Identification of a newly described OsHV-1 µvar from the North Adriatic Sea (Italy)

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Abstract

The surveillance activities for abnormal bivalve mortality events in Italy include the diagnosis of Ostreid herpesvirus type 1 (OsHV-1) in symptomatic oysters. OsHV-1-positive oysters (Crassostrea gigas) were used as a source for in vivo virus propagation and a virus-rich sample was selected to perform shotgun sequencing based on Illumina technology. Starting from this unpurified supernatant sample from gills and mantle, we generated 3.5 million reads (2 x 300 bp) and de novo assembled the whole genome of an Italian OsHV-1 microvariant (OsHV-1-PT). The OsHV-1-PT genome encodes 125 putative ORFs, 7 of which had not previously been predicted in other sequenced OsHV-1 variants. Overall, OsHV-1-PT displays typical microvariant OsHV-1 genome features, while few polymorphisms (0.08 %) determine its uniqueness. As little is known about the genetic determinants of OsHV-1 virulence, comparing complete OsHV-1 genomes supports a better understanding of the virus pathogenicity and provides new insights into virus–host interactions.

Ostreid herpesvirus type 1 (OsHV-1) was firstly detected from Crassostrea gigas larvae during mortality events in French hatcheries in 1991 [1, 2] and it was then progressively associated with the mass mortality of oyster spats and juveniles in Europe and other regions of the world [3–8]. More aggressive OsHV-1 indicated as microvariants of the reference OsHV-1 [9] (GenBank ID: AY509253) have increasingly been detected since 2008 in Europe (France, Ireland, United Kingdom, Netherlands, Spain, Portugal and Italy), as well as in other parts of the world, and at present they likely represent the prevailing OsHV-1 virus type [7, 10–13]. Most of the sequence data referring to OsHV-1 refer to diagnostic genome regions (i.e. ORF4, ORF36-37-38, ORF42-43, ORF88, ORF99 and ORF100) [4, 12, 14–17]. Up to now, the genomes of seven Malacoherpesviridae have been sequenced and comparatively reported, namely the reference OsHV-1 [9], two OsHV-1 µVars [13] and four OsHV-1 variants detected in other bivalve species [18–20] (KU096999, NCBI April 2016).

Italy is the third European producer of marine bivalves, with more than 100 000 tons estimated in 2015 [21]. The bivalve farming industry is economically relevant for the regions bordering the Adriatic Sea and, even if the production of the Pacific oyster is at its onset, the global diffusion of infectious OsHV-1 microvariants raises significant concern in the national authorities and farmers.

In this study, we investigated the identity and infectivity of an OsHV-1 virus detected in oysters, diploid C. gigas, produced and farmed in the North Adriatic Sea. Basically, a first supernatant from pooled virus-positive oysters allowed us to propagate the virus in nine subsequent in vivo infection trials, from which we selected a homogenate of pooled gills and mantle to purify total DNA and sequence the whole genome of a new OsHV-1 microvariant, applying short communication
In April 2016, initial signs of mortality were observed following an event of reduced water salinity (25 psu; 18–19 °C) in oysters no more than 4–5 months of age, farmed in the Porto Tolle area (Po Delta basin, North Adriatic Sea, Italy). No massive mortality appeared, but some moribund and asymptomatic individuals were found to be positive for the presence of OsHV-1 DNA and were used as a source for the subsequent inocula (Table 1). Details on the DNA extraction and quantitative real-time (qPCR) protocols are reported in the Supplementary Materials and methods. Following experimental infection models based on the intramuscular injection of OsHV-1 preparations [22–25], we set up an infection protocol that aimed to produce and maintain a suitable quantity of virus in vivo, in the absence of mollusk cell lines, and to characterize the Porto Tolle OsHV-1, hereafter referred to as OsHV-1-PT. A batch of about 300 native C. gigas of about 4 cm in shell length and 4–5 months of age, obtained from the Porto Tolle area (Consorzio Cooperative Pescatori del Polesine, Scardovari), was preliminarily demonstrated to be OsHV-1-negative through the testing of 30 individuals with the standard qPCR protocol that was then used to measure the OsHV-1 DNA in the injected oysters. As detailed in the Methods section (see also the Supplementary Materials and methods), up to 13 oysters per trial were tentatively infected (145 in total), while the negative controls (10 per trial) were injected with the same volume of sterile seawater. At the end of each trial, the gills and mantle [25 mg wet weight (w.w.) tissue] were sampled from individual oysters to assess the presence of OsHV-1 DNA (ORF100 region) by qPCR. Starting from the naturally infected oysters, all inocula were freshly prepared by homogenization of the pooled gills and mantle fragments of oysters showing viral titres above 10⁶ OsHV-1 copies µl⁻¹. Native OsHV-1-free oysters were experimentally injected with a minimum viral load of 10⁷ DNA copies.

In the first infection trial (I), the injection of 2.5–2.8 × 10⁸ OsHV-1 DNA copies caused 50 % mortality. In the subsequent eight infection trials (II–IX), the injection of 1.0 × 10⁶–1.0 × 10⁹ OsHV-1 DNA copies caused lower levels of oyster mortality (0–41.4 %). Relating the copy number of OsHV-1 DNA of each inoculum to the copy number of OsHV-1 DNA detected in moribund oysters, the greatest mortality levels were found to be associated with inocula with 1 × 10⁸ or more OsHV-1 DNA copies. Variable viral DNA titres were detected in both dead (0–1.8 × 10⁸ viral copies µl⁻¹) and surviving oysters (0–4.9 × 10⁷ viral copies µl⁻¹) (Table 1).

