

Isolation and characterization of iridoviruses from the giant toad *Bufo marinus* in Venezuela

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ABSTRACT: In this communication we describe for the first time the isolation of 7 iridoviruses from the toad *Bufo marinus* and an unknown species of frog *Leptodactylus* in Venezuela, South America. The viruses are icosahedral with electron-dense cores, each of which is surrounded by an inner membrane, capsid and a cell-derived envelope. The virus(es) have an average vertex to vertex diameter of 160 nm and replicate in the cytoplasm of a range of cell lines. Within the cytoplasm of infected cells, rarified areas could be observed; structures lacked cellular organelles and contained complete, empty and developing viruses. Results from antigen-capture enzyme-linked immunosorbent assays (ELISA) with polyclonal antibody raised against epizootic haematopoietic necrosis virus (EHNV) indicated cross-reactivity between these isolates, Bohle iridovirus (BIV) and frog virus 3 (FV3). Comparison of polypeptide and genomic profiles indicated that the Venezuelan viruses shared many polypeptides of equivalent molecular weight with type species FV3. There were, however, differences between the group of Venezuelan viruses and FV3 and BIV. The viruses belong to the family *Iridoviridae* and the genus *Ranavirus*.

KEY WORDS: *Bufo marinus* · Bohle virus · Frog virus 3 · Iridovirus · Ranavirus

INTRODUCTION

The family *Iridoviridae* encompasses 5 genera: *Iridovirus*, *Chloriridovirus*, *Ranavirus*, *Lymphocystivirus* and 'goldfish virus 1-like viruses' (Goorha 1995).

Many iridoviruses have been identified in both amphibian and piscine hosts. Piscine iridoviruses have been isolated from reelfin perch *Perca fluviatilis* (epizootic haematopoietic necrosis virus, EHNV) in Australia (Langdon et al. 1986), the catfish *Ictalurus melas* (Pozet et al. 1992) in France, the sheatfish *Silurus glanis* (Ahne et al. 1989) in Germany, the ornamental guppy *Poecilia reticulata* and doctor fish *Labroides dimidiatus* in south-east Asia (Hedrick & McDowell 1995) and a range of fish from Japan and Thailand (refer to

Miyata et al. 1997). The amphibian iridoviruses include Bohle iridovirus (BIV) that was isolated in Australia from the ornate burrowing frog *Limnodynastes ornatus* (Speare & Smith 1992, Hengstberger et al. 1993), an iridovirus from the common frog *Rana temporaria* in the United Kingdom (Cunningham et al. 1993, Drury et al. 1995), frog virus 3 (FV3) from the leopard frog *Rana pipiens* in North America (Granoff et al. 1966), tadpole edema virus (TEV) from the American bullfrog *Rana catesbeiana* (Wolf et al. 1968), and iridoviruses from *Rana esculenta* in Croatia (Fijan et al. 1991).

EHNV, FV3, TEV, BIV, sheatfish iridovirus, catfish iridovirus and iridoviruses from guppy and doctor fish cross-react in antigen-capture enzyme-linked immunosorbent assays (ELISAs) using antisera against EHNV (Essani & Granoff 1989, Hedrick et al. 1992, Hengstberger et al. 1993, Hedrick & McDowell 1995, Mao et

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al. 1997). The cross-reactivity of these viruses indicated that they probably belong to the same genus, namely *Ranavirus*. Recent data from Mao et al. (1997) confirm that the Australian iridoviruses, European iridoviruses, and iridoviruses from guppy and doctor fish belong to the genus *Ranavirus*. These findings suggest that ranaviruses are broadly distributed throughout the world and infect a range of anuran and piscine species.

To date only one iridovirus, an erythrocytic iridovirus, has been detected in *Bufo marinus* (Speare et al. 1991). This virus is not a recognised member of the *Ranavirus* genus; it belongs to an undescribed group of erythrocytic iridoviruses. In this report, we describe for the first time the isolation and characterisation of ranaviruses from free-ranging *B. marinus* in Venezuela, South America.

MATERIAL AND METHODS

Animals and processing of samples. A total of 206 free-ranging toads *Bufo marinus* and 13 frogs of an unknown species of *Leptodactylus* were collected from 45 locations in Venezuela during 1992 and 1995 (Table 1). The toad and frogs were sacrificed by cranial pithing. Samples of liver, lung, kidney and spleen were collected and macerated separately. Homogenates (in phosphate buffered saline) were centrifuged at $800 \times g$ for 15 min at 4°C and the resulting supernatant filtered through 0.45 µm cellulose membranes and inoculated (10% solution) onto tissue culture cells.

