

## New Viruses from *Lacerta monticola* (Serra da Estrela, Portugal): Further Evidence for a New Group of Nucleo-Cytoplasmic Large Deoxyriboviruses

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**Abstract:** Lizard erythrocytic viruses (LEVs) have previously been described in *Lacerta monticola* from Serra da Estrela, Portugal. Like other known erythrocytic viruses of heterothermic vertebrates, these viruses have never been adapted to cell cultures and remain uncharacterized at the molecular level. In this study, we made attempts to adapt the virus to cell cultures that resulted instead in the isolation of a previously undetected *Ranavirus* closely related to FV3. The *Ranavirus* was subsequently detected by polymerase chain reaction (PCR) in the blood of infected lizards using primers for a conserved portion of the *Ranavirus* major capsid protein gene. Electron microscopic study of the new *Ranavirus* disclosed, among other features, the presence of intranuclear viruses that may be related to an unrecognized intranuclear morphogenetic process. Attempts to detect by PCR a portion of the DNA polymerase gene of the LEV in infected lizard blood were successful. The recovered sequence had 65.2/69.4% nt/aa% homology with a previously detected sequence from a snake erythrocytic virus from Florida, which is ultrastructurally different from the studied LEV. These results further support the hypothesis that erythrocytic viruses are related to one another and may represent a new group of nucleo-cytoplasmic large deoxyriboviruses.

**Key words:** lizard erythrocytic virus, *Ranavirus*, *Lacerta monticola*, virus isolation, PCR

### INTRODUCTION

Erythrocytic viruses of heterothermic vertebrates (fish, amphibians, and reptiles) produce cytoplasmic inclusions in the infected cells (Johnston, 1975; Paperna & Alves de Matos, 1993). These are readily seen in Giemsa-stained smears and were once thought to represent protozoan parasites (Johnston, 1975). Morphologically the viruses have the traits commonly found among the nucleo-cytoplasmic large deoxyriboviruses (NCLDV), such as a cytoplasmic virus assembly site and complex large icosahedral virions reminiscent of the *Iridoviridae* or *Asfarviridae* (Devauchelle et al., 1985; Alves de Matos & Paperna, 1993). However, early attempts to isolate erythrocytic viruses have mostly failed, and molecular data were insufficient to evaluate their phylogeny (Gruia-Gray et al., 1989, 1992). A recent study of a rattlesnake erythrocytic virus (SEV) from Florida was first to describe a sequence from the viral DNA dependent DNA polymerase using a polymerase chain reaction (PCR) procedure designed for amplification of a conserved region of the DNA polymerases encoded by a broad spectrum of DNA viruses (Hanson et al., 2006). Results suggested that the virus could represent a new group of NCLDV, probably

belonging to a novel genus and species (Wellehan et al., 2008).

In previous studies, erythrocytic viruses were found in lizard populations of *Lacerta monticola* and *Lacerta schreiberi* from Serra da Estrela, Portugal, and their interaction with the infected cells were studied (Alves de Matos et al., 2002). However, the phylogeny of these viruses remained obscure because no information on their molecular features has ever been obtained.

Other NCLDV that have been described in reptiles are members of the *Ranavirus* genus of the *Iridoviridae* family. These viruses may induce highly lethal infections in a range of fish, amphibian, and reptilian species (Williams et al., 2005). *Ranaviruses* of reptiles have been found in turtles, snakes, and geckos (reviewed in Jancovich et al., 2010). Members of the insect iridoviruses (*Iridovirus* genus of the *Iridoviridae* family) have also been found to be able to infect captive lizards (Weinmann et al., 2007).

In this article, we report ultrastructural and molecular data on two viruses from *L. monticola* from Serra da Estrela Portugal. One of the viruses is a typical lizard erythrocytic virus (LEV) that has been reported elsewhere (Alves de Matos et al., 2002), but from which no molecular data were previously available. The second virus is a *Ranavirus* that was unexpectedly recovered from LEV-infected specimens

during unsuccessful attempts at LEV isolation. Our results reveal that LEV is related at the molecular level to SEV (Wellehan et al., 2008) but differs ultrastructurally from the latter. The collective data indicate that this may be a new group of NCLDVs.

## MATERIALS AND METHODS

### Samples

Specimens of *L. monticola* from Serra da Estrela, Portugal, known to be infected with LEVs (Alves de Matos et al., 2002), were captured during a study of the lizard population dynamics and were checked for haemoparasites by light microscopic examination of Giemsa stained blood smears. Several animals displayed red cytoplasmic inclusions compatible with the presence of LEV infections. A drop of blood from the clipped tip of the tail of infected animals was recovered and used for electron microscopy or kept frozen at  $-80^{\circ}\text{C}$  for virus isolation attempts and for molecular biology studies. After sample collection, the animals were released in the locality of capture.

### Light Microscopy

Prepared blood smears were dried in air for 1 h, fixed in absolute methanol, and stained with Giemsa diluted 1/10 in phosphate buffered saline, pH 7.0, for 1 h. Up to ten fields at  $400\times$  magnification were screened before the samples were considered negative. For this study, only samples containing more than 50% infected erythrocytes were used for transmission electron microscopy (TEM), virus isolation attempts, or PCR.

### Transmission Electron Microscopy

Small drops of blood were extracted from the tip of the clipped tail by capillary into the yellow point of an automatic pipette. The blood-filled tip was cut and immersed in primary glutaraldehyde fixative for 1 h. The hardened blood plug was extracted into new primary fixative and left for further fixation for up to 24 h.