Using the Kaplan–Meier method (reported in detail in the Supplementary Materials and methods) we estimated the oyster survival probability over time, which was found to be 97.3 % on the first day and 75 % on the sixth day post-injection, while no mortality was observed in the control animals (Fig. 1).

Amongst the samples generated during the nine infection trials, we selected one sample that was rich in OsHV-1-PT (1.8 × 10⁸ copies µl⁻¹) for confirmatory transmission electron microscopy (TEM). Tissues were prepared according to standard procedures, negatively stained with 2 % sodium phosphotungstate solution and finally observed via TEM (Philips 208S). Virions that were compatible with herpesvirus particles in terms of both size and shape were detected (Fig. S2).

The relative amounts of OsHV-1 DNA and C. gigas DNA in such a virus-rich sample were assessed by qPCR with the same set of primers used for the OsHV-1 DNA quantification and with a primer set designed for elongation factor (EF1α), a single-copy oyster gene (see details in Supplementary Materials and methods). The resulting ratio of 25:1 copy number between OsHV-1 and C. gigas made us confident in applying a direct next-generation sequencing (NGS) approach to the total DNA, purified from the above-

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**Table 1. Description of the nine virus propagation trials.** The oyster numbers per trial, together with the volume and viral load of the inoculum and the final viral load in the infected oysters are reported.

| Trial | No. of injected oysters | Inoculum vol. (µl) | Injected viral load (DNA copies) | Detected viral load (copies µl⁻¹) |
|-------|-------------------------|--------------------|----------------------------------|---------------------------------|
| 1     | 13                      | 100                | 2.5 × 10⁶–2.8 × 10⁸              | 0–2.4 × 10⁷                    |
| 2     | 12                      | 100                | 2.9 × 10⁶–1 × 10⁹               | 0–1 × 10⁸                     |
| 3     | 13                      | 150                | 1 × 10¹⁰                         | 0–8.3 × 10⁸                   |
| 4     | 13                      | 100                | 1.5 × 10⁸–3.3 × 10⁸              | 0–1.2 × 10⁸                   |
| 5*    | 12                      | 150                | 1.2 × 10⁸–3.8 × 10⁸              | 0–7.7 × 10⁷                   |
| 6†    | 10                      | 150                | 1.2 × 10⁸–3.8 × 10⁸              | 0–7.7 × 10⁷                   |
| 7*    | 13                      | 100                | 2.2 × 10⁸–7.7 × 10⁸              | 5.6 × 10⁷–1.8 × 10⁸           |
| 7†    | 10                      | 150                | 1 × 10⁷–3.3 × 10⁹               | 0–1.8 × 10⁸                   |
| 8*    | 13                      | 100                | 1.7 × 10⁹                        | 4.1 × 10⁷–1.9 × 10⁶           |
| 8†    | 10                      | 100                | 1.7 × 10⁹                        | 4.1 × 10⁷–1.9 × 10⁶           |
| 9     | 13                      | 100                | 1 × 10⁷–1 × 10¹⁰                | 0–4.9 × 10⁷                   |

* and † indicate oysters injected and kept in separate tanks.
mentioned virus-positive supernatant, without a virus purification step.

Following library preparation and Illumina sequencing, we generated 3,436,820 paired-end reads (2 x 300 bp), which allowed the recovery of 87,582 high-quality reads truly belonging to the order Herpesvirales (2.6% OsHV-1 DNA to exogenous DNA ratio). The OsHV-1 reads represented a 200-fold base pair sequence coverage and a 279-fold physical coverage of the OsHV-1 genome. The genome was assembled by applying a de novo approach tailored with a scaffolding step on the OsHV-1 reference genome (AY509253), which allowed us to produce five large contigs ranging in length from 2,684 to 164,511 bp and to merge them into a continuous sequence with three short ‘N’ stretches (64 ‘N’ bases in total), two of which were subsequently resolved by Sanger sequencing. The remaining ‘N’ stretch could not be resolved and its length was estimated solely on the basis of the scaffolding step. The final assembly was 203,983 bp long, with 1 ‘N’ stretch of 26 nucleotides (details are provided in the Supplementary Material).

The assembled OsHV-1-PT sequence showed a nucleotide composition of 38.6% G+C, which is comparable to that of the reference OsHV-1 genome (38.7%) and to the recently sequenced OsHV-1 µVar genome KY271630 (38.9%), with 99.80% and a 99.92% nucleotide sequence similarity to the reference OsHV-1 and the OsHV-1 µVar sequences, respectively.

The OsHV-1-PT genome displayed the same structure as OsHV-1 µVar [13], with an organization that can be represented as TR_L–U_L–IR_L–X–IR_S–U_S–TR_S–X’ or X’–TR_L–U_L–IR_L–X–IR_S–U_S–TR_S, due to the impossibility of placing the X region exactly. OsHV-1-PT is characterized by the five large deletions and the large insertion discriminating the OsHV-1 µVar from the OsHV-1 reference [13]. The latter insertion had previously been detected in both the acute viral necrosis virus (ANVN) and Scapharca broughtoni ostreid herpesvirus-1 (OsHV-1-SB) genomes [19, 20]. The 86 bp insertion, found in the OsHV-1 µVar genome when compared to the OsHV-1 reference, was missing in OsHV-1-PT genome; instead, in the inverted repeat IR_S/TR_S we found two additional deletions of 115 (starting at nucleotide 191,861 of IR_S and at nucleotide 199,578 of TR_S) and 235 bp (starting at nucleotide 192,125 of IR_S and at nucleotide 200,107 of TR_S). The assembled OsHV-1-PT shared 122 indels with the OsHV-1 µVar genomes, accounting for 1,363 nucleotides, the majority of which (82.8%) were short in length (<10 bp). The localization of most indels (82%) in repeated sequence motifs (TR/IR) is not surprising as the performance of the de novo approach is well known to be difficult to apply on repeat-containing regions.