Cells and media. Turtle heart subline B1(TH-1, ATCC CCL 50) cells were grown in Basal Eagle's Medium (BME), supplemented with 10% foetal bovine serum (FBS). Fathead minnow (FHM, ATCC CCL 42) cells were cultured in Eagle's Minimum Essential Medium (EMEM) and 10% FBS. A6 (kidney, South African clawed toad) cells (ATCC CCL 102) from *Xenopus laevis* were grown in 75% NCTC (National Cancer Tissue Culture) 109 medium supplemented with 10% FBS. Bluegill fry (BF-2 ATCC CCL 81), chinook salmon embryo (CHSE-214, ATCC CRL 1681) and Vero cells (ATCC CCL 81) were grown in EMEM with 10% FBS. Baby hamster kidney (BHK-21) cells, referred to hereafter as BHK (Commonwealth Serum Laboratories, Mel-

bourne, Australia), were grown in EMEM containing 10% FBS, 10 mM HEPES (N-2-hydroxyethylpiperazine-N ethanesulphonic acid) and 5% tryptose phosphate broth. All cell lines were kept at 24°C (room temperature, RT) and media were supplemented with 10 mM HEPES, 100 U of penicillin and 100 µg streptomycin.

Cell inoculations. The supernatants of filtered organ (liver, lung, kidney and spleen) homogenates (0.2 ml

Table 1. Origin, species and number (n) of animals examined

Origin (Venezuela)	Coordinates	Species	(n)
Altigracia de Orituco	9°51' N, 66°23' W	<i>Bufo marinus</i>	1
Guatopo National Park	10°12' N, 66°30' W	<i>B. marinus</i>	24
Boca de Uchire	10°8' N, 65°26' W	<i>B. marinus</i>	1
Bruzual	8°5' N, 69°21' W	<i>B. marinus</i>	2
Caicara del Orinoco	7°39' N, 66°11' W	<i>B. marinus</i>	2
Canta Los Gallo	11°7' N, 72°2' W	<i>B. marinus</i>	4
Choroni	10°31' N, 67°36' W	<i>B. marinus</i>	2
Clarines	9°57' N, 65°11' W	<i>B. marinus</i>	2
Coro	11°24' N, 69°41' W	<i>B. marinus</i>	8
Cuao (River Cuao)	5°19' N, 69°17' W	<i>Leptodactylus</i> sp.	12
Cumana	10°29' N, 64°12' W	<i>B. marinus</i>	30
Cuyagua	10°29' N, 67°47' W	<i>B. marinus</i>	2
Tucupita	9°3' N, 62°4' W	<i>B. marinus</i>	4
El Frio	7°24' N, 70°29' W	<i>B. marinus</i>	2
Elorza	7°3' N, 69°30' W	<i>B. marinus</i>	3
El Tigre	8°54' N, 69°15' W	<i>B. marinus</i>	3
El Vigia	8°37' N, 71°39' W	<i>B. marinus</i>	2
Falcon; Buena Vista	11°56' N, 69°58' W	<i>B. marinus</i>	4
Guiria Sucre	10°35' N, 62°18' W	<i>B. marinus</i>	2
Higuerote	10°29' N, 66°7' W	<i>B. marinus</i>	4
La Victoria	10°13' N, 67°13' W	<i>B. marinus</i>	2
Lagunilla	8°31' N, 71°24' W	<i>B. marinus</i>	2
Mapire	7°45' N, 64°43' W	<i>B. marinus</i>	5
Margarita (Macanao)	11°4' N, 64°16' W	<i>B. marinus</i>	8
Masaguaral Hata Flores	8°32' N, 67°28' W	<i>B. marinus</i>	1
Maturin	9°45' N, 63°10' W	<i>B. marinus</i>	10
Maturin	9°45' N, 63°10' W	<i>Leptodactylus</i> sp.	1
Merida (via Los Araquez)	8°31' N, 71°9' W	<i>B. marinus</i>	6
Paraguana	12°2' N, 70°3' W	<i>B. marinus</i>	1
Pinero (Hato Pinero)	9°42' N, 68°58' W	<i>B. marinus</i>	9
Puerto Ayacucho	5°40' N, 67°39' W	<i>B. marinus</i>	6
Puerto Columbiana	10°31' N, 67°36' W	<i>B. marinus</i>	22
Puerto la Cruz	10°32' N, 67°21' W	<i>B. marinus</i>	1
Puerto Piritu	10°3' N, 65°3' W	<i>B. marinus</i>	1
Quibor	9°55' N, 69°38' W	<i>B. marinus</i>	2
Sabana Mendoza	7°3' N, 71°40' W	<i>B. marinus</i>	2
Santa Elena	4°58' N, 60°55' W	<i>B. marinus</i>	2
San Felipe	5°11' N, 61°16' W	<i>B. marinus</i>	1
San Felipe (Apure)	6°53' N, 69°43' W	<i>B. marinus</i>	1
San Juan de los Morros	9°54' N, 67°22' W	<i>B. marinus</i>	4
San Luis	10°28' N, 64°11' W	<i>B. marinus</i>	2
Santa Teresa	10°14' N, 66°4' W	<i>B. marinus</i>	2
Temblador Monages	9°0' N, 62°38' W	<i>B. marinus</i>	2
Tinaguillo	9°54' N, 68°19' W	<i>B. marinus</i>	1
Trujillo	9°22' N, 70°26' W	<i>B. marinus</i>	2
Tucacas edo Falcon	10°47' N, 68°20' W	<i>B. marinus</i>	1
Universidad Simon Bolivar	10°30' N, 66°50' W	<i>B. marinus</i>	2
Uputa - Bolivar	8°1' N, 62°24' W	<i>B. marinus</i>	1
Urumaco	11°12' N, 70°15' W	<i>B. marinus</i>	1
Valle de la Pascua	9°12' N, 66°1' W	<i>B. marinus</i>	2
Zarasa	9°20' N, 65°19' W	<i>B. marinus</i>	2
Total			219