The primary fixative consisted of 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3. Following primary fixation for the periods indicated above, the samples were washed in cacodylate buffer and secondarily fixed for 3 h in 1% osmium tetroxide, in some cases containing 0.5% potassium ferricyanide in 0.1 M sodium cacodylate buffer, pH 7.3. The samples were washed in 0.1 M acetate acetic acid buffer, pH 5.0, and further fixed in 0.5% uranyl acetate in the same buffer for 1 h. Dehydration was carried out in increasing concentrations of ethanol. After passage through propylene oxide, the samples were embedded in Epon-Araldite, using SPI-Pon as an Epon 812 substitute. Thin sections were made with glass or diamond knives and stained with 2% aqueous uranyl acetate and Reynold's lead citrate. The stained sections were studied and photographed in a JEOL 100-SX electron microscope.

## Virus Isolation

### Virus Source

Infected blood from one lizard displaying close to 100% infected erythrocytes was frozen and thawed three times to rupture infected cells and then diluted ten times in Leibowitz L15 medium containing  $1,000\ \mu\text{g}/\text{mL}$  gentamycin. No attempt was made to filter the preparation due to the possibility of retention of the virions in the filter as a result of their entrapment by the membranes of the ruptured erythrocytes.

### Cells and Inoculation

Iguana heart (IgH-2) and viper heart (VH2) cell lines were propagated at  $28^{\circ}\text{C}$  in Dulbecco's modified Eagle (D-MEM) supplemented with 10% fetal calf serum and containing  $100\ \mu\text{g}/\text{mL}$  gentamycin. Vero cells were propagated at  $37^{\circ}\text{C}$  in the same medium.

Inoculation experiments were made at room temperature (approximately  $20\text{--}28^{\circ}\text{C}$ ) and  $28\text{--}30^{\circ}\text{C}$  in a  $\text{CO}_2$  incubator. Adsorption of the inoculum was carried out at  $28^{\circ}\text{C}$  for 1 h, after which D-MEM supplemented with 2% fetal calf serum and containing  $500\ \mu\text{g}/\text{mL}$  gentamycin was added.

At different post-infection times (p.i.t.), infected cell cultures were frozen and thawed three times to rupture the cells and stored at  $-80^{\circ}\text{C}$ . A suspension of thawed cells was used for the inoculation of new cells. Up to five blind passages were made before the onset of detectable cytopathic effects (CPE).

### Microscopic Examination

Samples of cells were collected for microscopic examination by light and electron microscopy at 24 h intervals beginning two days post-inoculation, until the first CPE was observed. Attached cells were detached with trypsin (0.25%)-EDTA (0.1%), and cells in suspension were centrifuged at  $2,000\times g$  for 10 min in a Shandon Cytospin II cytocentrifuge; both were stained for 20 min in 10% Giemsa in 0.01 M phosphate buffer pH 7.0 for light microscope examination. Pellets of cells were processed for TEM using the same procedure described for erythrocyte samples.

### PCR

PCR experiments to amplify a region of the *Ranavirus* major capsid protein (MCP) gene were carried out as described by Mao et al. (1997) with template DNA (either from blood samples or from virions) extracted and purified with QIAamp Ultrasens Virus Kit (Qiagen, Valencia, CA, USA). PCR products confirmed on 0.7% agarose gels were purified with JetQuick PCR product purification spin kit (Genomed GmbH, Löhne, Germany) and sent to be sequenced by a commercial lab (StabVida, Lisbon, Portugal).

PCR experiments to amplify a region of the DNA dependent DNA polymerase gene were carried out by a direct PCR protocol with Phusion high-fidelity DNA polymerase (Finnzymes Oy, Espoo, Finland). Briefly,  $1\ \mu\text{L}$  of the frozen blood sample diluted 1:20 in Phusion high-fidelity

DNA polymerase reaction buffer containing 200  $\mu\text{g}/\text{mL}$  proteinase K was incubated overnight at 65°C and then heated for 10 min at 100°C. After this procedure, the sample was cooled down and centrifuged at 12,000  $\times g$  for 5 min. The supernatant was saved and maintained at -20°C. For PCR amplifications, 1  $\mu\text{L}$  of this supernatant was added to 24  $\mu\text{L}$  of PCR mixture containing Phusion reaction buffer, 200  $\mu\text{M}$  dNTPs, 0.5  $\mu\text{M}$  of each primer, and 0.2  $\mu\text{L}$  of Phusion high-fidelity DNA polymerase. Forward and reverse primers were, respectively, HV primer and Cons lower primer described by Hanson et al. (2006). Identical PCR amplifications were carried out with 1  $\mu\text{L}$  of purified DNA extracted from virions of the *L. monticola* ranavirus produced in VERO cells.

Cycling conditions consisted of 3 min denaturation at 98°C followed by 35 cycles of 10 s at 98°C, 20 s at 45°C and 20 s at 72°C; and finally, 1 min at 72°C. PCR products were resolved on 0.7% agarose gels, gel purified with DNA gel extraction kit (Easy Spin) and sent to StabVida for sequencing.

### Sequencing

Direct sequencing was performed on an ABI automated DNA sequencer, by StabVida laboratories, using the big dye terminator kit. Raw sequences were edited, assembled, and compared using the STADEN Package version 2003.0 Pre-gap4 and Gap4 programs (Bonfield et al., 1995). The sequences were compared to data in GenBank (National Center for Biotechnology Information, Bethesda, MD, USA) online ([www.ncbi.nih.gov](http://www.ncbi.nih.gov)) using TBLASTX options. Multiple alignments of nucleotide sequences and deduced amino acid sequences were performed with the ClustalW algorithm of the BioEdit Sequence Alignment Editor program (Hall, 1999). This alignment was further used for phylogenetic analysis in the PHYLIP program Package version 3.6. (Felsenstein, 1989) comparing parsimony, maximum-likelihood, and distance-based methods to obtain an optimal tree. Bootstrap analysis of 100 replicates was carried out.