The open reading frame (ORF) prediction resulted in 125 different putative OsHV-1-PT proteins, including 111 unique ORFs and other 14 ORFs that were repeated twice in the genome because located in the IR regions (Table 2). We compared the ORFs of OsHV-1-PT (ORF^PT) with those already described for OsHV-1 µVar (ORF^V). The previously described indels led to the shortening of 4 ORFs (ORF^PT 3; 16; 114 and IN.4) down to 40 codons (Table 3), while 3 ORFs^PT, namely 90, 119 and IN.1, gained 56, 43 and 137 extra amino acids, respectively. Sixty-one nucleotide substitutions, mostly non-synonymous (67.2%), were found to be evenly distributed among 41 ORFs^PT. Seven new ORFs were predicted and newly named from PT1–PT7. The remaining

Fig. 1. Cumulative Kaplan–Meier curves describing the survival probability of oysters infected with OsHV-1-PT.
Table 2. ORF annotation and gene ontology classification for the µvar OsHV-1-PT

For each putative ORF, information regarding the genomic coordinates (start, end), strand (positive or negative) and, if they are present, gene ontology, EC number and InterPro GO is reported. The column 'Repeated' indicates whether the ORF is present in repeated regions, and therefore has an alternative start, end and strand. Putative ORFs called 'ORFnumber' correspond to proteins in the reference (GenBank ID: AY509253); putative ORFs called 'ORF1N.number' are proteins that are partially or completely within an insertion; and putative ORFs called 'ORF-PTnumber' are new proteins that have been predicted and are not present in the reference.