well⁻¹) from individual toads and frogs were added to monolayers of TH-1 and A6 cells in 24-well cluster plates (Nunc). Each inoculum was allowed to adsorb for 1 h at RT after which it was removed and cells replenished with the corresponding medium. Thereafter, cells were kept at RT and examined daily. At 5 d post-infection (pi) the culture supernatants were transferred to confluent FHM cell monolayers.

Supernatants from FHM infected cells which displayed a cytopathic effect (CPE) were inoculated onto FHM-, CHSE-, BHK-, Vero- and BF-2 cells. The supernatants were adsorbed for 1 h at RT and then replaced with the appropriate maintenance media. The supernatants were harvested 7 d pi and examined by ELISA (refer below).

ELISA. The EHNV-antigen capture ELISA was based on that described by Hyatt et al. (1991). The assay was used to determine whether the isolates from *Bufo marinus* and *Leptodactylus* sp. cross-reacted with antisera against EHNV and to evaluate the ability of the isolates to replicate in a range of cell lines (BHK, Vero, FHM, BF-2 and CHSE cells; Hengstberger et al. 1993). Briefly, the coating primary antibody was rabbit anti-EHNV (raised against gradient purified EHNV, isolated from rainbow trout *Oncorhynchus mykiss*), and the secondary antibody was sheep anti-EHNV (also raised against gradient purified EHNV isolated from rainbow trout) (Hyatt et al. 1991). The antigens (of known TCID₅₀ per ml, refer below) consisted of neat supernatants from FHM cells infected with each of the isolated viruses, BIV and FV3. The substrates and other specifics of the assay are described by Hyatt et al. (1991). Optical densities (OD) were read in a Lab-systems Multiscan MS plate reader at 450 nm, and recorded as: (sample OD) – (OD of uninfected cells). The control antigens were EHNV 86/8774 and were used at dilutions of 1:10, 1:100, 1:500 and 1:10000. The positive-negative values for the ELISA are approximated by the OD value of the 1:500 dilution of the EHNV control (A. D. Hyatt & R. J. Whittington unpubl. data). The ELISA was also used to assess the ability of the viruses to grow at 18, 24 (RT), 26, 30 and 37°C in Vero cells.