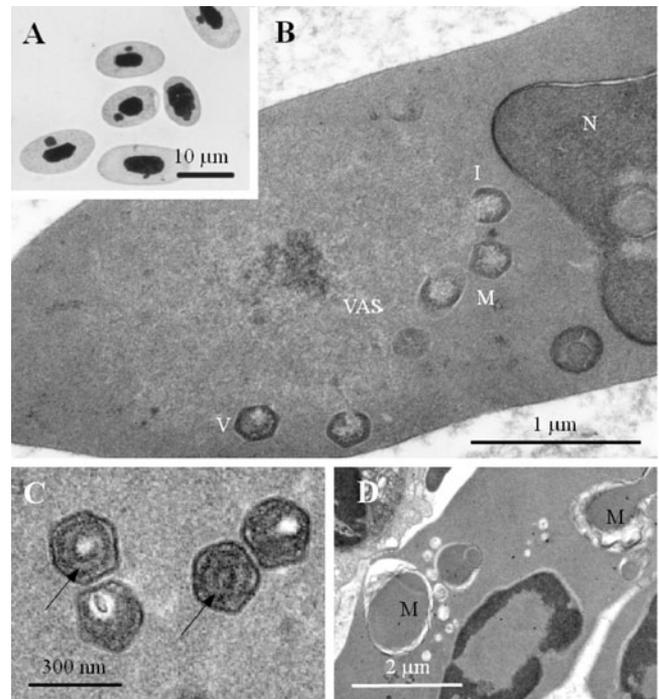
## RESULTS

### The Erythrocytic Viruses

Infected *L. monticola* displayed red round cytoplasmic inclusions in a few to up to 100% of the erythrocytes (Fig. 1A). TEM showed corresponding round cytoplasmic areas associated with virions or aberrant viruses (Fig. 1B). The virus particles displayed hexagonal or pentagonal profiles in sections of the capsid measuring 200–220 nm between faces ( $n = 50$ ). Inside the capsid, a nucleoid formed by a multilayered structure was observed (Fig. 1C). Multilamellar bodies consisting of concentric membranes were frequently detected in the erythrocytes of infected animals (Fig. 1D), some of them containing viruses. No other type of virus was observed within the erythrocytes.

### Isolation of a Lizard *Ranavirus*

Viper heart and Vero cells did not show any CPE upon repeated inoculation of virus infected material at room



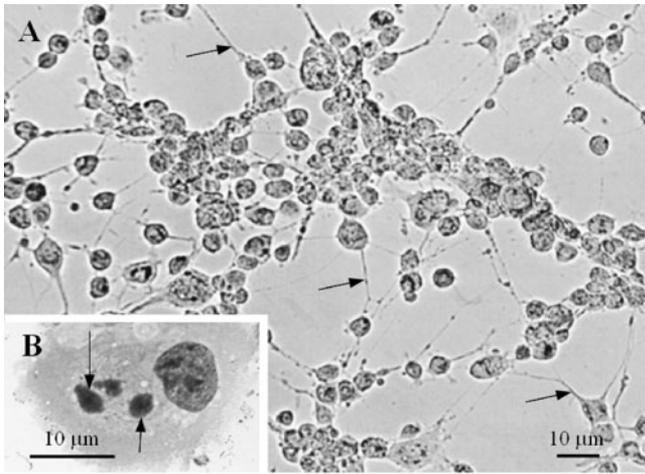
**Figure 1.** Lizard erythrocytic virus in *L. monticola* blood samples. **A:** Peripheral blood smear stained with Giemsa. Lizard erythrocytes display cytoplasmic inclusions. **B:** TEM of an infected erythrocyte. VAS associated with virions (V) and incomplete (I) or misformed capsids (M). Nucleus (N) at the right. **C:** Virions showing pentagonal or hexagonal sectional profiles and complex multilayered inner nucleoids (arrows). **D:** Multilayered membranous inclusions (M) in erythrocytes of infected blood.

temperature or at 30°C. Approximately two weeks after the first inoculation, IgH-2 cells inoculated at room temperature showed rounding of some cells, formation of a few localized patches of detached cells, and detachment of parts of the intact cell layer from the culture flask. No similar changes were observed in control cells.

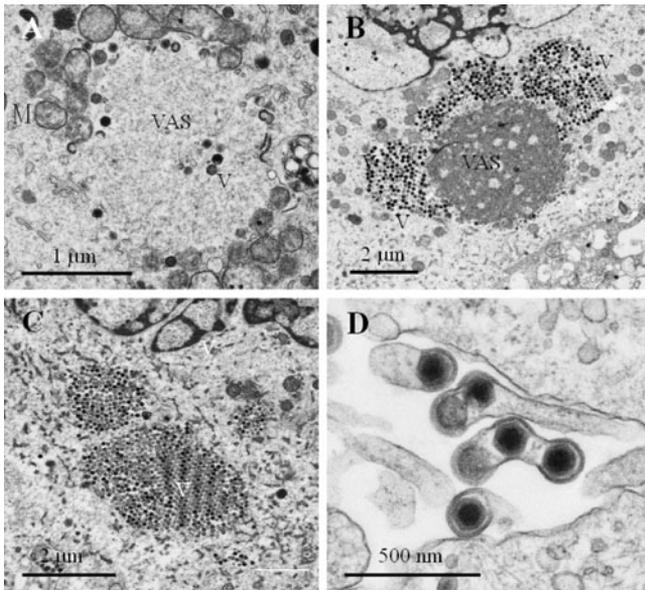
In the fifth passage, the infected cells showed clear CPE consisting of rounding of the cell bodies and formation of elongated projections, three days after inoculation. Patches of detached cells showing rounded cells at the border increased rapidly in size, and the monolayer was destroyed in the subsequent two days. With further passages, the CPE developed faster and became more diffuse, affecting all cells. No localized infection patches were then identifiable (Fig. 2A). Giemsa staining of infected cells showed the presence of large cytoplasmic inclusions in all cells (Fig. 2B). With further passages, the virus was adapted to grow in Vero cells.