| Sequence | Start  | End    | Strand | Repeated | Description                                      | GO IDs                                   | EC IDs | EC names                                      | InterPro GO IDs | InterPro GO names                                      |
|----------|--------|--------|--------|----------|-------------------------------------------------|------------------------------------------|--------|-----------------------------------------------|----------------|--------------------------------------------------------|
| ORF1     | 77     | 178159 | 523    | Yes      | DNA replication origin-binding helicase         | F:GO:00036779; P:GO:0009058; C:GO:0005694; P:GO:0006259; C:GO:0005730 |        | F:DNA replication activity; P:biosynthetic process; C:chromosome; P:DNA metabolic process; C:ribosome |                |                                                        |
| ORF2     | 641    | 177538 | 1144   | Yes      | RNase H reverse transcriptase                   | F:GO:0043167                             |        | F:Exonuclease activity; P:DNA replication, synthesis of RNA primer |                |                                                        |
| ORF3     | 1845   | 176073 | 2609   | Yes      | 5' RNA cap maturation initiation helicase       | F:GO:00044281; P:GO:0005491; C:GO:0005829; P:GO:0009058; C:GO:0006259; P:GO:00044711 |        | P:ribonucleoside-diphosphate reductase subunit M2 |                |                                                        |
| ORF4     | 3336   | 174297 | 4385   | Yes      | 5' RNA cap maturation initiation helicase       | F:GO:00044281; P:GO:0005491; C:GO:0005829; P:GO:0009058; C:GO:0006259; P:GO:00044711 |        | P:ribonucleoside-diphosphate reductase subunit M2 |                |                                                        |
| ORF6     | 6361   | 8391   | 8391   | No       | DNA replication origin-binding helicase         | F:GO:00036779; P:GO:0009058; C:GO:0005694; P:GO:0006259; C:GO:0005730 |        | F:DNA replication activity; P:biosynthetic process; C:chromosome; P:DNA metabolic process; C:ribosome |                |                                                        |
| ORF7     | 8568   | 12113  | 12113  | No       | DNA replication origin-binding helicase         | F:GO:00036779; P:GO:0009058; C:GO:0005694; P:GO:0006259; C:GO:0005730 |        | F:DNA replication activity; P:biosynthetic process; C:chromosome; P:DNA metabolic process; C:ribosome |                |                                                        |
| ORF8     | 12152  | 13111  | 13111  | No       | DNA polymerase                                  | F:GO:0043167                             |        | F:Exonuclease activity; P:DNA replication, synthesis of RNA primer |                |                                                        |
| ORF9     | 13217  | 14977  | 14977  | No       | DNA polymerase                                  | F:GO:0043167                             |        | F:Exonuclease activity; P:DNA replication, synthesis of RNA primer |                |                                                        |
| ORF10    | 15237  | 16265  | 16265  | No       | DNA polymerase                                  | F:GO:0043167                             |        | F:Exonuclease activity; P:DNA replication, synthesis of RNA primer |                |                                                        |
| ORF11    | 16530  | 18431  | 18431  | No       | DNA polymerase                                  | F:GO:0043167                             |        | F:Exonuclease activity; P:DNA replication, synthesis of RNA primer |                |                                                        |
| ORF12    | 18537  | 19124  | 19124  | No       | DNA polymerase                                  | F:GO:0043167                             |        | F:Exonuclease activity; P:DNA replication, synthesis of RNA primer |                |                                                        |
| ORF13    | 19173  | 19490  | 19490  | No       | DNA polymerase                                  | F:GO:0043167                             |        | F:Exonuclease activity; P:DNA replication, synthesis of RNA primer |                |                                                        |
| ORF14    | 20042  | 20626  | 20626  | No       | DNA polymerase                                  | F:GO:0043167                             |        | F:Exonuclease activity; P:DNA replication, synthesis of RNA primer |                |                                                        |
| ORF15    | 20973  | 21617  | 21617  | No       | DNA polymerase                                  | F:GO:0043167                             |        | F:Exonuclease activity; P:DNA replication, synthesis of RNA primer |                |                                                        |
| ORF16    | 21698  | 21922  | 21922  | No       | DNA polymerase                                  | F:GO:0043167                             |        | F:Exonuclease activity; P:DNA replication, synthesis of RNA primer |                |                                                        |
| ORF17    | 22076  | 22423  | 22423  | No       | DNA polymerase                                  | F:GO:0043167                             |        | F:Exonuclease activity; P:DNA replication, synthesis of RNA primer |                |                                                        |
| ORF18    | 22475  | 22756  | 22756  | No       | DNA polymerase                                  | F:GO:0043167                             |        | F:Exonuclease activity; P:DNA replication, synthesis of RNA primer |                |                                                        |
| ORF19    | 22802  | 24010  | 24010  | No       | DNA polymerase                                  | F:GO:0043167                             |        | F:Exonuclease activity; P:DNA replication, synthesis of RNA primer |                |                                                        |
| ORF20    | 24090  | 25829  | 25829  | No       | DNA polymerase                                  | F:GO:0043167                             |        | F:Exonuclease activity; P:DNA replication, synthesis of RNA primer |                |                                                        |
| ORF21    | 25900  | 28854  | 28854  | No       | DNA polymerase                                  | F:GO:0043167                             |        | F:Exonuclease activity; P:DNA replication, synthesis of RNA primer |                |                                                        |
| ORF22    | 29018  | 33916  | 33916  | No       | DNA polymerase                                  | F:GO:0043167                             |        | F:Exonuclease activity; P:DNA replication, synthesis of RNA primer |                |                                                        |
| ORF23    | 34047  | 37865  | 37865  | No       | DNA polymerase                                  | F:GO:0043167                             |        | F:Exonuclease activity; P:DNA replication, synthesis of RNA primer |                |                                                        |
| ORF24    | 37962  | 39095  | 39095  | No       | DNA polymerase                                  | F:GO:0043167                             |        | F:Exonuclease activity; P:DNA replication, synthesis of RNA primer |                |                                                        |
| ORF25    | 39178  | 39843  | 39843  | No       | DNA polymerase                                  | F:GO:0043167                             |        | F:Exonuclease activity; P:DNA replication, synthesis of RNA primer |                |                                                        |
| Sequence | Start  | End    | Strand   | Repeated | Description                          | GO IDs                                                                 | GO names                                                                 | EC IDs                                                                 | EC names                                                                 | InterPro GO IDs                  | InterPro GO names                      |
|----------|--------|--------|----------|----------|--------------------------------------|------------------------------------------------------------------------|--------------------------------------------------------------------------|------------------------------------------------------------------------|--------------------------------------------------------------------------------|-------------------------------------|----------------------------------------|
| ORF26    | 39891  | 41078  | Positive | No       | ORF26                                | P: small molecule metabolic process; P: cellular nitrogen compound metabolic process; F: molecular_function | C: membrane; C: integral component of membrane; P: dUTP metabolic process; F: hydrolase activity |
| ORF27    | 41188  | 41988  | Negative | No       | ORF27                                | PGO:0044281; PGO:0034643; FGO:0003674                                    |                                                                          |                                                                        |                                                                                |                                    |
| ORF28    | 42073  | 44634  | Negative | No       | ORF28                                | CGO:0016020; CGO:0016021; PGO:0046080; FGO:0016787                      |                                                                          |                                                                        |                                                                                |                                    |
| ORF29    | 44324  | 44929  | Negative | No       | ORF29                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
| ORF30    | 45032  | 45778  | Negative | No       | ORF30                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
| ORF31    | 45832  | 46389  | Negative | No       | ORF31                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
| ORF32    | 47184  | 48842  | Positive | No       | ORF32                                | Y088_OSHVF AME: full=transmembrane ORF88 FLAGS: precursor ORF33         |                                                                          |                                                                        |                                                                                |                                    |
| ORF33    | 49157  | 49744  | Negative | No       | ORF33                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
| ORF34    | 49824  | 50198  | Negative | No       | ORF34                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
| ORF35    | 50284  | 50874  | Negative | No       | ORF35                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
| ORF36    | 51579  | 52970  | Negative | No       | ORF36                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
| ORF37    | 53853  | 56774  | Positive | No       | ORF37                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
| ORF38    | 55301  | 58624  | Negative | No       | ORF38                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
| ORF39    | 58023  | 58634  | Positive | No       | ORF39                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
| ORF40    | 61657  | 62583  | Negative | No       | ORF40                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
| ORF41    | 62727  | 63452  | Positive | No       | ORF41                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
| ORF42    | 51388  | 51972  | Negative | No       | ORF42                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
| ORF43    | 51975  | 53702  | Negative | No       | ORF43                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
| ORF44    | 53853  | 56774  | Positive | No       | ORF44                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
| ORF45    | 55301  | 58634  | Negative | No       | ORF45                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
| ORF46    | 61657  | 62583  | Negative | No       | ORF46                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
| ORF47    | 62727  | 63452  | Positive | No       | ORF47                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
| ORF48    | 68707  | 72123  | Positive | No       | ORF48                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
| ORF49    | 73476  | 74777  | Negative | No       | ORF49                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
| ORF50    | 75289  | 77796  | Negative | No       | ORF50                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
| ORF51    | 75289  | 77796  | Negative | No       | ORF51                                |                                                                         |                                                                          |                                                                        |                                                                                |                                    |
Table 2. cont.