Electron microscopy. TH-1 cells infected with second passage supernatant were scraped and pelleted at 48 h pi. Pellets were fixed in phosphate (Sörensen's) buffered 2.5% (v/v) glutaraldehyde, washed with Sörensen's buffer (3 × 20 min), post-fixed in 1% (w/v) osmium tetroxide, dehydrated in ethanol (70 to 100%), infiltrated with propylene oxide and embedded in Medcast Epon resin. Ultra-thin sections were double-stained in uranyl acetate and lead citrate. Intracellular viruses located within cytoplasmic aggregates and exhibiting a hexagonal outline were measured vertex to vertex. The dimensions of the particles were calculated using a calliper (Mitutoyo Corp. model CD-6" P).

All Venezuelan isolates were inoculated onto grid-cell FHM cultures (Hyatt et al. 1987). At 18 h pi the cultures were negatively stained with 2% phosphotungstic acid (PTA) adjusted to pH 6.8 with 1 M KOH.

Specimens were examined with a Philips CM10 transmission electron microscope at 80 kV or a Hitachi H7000 transmission electron microscope at 75 kV. The H7000 (used for determining the diameter of the viruses) was calibrated with a 2100 lines mm⁻¹ replica standard.

Pulse radiolabelling and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). FHM cells were seeded at 2 × 10⁵ cells well⁻¹ in 24-well Linbro tissue culture (TC) plates. Cells were mock treated or inoculated (1 h) with tissue culture-passaged viruses (7 South American isolates, BIV, and FV3). The infective dose of each virus stock was determined empirically as to produce analogous polypeptide (band) densities in the SDS-PAGE. At 29 h pi, cells were washed and incubated 30 min in Met-, Cys-deficient EMEM (ICN Pharmaceuticals, Costa Mesa, CA, USA); this was replaced by deficient EMEM which contained 200 µCi ml⁻¹ of a ³⁵S-Met/Cys mixture (Tran³⁵S-Label, ICN Pharmaceuticals), and metabolic labelling was performed from 30 to 32 h pi. Cells were harvested by centrifugation (14 000 × g, 2 min). Supernatants and cell pellets were frozen in liquid nitrogen and stored at -20°C until separated by SDS-PAGE on 8 to 16% Tris-glycine linear gradient gels (Novex, San Diego, CA, USA) at 120 V. Cell pellets were prepared by resuspension in 20 µl of 10 mM Tris-HCl, 1 mM EDTA (TE buffer, pH 7.2) containing 2 mM PMSF (phenylmethylsulfonyl fluoride; Sigma Chemical Company, St. Louis, MO, USA), followed by addition of 10 µl of 3 × electrophoresis sample buffer (ESB; New England Biolabs, Beverly, MA, USA). Viscosity of lysed cells was reduced by sonication (Braunsonic Model 720) and heated (100°C, 5 min), microfuged briefly, and the cell extract (equivalent to 5 × 10⁴ cells) was loaded into the wells of the gell for electrophoresis. Broad range pre-stained molecular weight standards (New England Biolabs) were used for calibration. Gels were subsequently stained with Commassie Brilliant Blue, then dried and exposed on Kodak BioMax autoradiography film.

DNA purification. Monolayers of CHSE cells (one 150 cm² flask per virus) were infected with (10⁶ TCID₅₀ per ml) FV3, BIV and the 7 South American viruses. When CPEs had reached 70 to 80%, cells were removed from the flask, pelleted (800 × g for 10 min) and the pellets frozen at -20°C. Pellets were thawed, resuspended in 1 ml of TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8) and the virus released from the cell by shearing in a motor-driven Dounce homogeniser (Kontes Glass Co, Vineland, NJ, USA). The resultant homogenate was pelleted (800 × g, 10 min) and virus DNA purified from the supernatant (0.4 ml) using a genomic

DNA purification kit (Qiagen, USA). Isopropanol ($\times 0.7$ vol, RT) was added to the eluted samples. Tubes were inverted 10 times, centrifuged ($10\,000 \times g$) for 15 min and the supernatants carefully removed. Viral DNA was washed with 1 ml of cold 70% ethanol followed by air drying for 10 min and resuspended in 50 μ l of the TE buffer with RNAase A (10 μ g ml⁻¹, DNase-free Genesearch). To determine the concentration and yield of DNA, 2 μ l of each sample was diluted in 1 ml of TC water [reverse osmosis, sterilised (121°C, 15 min), 18.3 M Ω]. The spectrophotometer was calibrated with TC water and the OD of samples measured at A₂₆₀ and A₂₈₀. Equivalent concentrations (determined by absorbance, refer above) of DNA were used in the following experiments.