### TEM of the Isolated Viruses

Cultured cells displaying CPE had round fibrillar virus assembly sites (VAS) devoid of cell organelles and associated with complete and incomplete virions. Mitochondria were often concentrated around the VAS (Fig. 3A). VAS were first observed at 2 p.i.t. In more advanced stages of the infection (4 and 5 p.i.t.), the matrix of the inclusions became more



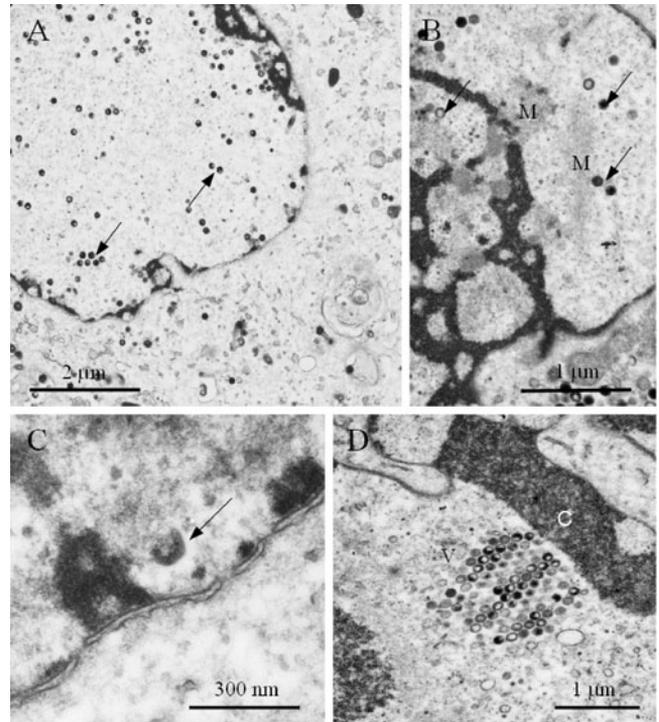
**Figure 2.** Cytopathic effect in infected IgH-2 cell cultures. **A:** Rounding of the cell bodies and elongated projections of the infected cells (arrows). **B:** Giemsa stained infected cell showing large cytoplasmic inclusions (arrows).



**Figure 3.** TEM of infected IgH-2 cells. **A:** VAS at 3 p.i.t. surrounded by mitochondria (M) and associated with virus particles (V). **B:** Accumulation of virions (V) around the virus assembly site, which shows densification of the matrix (VAS). 4 p.i.t. **C:** Paracrystalline aggregate of virus particles (V). 5 p.i.t. **D:** Budding of virions at the cell membrane. Some of the projections contain multiple virus particles.

electron dense. Complete virions accumulated at the periphery of the VAS (Fig. 3B) and in large cytoplasmic paracrystalline aggregates (Fig. 3C).

Profiles of sectioned virions were hexagonal or pentagonal with 130 nm face to face ( $n = 50$ ). The complete virions had a dense spherical nucleoid 90 nm in diameter. Many virions were observed budding at the cell membrane. The latter were projections of the cell surface containing one or more virions (Fig. 3D).



**Figure 4.** TEM of intranuclear virus particles. **A:** Nucleus of infected cell containing many virus particles (arrows). **B:** Condensed chromatin in infected cell interrupted by medium density material (M). Intranuclear virus particles are often found at the periphery of the medium density material (arrows). **C:** Incomplete particle at the periphery of the medium density material (arrow). **D:** Paracrystalline virus accumulation inside the nucleus (V). Chromatin (C).

Up to 20% of the nuclei of infected cells also contained virions (Fig. 4A). Chromatin of infected cell nuclei was condensed forming a network of dense strands, interrupted at several places by masses of medium density fibrillar material (Fig. 4B). Complete and incomplete virus particles were associated with the surface of this material (Fig. 4C) and accumulated within the infected cell nuclei, occasionally forming aggregates (Fig. 4D).

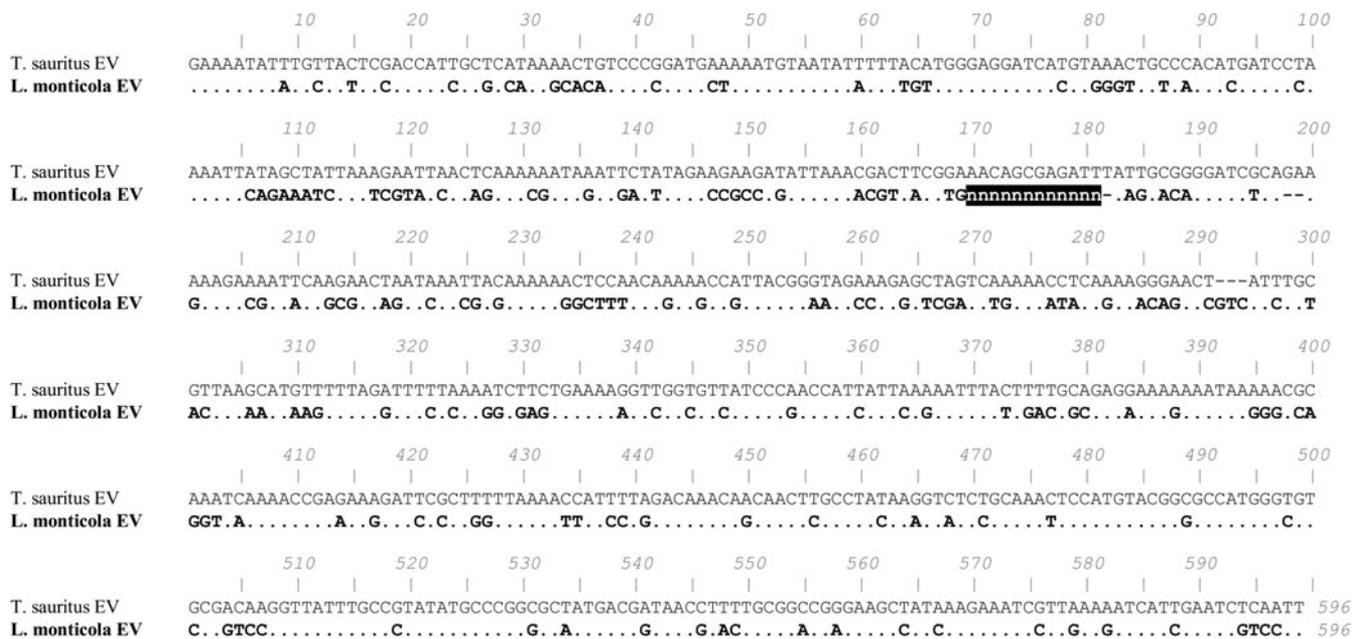
## PCR Amplifications of Erythrocytic Samples

