front and stored in alcohol were also positive for the virus. Finally, no viral or bacterial transcript other than those from BBSV were identified by our RNA-seq approach. Thus, we believe that the evidence provided in this study is sufficient to establish a very likely association between the presence of this virus and the mass mortality event observed in the Bonneille brook.

Our survey of the mortality front in Bonneille in 2017 and 2018 indicates that the virus is quite virulent. Indeed, we observed that the disease front progressed upstream by 1 km between the 24th of July 2017 and the end of August 2017. One year later (10th of September 2018), we observed moribund crayfish showing large brown spots on the cuticle in places located another 1,5 km upstream of the 2017 mortality front. The BBSV seems nevertheless less virulent than the crayfish plague (*A. astaci*), which also causes mass mortality events in wild populations of white-clawed crayfish (Collas et al., 2016). For example, Collas et al. (2016) reported a disease front that was progressing upstream of 3 km in ten days during July based on surveys performed every two days. In crayfish experimentally infected by the plague, the death occurred within 7–10 days when virulent strains are involved (Becking et al., 2015). Time to mortality is at least 3 to 10 times longer after infection by BBSV as infected specimens sampled on the 10th of September 2018 and maintained in aquarium died respectively after 20 days, 35 days and 3 months. Additional experimentations are required to better characterize the virulence and transmission mode of this virus. In the future we will systematically check for the presence of this virus in natural populations, particularly those suffering from epidemiological outbreaks. In this regard, it is noteworthy that 50% of crayfish outbreaks in the wild remain unexplained as specific molecular diagnostics have failed to detect infection by *A. astaci* (Grandjean, pers. com.). Ultimately, the discovery and characterization of BBSV thus has the potential to contribute to the management of wild white-clawed crayfish populations and to enhance conservation efforts aiming at preserving this endangered species (Füreder et al., 2010).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.05.014.

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