| Sequence | Start | End   | Strand | Repeated | Description | GO IDs                          | GO names                  | EC IDs   | EC names       | InterPro GO IDs | InterPro GO names                  |
|----------|-------|-------|--------|----------|-------------|---------------------------------|---------------------------|----------|---------------|----------------|--------------------------------------|
| ORF52    | 77913 | 78455 | Positive| No       | ORF52       |                                 |                           |          |               |                |                                      |
| ORF53    | 78539 | 80086 | Positive| No       | ORF53       |                                 |                           |          |               |                |                                      |
| ORF54    | 80145 | 82568 | Positive| No       | Y068_OSHVF AME: full=transmembrane ORF68 FLAGs: precursor | C:GO:0005575            | C:cellular_component |          |               |                | P:biosynthetic process; P:DNA metabolic process |
| ORF55    | 82645 | 83064 | Negative| No       | ORF55       |                                 |                           |          |               |                |                                      |
| ORF56    | 83338 | 84186 | Positive| No       | ORF56       |                                 |                           |          |               |                |                                      |
| ORF57    | 83936 | 84886 | Positive| No       | Chloride channel CLIC1 ORF58     | C:GO:0005573; C:GO:0043226 | C:cytoplasm; C:organelle |          |               |                |                                      |
| ORF58    | 84932 | 86497 | Positive| No       | ORF58       |                                 |                           |          |               |                |                                      |
| ORF59    | 86590 | 89832 | Positive| No       | Hypothetical protein             | C:GO:0005575            | C:cellular_component |          |               |                |                                      |
| ORF60    | 89885 | 91120 | Negative| No       | ORF60       |                                 |                           |          |               |                |                                      |
| ORF61    | 91298 | 93034 | Positive| No       | ORF61       |                                 |                           |          |               |                |                                      |
| ORF64    | 93453 | 94649 | Negative| No       | RNA ligase F:GO:00016874      |                                 |                           |          |               |                |                                      |
| ORF66    | 96797 | 100189| Negative| No       | ORF66       | F:GO:00016779; F:GO:00009058; C:GO:0005694; F:GO:00066259; C:GO:00055730 | F:ligase activity     |          |               |                |                                      |
| ORF67    | 100603| 102393| Positive| No       | DEAD-box ATP-dependent RNA helicase | F:GO:00036677; F:GO:00005524; F:GO:0004366; F:GO:00016787; C:GO:0005575 | F:DNA-directed RNA polymerase | EC2.7.7.6 |               |                | DNA primase activity; DNA replication, synthesis of RNA primer |
| ORF68    | 102474| 104555| Negative| No       | Y068_OSHVF AME: full=transmembrane ORF68 FLAGs: precursor | C:GO:0005575            | C:cellular_component |          |               |                |                                      |
| ORF69    | 104623| 106014| Negative| No       | ORF69       |                                 |                           |          |               |                |                                      |
| ORF70    | 106498| 107100| Negative| No       | ORF70       |                                 |                           |          |               |                |                                      |
| ORF71    | 107387| 108748| Positive| No       | ORF71       |                                 |                           |          |               |                |                                      |
| ORF72    | 108646| 109212| Positive| No       | ORF72       | C:GO:0005575            | C:cellular_component |          |               |                |                                      |
| ORF74    | 111208| 111564| Negative| No       | ORF74       |                                 |                           |          |               |                |                                      |
| ORF75    | 111620| 112330| Negative| No       | ORF75       | P:GO:0044281; P:GO:00036461; F:GO:0003674 | P:small molecule metabolic process; P:cellular nitrogen compound metabolic process; P:molecular function |          |               |                |                                      |
| ORF76    | 113161| 115197| Positive| No       | ORF75       |                                 |                           |          |               |                |                                      |
| ORF77    | 115315| 119109| Positive| No       | Hypothetical protein             | C:GO:0005575            | C:cellular_component |          |               |                |                                      |
| ORF78    | 119163| 122618| Positive| No       | ORF78       |                                 |                           |          |               |                |                                      |
| ORF79    | 122633| 123073| Positive| No       | ORF79       |                                 |                           |          |               |                |                                      |
| ORF80    | 123137| 123487| Positive| No       | Hypothetical protein             | C:GO:0005575            | C:cellular_component |          |               |                |                                      |
| ORF81    | 123611| 124252| Positive| No       | ORF81       |                                 |                           |          |               |                |                                      |
| ORF82    | 124200| 125090| Positive| No       | ORF82       |                                 |                           |          |               |                |                                      |
| Sequence | Start | End   | Strand   | Repeated | Description                  | GO IDs       | GO names                        | EC IDs     | EC names                  | InterPro GO IDs | InterPro GO names                                      |
|----------|-------|-------|----------|----------|------------------------------|--------------|--------------------------------|------------|---------------------------|-----------------|---------------------------------------------------------|
| ORF83    | 125185| 126291| Negative | No       | ORF83                        | C:GO:0005575 | C:cellular_component            |            |                           |                 |                                                         |
| ORF84    | 126297| 126653| Negative | No       | ORF83                        |              |                                 |            |                           |                 |                                                         |
| ORF85    | 126658| 128661| Positive  | No       | ORF85                        |              |                                 |            |                           |                 |                                                         |
| ORF86    | 128665| 129072| Positive  | No       | ORF86                        |              |                                 |            |                           |                 |                                                         |
| ORF87    | 129087| 129599| Positive  | No       | E3 ubiquitin ligase XIAP      | F:GO:0008270; F:GO:0046872; FGO:0042981 | F:zinc ion binding; F:metal ion binding; P:regulation of apoptotic process |            |                           |                 |                                                         |
| ORF88    | 129693| 131939| Positive  | No       | Y088_OSHVF AME: full=transmembrane ORF88_FLAGs precursor ORF89         | C:GO:0005575 | C:cellular_component            |            |                           |                 |                                                         |
| ORF89    | 131991| 132725| Positive  | No       | ORF89                        |              |                                 |            |                           |                 |                                                         |
| ORF90    | 132786| 133838| Negative  | No       | ORF90                        |              |                                 |            |                           |                 |                                                         |
| ORF91    | 133876| 134868| Negative  | No       | ORF91                        | F:GO:0016020; F:GO:0016021 | C:membrane; C:intermediate component of membrane |            |                           |                 |                                                         |
| ORF92    | 134917| 135606| Negative  | No       | ORF92                        |              |                                 |            |                           |                 |                                                         |
| ORF93    | 135542| 136756| Negative  | No       | ORF93                        |              |                                 |            |                           |                 |                                                         |
| ORF94    | 136761| 137804| Positive  | No       | ORF94                        | F:GO:0008168; F:GO:0006259; F:GO:0046872; | F:methyltransferase activity; F:DNA metabolic process; P:response to stress F:DNA binding; F:nuclease activity; P:DNA metabolic process; |            |                           |                 |                                                         |
| ORF95    | 137889| 138821| Positive  | No       | Exonuclease V                | F:GO:0003677; F:GO:004518; F:GO:0003887 | F:DNA binding; F:nuclease activity; P:nucleobase-containing compound catalytic process; P:DNA metabolic process; |            |                           |                 |                                                         |