### *Amplification with Primers for the MCP Gene of Ranavirus and Sequence Analysis*

DNA extracted from blood samples of LEV-infected lizards, when amplified with primers for a conserved region of the *Ranavirus* MCP gene produced a 500 bp product. This product was sequenced and showed close identity with the corresponding region of the FV3 genome, differing in just five nucleotides (data not shown).

### *Amplification with Primers for the DNA Polymerase Genes and Sequence Analysis*

Direct PCR applied to blood samples from a LEV-infected animal with degenerate primers targeting genes of the DNA dependent DNA polymerases (Hanson et al., 2006) resulted in several bands. The band of about 800 nt was gel purified



**Figure 5.** ClustalW nucleotide alignment of *L. monticola* erythrocytic virus partial DNA dependent DNA polymerase sequence compared to the corresponding sequence of the peninsula ribbon snake (*T. sauritus*) erythrocytic virus (GenBank Acc. No.: EF608450). Dots indicate identical nucleotides; dashes show insertions. (Please note that the *L. monticola* EV sequence is missing 12 nucleotides from position 169, due to poor sequence read quality. This region was omitted in the further calculations.)

and sequenced. The products resulting from three independent amplifications were sequenced from both directions.

The corresponding products had a similar sequence pattern, yet some contained ambiguities. After editing all of the obtained sequences, a consensus sequence containing 12 uncertain positions with a length of 596 nt (not the full amplicon length) was assembled (Fig. 5). The assembled sequence has been submitted to the GenBank and received the accession number HQ123319. The evaluated sequence has 65.2% nt and 69.4% deduced amino acid identity values to the corresponding *Thamnophis sauritus* erythrocytic virus (snake EV) partial polymerase sequence (Wellehan et al., 2008). An amino acid alignment with other members of the family *Iridoviridae* and an ascovirus is shown in Figure 6.

Phylogenetic trees constructed using different methods had similar topologies (not all shown). All earlier GenBank sequences clustered with high bootstrap values according to the corresponding accepted genera of the family *Iridoviridae*. Our LEV sequence together with the Snake EV (Wellehan et al., 2008) seemed to form a new group, outside these genera. The distance matrix based tree is shown in Figure 7.

### PCR Amplifications of the Isolated *Ranavirus*

#### *Amplification with Primers for the MCP Gene of Ranavirus and Sequence Analysis*

The expected 500 bp PCR product was observed in agarose gels. This amplicon was sequenced and showed to be identical to the amplification product recovered from blood samples.

#### *Amplification with Primers for DNA Polymerase Genes and Sequence Analysis*

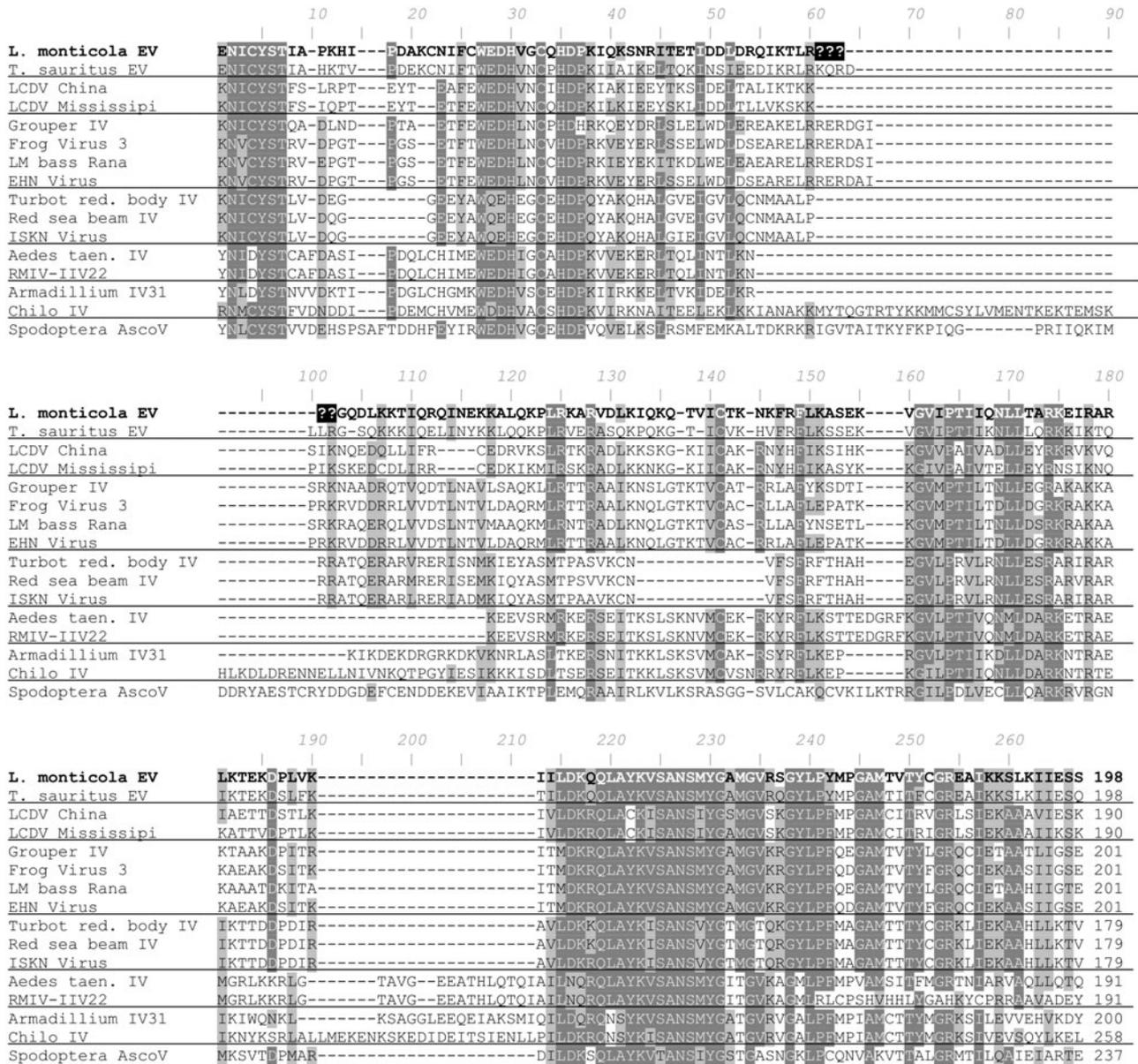
Two PCR products were observed in agarose gels. A band of about 800 bp was excised from the gel and sequenced. 859 nt of the reverse sequence showed 100% identity with the corresponding FV3 sequence (accession number AY 548484.1).