Restriction endonuclease hybridisation analyses. Restriction endonuclease (RE) digestion (with *Hpa*II, *Msp*I, *Bam*HI, *Hind*III and *Xba*I) and agarose gel electrophoresis were carried out as described by Coupar et al. (1990) and Hengstberger et al. (1993). For hybridisation studies, 3-fold dilutions of sonicated DNA [approximately 0.6 μ g (initial amount) for each virus isolate, in addition to vaccinia virus (negative control)] in $10 \times$ SSC (saline sodium citrate buffer) were denatured at 95°C for 10 min, cooled on ice and loaded in equivalent concentrations onto duplicate Gene Screen Plus (NEN) membranes. Membranes were hybridized with nick translated ³²P-labelled FV3 or the isolate GV overnight at 42°C in buffer containing 50% formamide. Membranes were washed with $2 \times$ SSC/0.1% sodium dodecyl sulphate (SDS) followed by $0.1 \times$ SSC/0.1% SDS at 60°C and exposed to X-ray film.

RESULTS

Isolation of viruses

Animals exhibited no external lesions or internal signs of disease. No histopathology was performed.

CPE was observed from TH-1 cells inoculated with homogenates from 6 individual *Bufo marinus* toads and 1 unknown species of *Leptodactylus*. The isolates are referred to as Cum5, Cum6, Cum30, Mat1, Mat2, GV and Mg1 (Table 2). The CPE was generally observed 4 d pi in TH-1 cells inoculated with tissue homogenates. CPE was initially identified by the presence of scattered clusters of round and refringent cells followed by the formation of punctate plaques. The CPE expanded over the course of infection until the remaining cells detached from the substrate. In contrast, the CPE in A6 cells took longer, generally 6 d pi, and consisted of patches of rounded cells forming indistinct plaques. CPE in the form of punctate plaques was also identified after 2 passages in FHM cells.

Table 2. Species and geographical locations from which viruses were isolated

Species	Name of isolate	Name of location in Venezuela
<i>Bufo marinus</i>	Cum5	Cumana
<i>Bufo marinus</i>	Cum6	Cumana
<i>Bufo marinus</i>	Cum30	Cumana
<i>Leptodactylus</i> sp.	Mat1	Maturin
<i>Bufo marinus</i>	Mat2	Maturin
<i>Bufo marinus</i>	GV	Guatopo National Park
<i>Bufo marinus</i>	Mg1	Margarita Island

Electron microscopy

Thin section analysis of glutaraldehyde-fixed infected TH-1 cells revealed the presence of cytoplasmic paracrystalline arrays of icosahedral viruses (Fig. 1A). The arrays were located both within rarified areas of the cytoplasm and in the surrounding cytoplasm. The cytoplasmic viruses were observed at different stages of assembly, ranging from complete icosahedral particles containing electron-dense cores to incomplete particles containing empty to partially full cores. The average diameter for each of the intracellular viruses of *Bufo marinus* (determined from ultra-thin sections of infected cells) was approximately 160 nm (Table 3) from vertex to vertex. The average diameter of mature viruses within the unknown *Leptodactylus* species was 168 ± 8 nm ($n = 102$) from vertex to vertex (Table 3). Viruses were also observed budding from the infected cells (Fig. 1B), where they acquired a host-derived membrane that encapsulated the virus. No particles were observed inside the cell nuclei. Examination of infected grid-cell cultures (Fig. 1C) revealed the presence of large numbers of icosahedral viruses adsorbed to the grid substrate. The viruses possessed an outer envelope, a capsid that, in turn, was surrounded by an inner membrane.

ELISA

FHM, Vero, BHK, BF-2 and CHSE cells were inoculated with each of the virus isolates (Table 2) and control virus (FV3). At 7 d pi the supernatants were examined in the EHNv-capture ELISA. TCID₅₀ values per ml for GV, Mat1, Mat2, Cum5, Cum6, Cum30, Mg1 and FV3 were $10^{5.2}$, $10^{5.9}$, $10^{5.5}$, $10^{5.0}$, $10^{6.5}$, $10^{5.2}$, $10^{6.7}$ and $10^{4.7}$ respectively. The ELISA results (Fig. 2) show that most virus isolates replicate (OD positive-negative cutoff 0.3, data not shown) in each of the cell lines at 24°C (RT). The one exception was FV3 in BF-2 cells.