| ORF96    | 138886| 139608| Negative  | No       | ORF96                        | F:GO:0043167 | F:ion binding                   |            |                           |                 |                                                         |
| ORF97    | 139689| 140234| Negative  | No       | ORF97                        | F:GO:0043167 | F:ion binding                   |            |                           |                 |                                                         |
| ORF98    | 140737| 142491| Positive  | No       | ORF98                        |              |                                 |            |                           |                 |                                                         |
| ORF99    | 142863| 143615| Negative  | No       | Inhibitor of apoptosis       | F:GO:0008270; F:GO:0046872; F:GO:0003676; F:GO:0000166; F:GO:004518; F:GO:0003887 | F:zinc ion binding; F:metal ion binding; P:modulation by virus of host apoptotic process F:DNA binding; F:DNA-directed DNA polymerase activity |            |                           |                 |                                                         |
| ORF100   | 144107| 149743| Positive  | No       | DNA polymerase delta catalytic | F:GO:0003677; F:GO:0046872; F:GO:0003676; F:GO:0000166; F:GO:004518; F:GO:0003887 | F:DNA binding; F:DNA-directed DNA polymerase activity |            |                           |                 |                                                         |
| ORF101   | 149830| 150459| Positive  | No       | ORF101                       |              |                                 |            |                           |                 |                                                         |
| ORF102   | 150499| 152787| Negative  | No       | ORF102                       |              |                                 |            |                           |                 |                                                         |
| ORF103   | 152800| 154071| Positive  | No       | Y103_OSHVF AME: full=transmembrane ORF103_FLAGs precursor ORF104         | C:GO:0005575 | C:cellular_component            |            |                           |                 |                                                         |
| ORF104   | 154220| 157822| Positive  | No       | ORF104                       |              |                                 |            |                           |                 |                                                         |
| ORF106   | 159718| 161115| Negative  | No       | Inhibitor of apoptosis 1     | F:GO:0043167 | F:ion binding                   |            |                           |                 |                                                         |
| Sequence | Start    | End      | Strand | Repeated | Description                           | GO IDs        | GO names                      | EC IDs | EC names                      | InterPro GO IDs | InterPro GO names          |
|----------|----------|----------|--------|----------|---------------------------------------|---------------|--------------------------------|--------|--------------------------------|----------------|-----------------------------|
| ORF107   | 161239   | 163308   | Positive | No       | ORF107                               |               |                                |        |                               |                |                             |
| ORF108   | 163525   | 164337   | Positive | No       | ORF108                               |               |                                |        |                               |                |                             |
| ORF109   | 164390   | 167014   | Positive | No       | DNA packaging terminase subunit 1      | PGO:0051276   | P:chromosome organization     |        |                               | PGO:0006323   | P:DNA packaging           |
| ORF110   | 165109   | 167894   | Positive | No       | ORF110                               |               |                                |        |                               |                |                             |
| ORF111   | 167983   | 168852   | Negative | No       | Y111_OSHVF_AME:full=transmembrane     | C:GO:0005575  | C:cellular_component          |        |                               |                |                             |
| ORF112   | 168976   | 170364   | Positive | No       | ORF111                               |               |                                |        |                               |                |                             |
| ORF113   | 170371   | 171327   | Positive | No       | ORF113                               |               |                                |        |                               |                |                             |
| ORF114   | 171473   | 172321   | Positive | No       | ORF114                               |               |                                |        |                               |                |                             |
| ORF115   | 178794;203136 | 179555;203897 | Positive; negative | Yes | ORF115                               | F:GO:0003677;F:GO:0043167;F:GO:0009058;P:GO:0006259;C:GO:0005575 | F:DNA binding;F:ion binding;P:biosynthetic process;P:DNA metabolic process;C:cellular_component |        |                               |                |                             |
| ORF116   | 181293;200628 | 182063;201398 | Positive; negative | Yes | ORF116                               |               |                                |        |                               |                |                             |
| ORF117   | 182787;198789 | 183902;199904 | Negative; positive | Yes | RING finger                          | F:GO:0043167  | F:ion binding                  |        |                               |                |                             |
| ORF118   | 184136;197887 | 184804;198555 | Negative; positive | Yes | RING finger                          | F:GO:0043167  | F:ion binding                  |        |                               |                |                             |
| ORF119   | 185173;196940 | 185751;197518 | Positive; negative | Yes | ORF119                               |               |                                |        |                               |                |                             |
| ORF120   | 186193;196175 | 186516;196498 | Positive; negative | Yes | ORF120                               |               |                                |        |                               |                |                             |
| ORF121   | 186637;195407 | 187284;196054 | Negative; positive | Yes | RING finger                          | F:GO:0043167  | F:ion binding                  |        |                               |                |                             |
| ORF122   | 187985;193552 | 189139;194706 | Negative; positive | Yes | ORF122                               |               |                                |        |                               |                |                             |
| ORF123   | 189857   | 190768   | Negative | No       | ORF123                               |               |                                |        |                               |                |                             |
| ORF124   | 191371   | 192795   | Negative | No       | ORF124                               |               |                                |        |                               |                |                             |
| ORF125   | 58692    | 59627    | Positive | No       | ORF125                               |               |                                |        |                               |                |                             |
| ORF126   | 60105    | 60578    | Positive | No       | ORF126                               |               |                                |        |                               |                |                             |
| ORF127   | 60601    | 61521    | Positive | No       | ORF127                               | C:GO:0005575  | C:cellular_component          |        |                               |                |                             |
| ORF-PT1  | 4503;173442 | 5240;174179 | Negative; positive | Yes | Hypothetical protein                 | C:GO:0016020;C:GO:0016021;C:GO:003644 | C:membrane;C:integral component of membrane;C:host cell membrane |        |                               |                |                             |
| ORF-PT2  | 5765;172540 | 6142;172917 | Negative; positive | Yes | NA                                   |               |                                |        |                               |                |                             |
| ORF-PT3  | 106114   | 106497   | Positive | No       | NA                                   |               |                                |        |                               |                |                             |
| ORF-PT4  | 109747   | 110373   | Positive | No       | NA                                   |               |                                |        |                               |                |                             |
| ORF-PT5  | 110433   | 111110   | Positive | No       | NA                                   |               |                                |        |                               |                |                             |
| ORF-PT6  | 157959   | 158690   | Negative | No       | NA                                   |               |                                |        |                               |                |                             |
| ORF-PT7  | 158633   | 159138   | Negative | No       | Hypothetical protein                 | AbHV_ORF44    |                                |        |                               |                |                             |
Table 3. Differences between ORFs found in OsHV-1 µvar-PT and OsHV-1 µVar (KY271630, [13]).