## DISCUSSION

### The Erythrocytic Viruses

The red inclusions found in the erythrocytes of infected *L. monticola*, a species that replaces other lacertid species at altitudes above 1,600 m on the Serra da Estrela mountain, Portugal, are similar to those reported in a previous study involving animals from the same population (Alves de Matos et al., 2002). TEM data on the inclusions also confirm their association with large icosahedral particles, showing pentagonal or hexagonal profiles in sections that, in previous works, were tentatively assigned to the *Iridoviridae* (Telford & Jacobson, 1993). However, these viruses were never isolated, and only recently some success in the partial characterization of a similar virus from a snake based on a 628 bp sequence of the DNA dependent DNA polymerase gene was reported. The study of this sequence showed that the virus is significantly different from other known *Iridoviridae* and probably belongs to a new genus (Wellehan et al., 2008).

The viruses described in snakes, however, are considerably different from the ones found in *L. monticola*. The

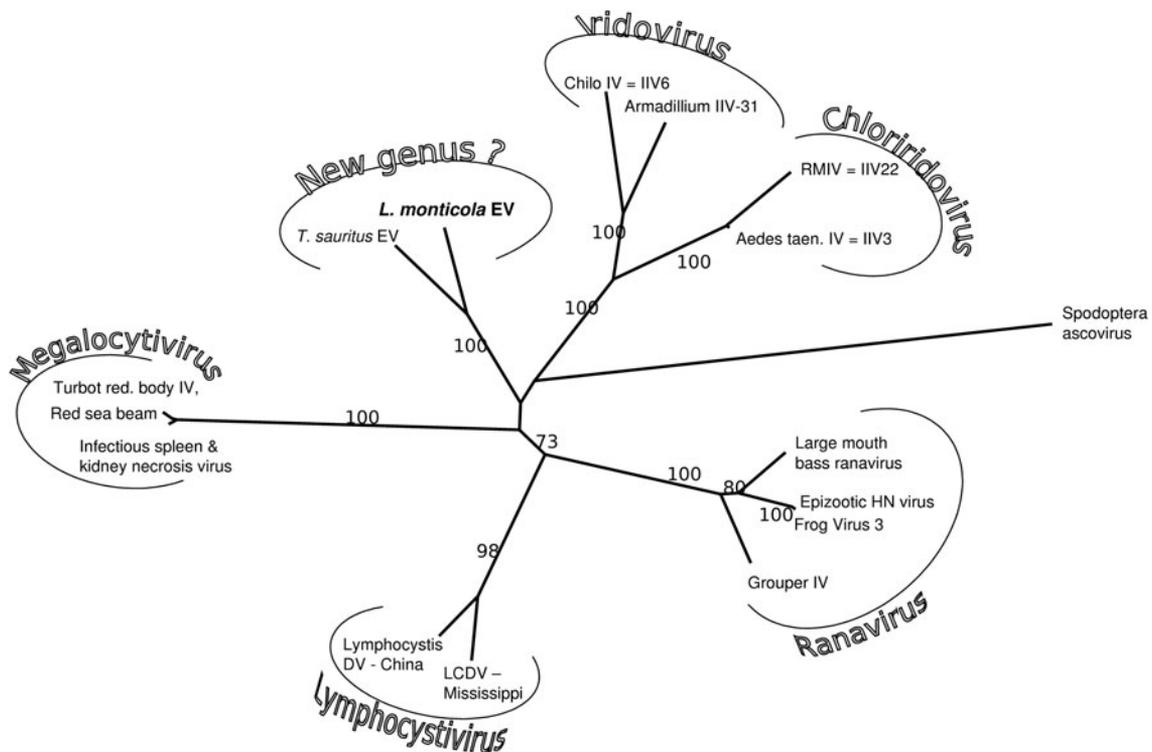


**Figure 6.** ClustalW alignment of deduced partial DNA dependent DNA polymerase amino acid sequences of different iridoviruses (IV). *Spodoptera* ascovirus was used as an outgroup. Identity and similarity shading refers to 80% among the presented sequences. Virus genera are separated by lines, *L. monticola* erythrocytic virus is in bold. (Please note that the *L. monticola* EV sequence is missing five amino acids at position 57-61, due to poor sequence read quality. This region as well as large gaps were omitted in the phylogenetic calculations.) GenBank Accession Numbers are: *T. sauritus* EV (EF608450), Frog Virus 3 (AY548484), Lymphocystis Disease Virus strain Mississippi (DQ159939), LCDV China (AY380826), Turbot reddish body IV (GQ273492), Chilo IV (AF303741), Infectious Spleen and Kidney Necrosis Virus (FN429981), Epizootic Haematopoietic Necrosis Virus (FJ433873), Red Sea Bream IV (AB007366), Grouper IV (AY666015), *Aedes taeniorhynchus* iridescent virus (NC\_008187), RMIV (CAC84133), *Armadillium* IV31 (CAC19196), Large Mouth bass Ranavirus (ABA41591), *Spodoptera ascovirus* (AAC54632)

internal structure of the virions of SEV is described as a solid dense core, while the LEV particle has a multilayered dense structure. Also SEV induces crystalline inclusions in infected cells (Johnsrude et al., 1997; Wellehan et al., 2008) that are not found in LEV-infected erythrocytes. Instead, multilamellar bodies are seen in the infected cells.