The Venezuelan viruses, represented by GV, replicated at 18 to 30°C, but not at 37°C (Fig. 3). These

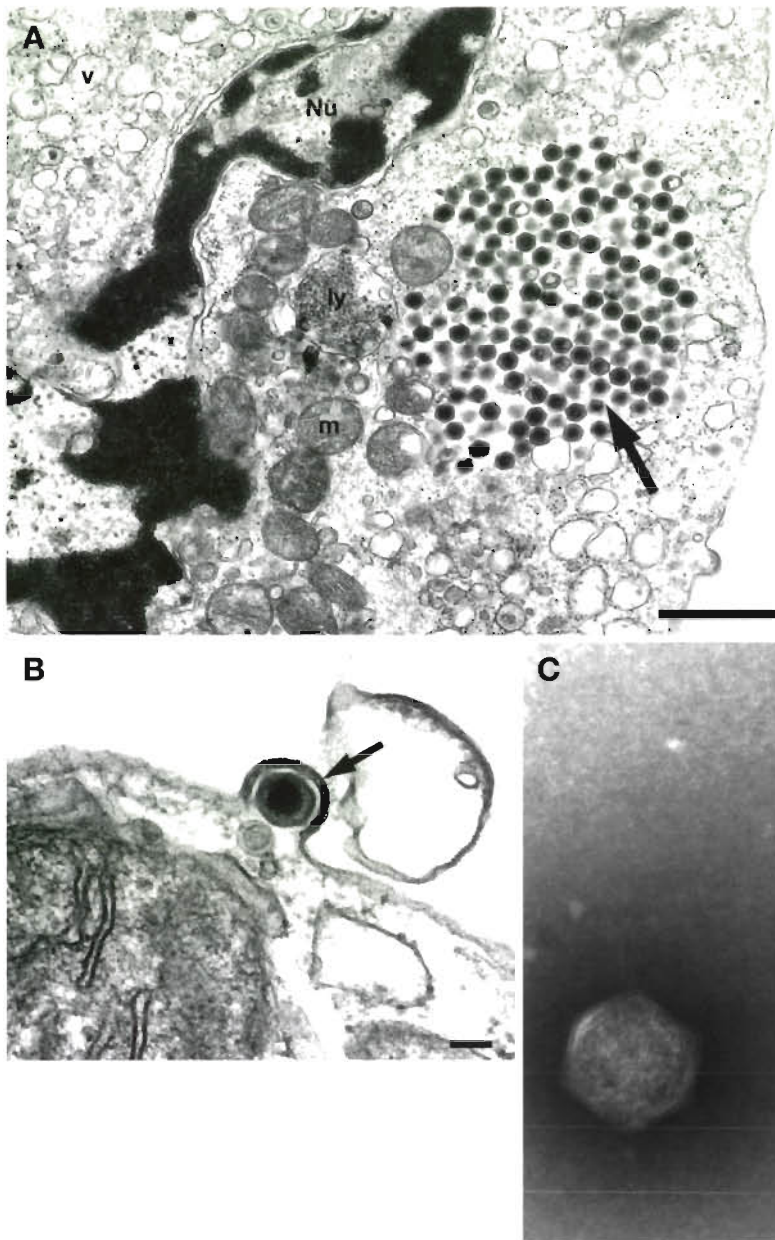


Fig. 1 Transmission electron micrographs of Guatopo virus-infected cells. (A) Cytoplasmic array of icosahedral viruses (large arrow). Nu: nucleus; m: mitochondrion; ly: lysosome; v: distended vesicles of the endoplasmic reticulum. Scale bar = 1 µm. (B) Virus budding (arrow) from the plasma membrane of an infected cell. Scale bar = 100 nm. (C) Negative staining of GV. Arrowhead: cell-derived envelope; small arrow: capsid; large arrow: inner de novo membrane. Scale bar = 100 nm

Table 3. Average diameter (vertex to vertex) of BIV, FV3 and the Venezuelan isolates. All measurements were derived from complete virions within cytoplasmic virus arrays. n: number of viruses analysed; SD: standard deviation

Name of isolate	n	Average diameter (nm) ± SD
Cum5	91	156 ± 6
Cum6	90	157 ± 9
Cum30	62	154 ± 7
Mat1	102	168 ± 8
Mat2	96	157 ± 8
GV	78	160 ± 8
Mg1	73	153 ± 8
BIV	73	155 ± 8
FV3	100	168 ± 6

results are similar to those derived from FV3-infected cells incubated at similar temperatures (Fig. 3). The replication of FV3 differed from GV in that the OD from infected cells was comparatively less at 18°C (Fig. 3).