| ORF ID | OsHV-1 µvar-PT | OsHV-1 (KY271630) | Nucleotide similarity | No. of SNP, nsSNP | Length difference (AA) | Function/family/domain |
|--------|----------------|-------------------|-----------------------|------------------|-----------------------|------------------------|
| ORF1   | 447            | 149               | 99.55                 | 2, 1             | 0                     | Unknown                |
| ORF2   | 504            | 168               | 99.8                  | 1, 1             | 0                     | Unknown                |
| ORF3   | 765            | 255               | 99.35                 | 2, 1             | -1                    | Unknown                |
| ORF4   | 1050           | 350               | 99.81                 | 2, 2             | 0                     | Unknown                |
| ORF6   | 2031           | 677               | 99.95                 | 1, 0             | 0                     | Unknown                |
| ORF7   | 3546           | 1182              | 99.94                 | 2, 2             | 0                     | Putative helicase      |
| ORF11  | 1902           | 634               | 99.89                 | 2, 1             | 0                     | Unknown                |
| ORF16  | 225            | 75                | 97.4                  | 1, 1             | -2                    | Membrane protein       |
| ORF17  | 348            | 116               | 99.71                 | 1, 1             | 0                     | Secreted               |
| ORF20  | 1740           | 580               | 99.94                 | 1, 1             | 0                     | Ribonucleotide reductase small subunit |
| ORF21  | 2955           | 985               | 99.97                 | 1, 1             | 0                     | Unknown                |
| ORF22  | 4899           | 1633              | 99.98                 | 1, 1             | 0                     | Transmembrane protein  |
| ORF23  | 3819           | 1273              | 99.97                 | 1, 1             | 0                     | Unknown                |
| ORF28  | 2562           | 854               | 99.96                 | 1, 1             | 0                     | Unknown                |
| ORF32  | 1659           | 553               | 99.82                 | 1, 1             | 0                     | Transmembrane glycoprotein |
| ORF34  | 375            | 125               | 99.73                 | 1, 1             | 0                     | Unknown                |
| ORF41  | 2922           | 974               | 99.9                  | 1, 1             | 0                     | Transmembrane protein  |
| ORF43  | 612            | 204               | 99.84                 | 1, 0             | 0                     | Unknown                |
| ORF47  | 4239           | 1413              | 99.93                 | 3, 1             | 0                     | Unknown                |
| ORF49  | 3417           | 1139              | 99.91                 | 1, 1             | 0                     | Unknown                |
| ORF50  | 1302           | 434               | 99.91                 | 1, 1             | 0                     | Unknown                |
| ORF59  | 3243           | 1081              | 99.97                 | 1, 1             | 0                     | Transmembrane glycoprotein |
| ORF68  | 2082           | 694               | 99.95                 | 1, 0             | 0                     | Transmembrane protein  |
| ORF71  | 1362           | 454               | 99.93                 | 1, 0             | 0                     | Unknown                |
| ORF76  | 2037           | 679               | 99.9                  | 2, 2             | 0                     | Unknown                |
| ORF77  | 3795           | 1265              | 99.97                 | 1, 1             | 0                     | Transmembrane glycoprotein |
| ORF78  | 3456           | 1152              | 99.94                 | 2, 1             | 0                     | Unknown                |
| ORF80  | 351            | 117               | 99.72                 | 1, 0             | 0                     | Transmembrane glycoprotein |
| ORF88  | 2247           | 749               | 99.82                 | 4, 4             | 0                     | Transmembrane glycoprotein |
| ORF89  | 735            | 245               | 99.86                 | 1, 0             | 0                     | Unknown                |
| ORF90  | 1053           | 351               | 99.97                 | 1, 1             | 0                     | Unknown                |
| ORF94  | 1044           | 348               | 99.9                  | 1, 1             | 0                     | Unknown                |
| ORF98  | 1755           | 585               | 99.94                 | 1, 0             | 0                     | Unknown                |
| ORF10  | 5637           | 1879              | 99.96                 | 2, 2             | 0                     | Catalytic subunit DNA polymerase |
| ORF101 | 630            | 210               | 99.84                 | 1, 0             | 0                     | Unknown                |
| ORF106 | 1398           | 466               | 99.93                 | 1, 0             | 0                     | Zinc-finger, ring type, BIR domain |
| ORF107 | 2070           | 690               | 99.9                  | 2, 2             | 0                     | Unknown                |
| ORF112 | 1389           | 463               | 99.93                 | 1, 0             | 0                     | Unknown                |
| ORF114 | 849            | 283               | 99.9                   | 0               | -40                   | Unknown                |
| ORF115 | 762            | 254               | 99.48                 | 2, 1             | 0                     | Replication origin-binding protein |
| ORF116 | 771            | 257               | 99.48                 | 4, 4             | 0                     | Unknown                |
| ORF119 | 579            | 193               | 99.33                 | 0               | 43                    | Unknown                |
| ORF120 | 324            | 108               | 99.69                 | 1, 1             | 0                     | Secreted               |
| ORF121 | 648            | 216               | 99.85                 | 1, 0             | 0                     | Zinc-finger, ring type |
| ORF123 | 912            | 304               | 99.78                 | 2, 1             | 0                     | Unknown                |
| ORF124 | 1425           | 475               | 99.79                 | 3, 2             | 0                     | Unknown                |
| ORF IN.1 | 936        | 312               | 99.68                 | 0               | 137                   | Secreted               |
| ORFIN.4 | 921          | 307               | 98.35                 | 2, 2             | -20                   | Transmembrane glycoprotein, 1 helix |
| ORF-PT1 | 738           | 246               | \                     | \               | \                     | Membrane protein       |
| ORF-PT2 | 375           | 126               | \                     | \               | \                     | Unknown                |
70 predicted proteins were found to be completely conserved when compared to the ORFs of OsHV-1 µVar with the GenBank accession number KY271630 (BLASTN E-value=0 and similarity=100%). All putative ORFs were functionally annotated using the NCBI NR protein database, Gene Ontology (GO) [26, 27] and the Kyoto Encyclopedia of Genes and Genomes (Table 2). It was possible to assign definitions [28] to 119 of the ORFs (95.2%) by homology search. We also assigned GO terms to 45 ORFs (36%), Enzyme Commission numbers and InterPro GO terms to 7 and 8 ORFs, respectively. As a result, it was possible to assign a definition to two of the seven new predicted ORFs (PT1–PT7) and to assign GO terms to PT1, revealing its putative function as an integral membrane protein.