PCR amplifications targeting the same DNA polymerase sequence recovered from SEV, but using LEV-infected

blood samples were successful and sequences with 65.2/69.4% nt/aa identity with the one found in SEV were recovered. The combined ultrastructural and molecular data thus indicate that LEV and SEV are different but related viruses and lends further support for the existence of a large and diverse group of erythrocytic viruses infecting several species of heterothermic vertebrates (Alves de Matos & Paperna, 1993; Paperna & Alves de Matos, 1993) that may



**Figure 7.** Phylogenetic distance tree based on the deduced partial DNA dependent DNA polymerase amino acid sequences (173-193 aa) of different iridoviruses (IV). *S. ascovirus* (195 aa) was used as an outgroup. (Uncertain region of the *L. monticola* EV sequence as well as large gaps were omitted in the phylogenetic calculations.) Sequences were analyzed using the PHYLIP program package (DNA distance followed by FITCH). Bootstrap values over 60 from 100 resamplings of the FITCH are indicated beside the nodes. For GenBank accession numbers, see legend of Figure 6.

be related to each other and constitute a new genus within the *Iridoviridae* family (Fig. 7).

### The *Ranavirus*

Attempts to adapt the LEV to cell cultures were not successful, in agreement with the results of other authors working with large icosahedral erythrocytic viruses (Gruia-Gray et al., 1989). However, during the isolation attempts, a second virus, morphologically similar to the known *Ranavirus*, was recovered.

A PCR carried out for the detection of the *Ranavirus* MCP gene was positive for the isolated virus as well as for blood samples of LEV-infected animals, and the sequences of the PCR products were similar, confirming that the isolated virus is infecting wild lizards. This indicates that infection with the *Ranavirus* is probably common among lizards of the studied population.

Ranaviruses are emerging disease agents known to infect amphibians and fish (Chinchar et al., 2009), where they may contribute to the decline of amphibian populations (Gray et al., 2009) and are known to induce severe outbreaks of high mortality disease among aquaculture facilities (Whittington et al., 2010). Although most ranaviruses have been found in amphibians and fish, some reptile infections are also known (Hyatt et al., 2002). In lizards, there is only one report of a *Ranavirus* infection in a gecko (Marschang et al., 2005). In that case, only a single animal

out of a mixed collection of amphibians and reptiles died and was found to be *Ranavirus* positive. In addition, insect iridoviruses have been detected in various lizards in Germany (Just et al., 2001; Weinmann et al., 2007).

The partial sequence characterization of the *Ranavirus* detected in these animals indicated that it was closely related to FV3, the type species of this genus. This has been true for all ranaviruses detected in reptiles and partially characterized to date. It has been discussed whether these viruses switched host species from fish or from amphibians (Jancovich et al., 2010). The host specificity of these “reptilian” ranaviruses is unknown. Many ranaviruses have very wide host ranges. The presence of this ranavirus in wild *L. monticola* in Portugal could therefore have ramifications for a variety of lower vertebrate species in the mountain regions where these lizards live.

Ultrastructural details of the cell culture-adapted virus showed features similar to known *Ranavirus* ultrastructural features, such as the presence of a VAS surrounded by mitochondria, accumulation of paracrystalline groups of virions in the cytoplasm, and virion release by budding (Devauchelle et al., 1985; Chinchar et al., 2009). New features such as the densification of the VAS in late stages of infection do occur.

A striking feature of this isolate was the presence of intranuclear viruses in about 20% of the nuclei of infected cells. This feature was previously seen by Kelly and Atkinson (1975), but to our knowledge has not been further reported.

The involved nuclei have a high content of virions that may form paracrystalline aggregates, and virion concentration may be much higher in the nucleus than in the surrounding cytoplasm. Interestingly, the presence of incomplete capsids suggests that a morphogenetic process may be occurring within the nucleus. These particles often associate with medium density material of unknown nature that interrupts the dense strands of condensed chromatin. These images suggest the existence of an additional intranuclear morphogenetic process.

It is known that members of the NCLDV have large genomes that codify for many of the tasks needed for DNA replication and virion assembly with a high degree of independence of the infected cell (Yutin et al., 2009). Also, DNA of FV3 is known to have an intranuclear replication and methylation step, but it is thought to migrate to the cytoplasm to be encapsidated (Schetter et al., 1993; Chinchar et al., 2009). Our observations, following those of Kelly and Atkinson (1975) suggest that, under some circumstances, not only DNA replication but also morphogenesis of the virus and DNA encapsidation may occur within the nucleus that may constitute an important phenomenon for understanding the biology of the virus.