To better investigate the genotype of the Italian OsHV-1-PT, a phylogenetic analysis based on the C region, currently regarded as the most variable region, was performed according to previously published studies [4, 12]. The OsHV-1-PT sequence of the C region, including ORFs 4/5, was compared with all OsHV-1 sequences retrieved from GenBank and representing different geographical areas. As expected, all the OsHV-1 microvariant sequences and OsHV-1-PT clustered together, although with a bootstrap value lower than 70 (data not shown). The progressive whole-genome sequencing of new Malacoherpesviridae viruses should produce a more refined phylogenetic classification and provide support for the functional characterization of the OsHV-1 variants currently affecting bivalve hosts.

In conclusion, we demonstrated that the next generation sequencing and subsequent de novo assembly approach represent a valid strategy for reconstructing the genome of a dsDNA virus, such as OsHV-1, with high-confidence, even in case of non-enriched, unpurified samples at relatively low sequencing depth. The availability of Malacoherpesviridae genomes can lead to a real understanding of functional virus features, i.e. the identification of virulence factors in OsHV-1 variants, as well as phylogenetic relationships and the evolutionary origin of mollusk viruses. Owing to the reported sequence features, we propose the Porto Tolle OsHV-1 virus as a new microvariant. Needless to say, additional studies that relate the pathogenic occurrence of OsHV-1 to developmental stages and environmental conditions are needed to fully characterize the pathogenicity of the Italian OsHV-1-PT virus.

**Table 3. cont.**

| ORF ID | OsHV-1 µvar-PT | OsHV-1 (KY271630) |
|--------|-----------------|-------------------|
|        | Length (bp)     | Length (codons)   | Nucleotide similarity | No. of SNP, nsSNP | Length difference (AA) | Function/family/domain |
| ORF-PT3 | 378            | 128              | \                     | \               | \                   | Unknown                |
| ORF-PT4 | 384            | 209              | \                     | \               | \                   | Unknown                |
| ORF-PT5 | 378            | 226              | \                     | \               | \                   | Unknown                |
| ORF-PT6 | 627            | 244              | \                     | \               | \                   | Unknown                |
| ORF-PT7 | 375            | 162              | \                     | \               | \                   | Unknown                |

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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