## REFERENCES

- ALVES DE MATOS, A.P. & PAPERNA, I. (1993). Ultrastructural study of *Pirhemocytion* virus in lizard erythrocytes. *Ann Parasitol Hum Comp* **68**, 24–33.
- ALVES DE MATOS, A.P., PAPERNA, I. & CRESPO, E. (2002). Experimental Infection of lacertids with lizard erythrocytic viruses. *Intervirology* **45**, 150–159.
- BONFIELD, J.K., SMITH, K.F. & STADEN, R. (1995). A new DNA sequence assembly program. *Nucleic Acids Res* **24**, 4992–4999.
- CHINCHAR, V.G., HYATT, A., MIYAZAKI, T. & WILLIAMS, T. (2009). Family Iridoviridae: Poor viral relations no longer. *Curr Top Microbiol Immunol* **328**, 123–170.
- DEVAUCHELLE, G., STOLTZ, D.B. & DARCY-TRIPIER, F. (1985). Comparative ultrastructure of Iridoviridae. *Curr Top Microbiol Immunol* **116**, 1–21.
- FELSENSTEIN, J. (1989). PHYLIP—Phylogeny Inference Package. *Cladistics* **5**, 164–166.
- GRAY, M.J., MILLER, D.L. & HOVERMAN, J.T. (2009). Ecology and pathology of amphibian ranaviruses. *Dis Aquat Organ* **87**, 243–66.
- GRUIA-GRAY, J., PETRIC, M. & DESSER, S.S. (1989). Ultrastructural, biochemical, and biological properties of an erythrocytic virus of frogs from Ontario, Canada. In *Viruses of Lower Vertebrates*, Ahne, W. & Kurstak, E. (Eds.), pp. 69–78. Berlin: Springer-Verlag.
- GRUIA-GRAY, J., RINGUETTE, M. & DESSER, S.S. (1992). Cytoplasmic localization of the DNA virus frog erythrocytic virus. *Intervirology* **33**, 159–164.
- HALL, T.A. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser* **41**, 95–98.
- HANSON, L.A., RUDIS, M.R., VASQUEZ-LEE, M. & MONTGOMERY, R.D. (2006). A broadly applicable method to characterize large DNA viruses and adenoviruses based on the DNA polymerase gene. *Virology J* **3**, 28–37.
- HYATT, A.D., WILLIAMSON, M., COUPAR, B.E., MIDDLETON, D., HENGSTBERGER, S.G., GOULD, A.R., SELLECK, P., WISE, T.G., KATTENBELT, J., CUNNINGHAM, A.A. & LEE, J. (2002). First identification of a ranavirus from green pythons (*Chondropython viridis*). *J Wildl Dis* **38**, 239–252.
- JANCOVICH, J.K., BREMONT, M., TOUCHMAN, J.W. & JACOBS, B.L. (2010). Evidence for multiple recent host species shifts among the Ranaviruses (family Iridoviridae). *J Virol* **84**, 2636–2647.
- JOHNSRUDE, J.D., RASKIN, R.E., HOGE, A.Y.A. & ERDOS, G.W. (1997). Intraerythrocytic inclusions associated with iridoviral infection in a fer de lance (*Bothrops moojeni*) snake. *Vet Pathol* **34**, 235–238.
- JOHNSTON, M.R.L. (1975). Distribution of *Pirhemocytion* Chatton and Blanc and other, possibly related, infections of poikilotherms. *J Protozool* **22**, 529–535.
- JUST, F., ESSBAUER, S., AHNE, W. & BLAHAK, S. (2001). Occurrence of an invertebrate iridescent-like virus (Iridoviridae) in reptiles. *J Vet Med B Infect Dis Vet Public Health* **48**, 685–694.
- KELLY, D.C. & ATKINSON, M.A. (1975). FV3 replication: Electron microscopic observations on the terminal stages of infection in chronically infected cell cultures. *J Gen Virol* **28**, 391–407.
- MAO, J., HEDRICK, R.P. & CHINCHAR, G. (1997). Molecular characterization, sequence analysis and taxonomic position of newly isolated fish iridoviruses. *Virology* **229**, 212–220.
- MARSCHANG, R.E., BRAUN, S. & BECHER, P. (2005). Isolation of a ranavirus from a gecko (*Uroplatus fimbriatus*). *J Zoo Wildl Med* **36**, 295–300.
- PAPERNA, I. & ALVES DE MATOS, A.P. (1993). Erythrocytic viral infections of lizards and frogs: New hosts, geographical locations and description of the infection process. *Ann Parasitol Hum Comp* **68**, 11–23.
- SCHETTER, C., GRÜNEMANN, B., HÖLKER, I. & DOERFLER, W. (1993). Patterns of frog virus 3 DNA methylation and DNA methyltransferase activity in nuclei of infected cells. *J Virol* **67**, 6973–6978.
- TELFORD, S.R. & JACOBSON, E.R. (1993). Lizard erythrocytic virus in east African chameleons. *J Wildl Dis* **29**, 57–63.
- WEINMANN, N., PAPP, T., ALVES DE MATOS, A.P., TEIFKE, J.P. & MARSCHANG, R.E. (2007). Experimental infection of crickets (*Gryllus bimaculatus*) with an invertebrate iridovirus isolated from a high-casqued chameleon (*Chamaeleo hoehnelii*). *J Vet Diagn Invest* **19**, 674–679.
- WELLEHAN, J.F., JR., STRIK, N.I., STACY, B.A., CHILDRESS, A.L., JACOBSON, E.R. & TELFORD, S.R., JR. (2008). Characterization of an erythrocytic virus in the family Iridoviridae from a peninsula ribbon snake (*Thamnophis sauritus sackenii*). *Vet Microbiol* **131**, 115–122.
- WHITTINGTON, R.J., BECKER, J.A. & DENNIS, M.M. (2010). Iridovirus infections in finfish—Critical review with emphasis on ranaviruses. *J Fish Dis* **33**, 95–122.
- WILLIAMS, T., BARBOSA-SOLOMIEU, V. & CHINCHAR, V.G. (2005). A decade of advances in iridovirus research. *Adv Virus Res* **65**, 173–248.
- YUTIN, N., WOLF, Y.I., RAOULT, D. & KOONIN, E.V. (2009). Eukaryotic large nucleo-cytoplasmic DNA viruses: Clusters of orthologous genes and reconstruction of viral genome evolution. *Virology J* **6**, 223–235.