Protein composition of purified South American viruses

Fig. 4 shows intracellular virus-specific proteins of FV3, BIV and the 7 Venezuelan virus isolates metabolically labelled at late times pi. Each virus isolate possessed a minimum of 20 distinguishable polypeptides ranging from approximately 8 to 121 kDa. The poly-

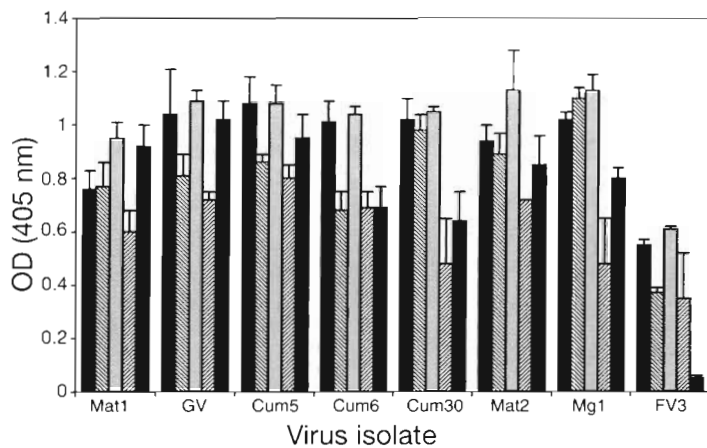


Fig. 2. Results (optical densities, OD) from an EHNv antigen-capture ELISA in which the supernatants from infected (Venezuelan viruses and FV3) CHSE-, FHM-, Vero-, BHK-, and BF-2 cells were analysed. Cells were maintained at 24°C (RT). Supernatants were harvested 7 d pi. (■) Vero, (▨) CHSE, (□) BHK, (▤) FHM, (■) BF-2 cells

peptide profiles for the Venezuelan virus isolates were very similar and each possessed a major polypeptide of approximately 49 kDa that is characteristic for rana-viruses. Whilst there was an overall similarity between isolates, Cum30 and Mat1 differed in that both possessed a 78 kDa polypeptide; Mat1, however, lacked a 29 kDa polypeptide, which was present in Cum30, BIV and FV3.

Collectively, the polypeptide patterns of the Venezuelan isolates were similar to the amphibian viruses BIV and FV3, i.e. all viruses possessed many polypeptides of equivalent molecular weights (e.g. 65, 59, 49, 45, 41, 34, 32, 28, 24, 17 and 12 kDa). The Venezuelan isolates, however, differed to BIV by the absence of a

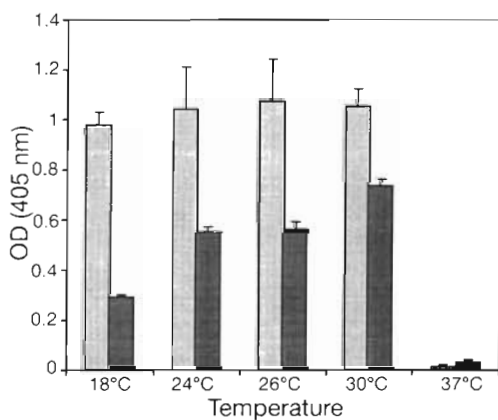


Fig. 3. Results (optical densities, OD) from an EHNv antigen-capture ELISA in which the supernatants from infected (GV and FV3) Vero cells were analysed. The cells were maintained at 18, 24 (RT), 26, 30 and 37°C. Supernatants were harvested 7 d pi. (□) GV; (■) FV3

prominent 72 kDa polypeptide and to both BIV and FV3 in the polypeptide pattern within the region of 103 and 78 kDa. In this band of polypeptides, the Venezuelan viruses possessed a similar profile (90, 98, 103 and 121 kDa). Most Venezuelan isolates also possessed a 76 kDa protein (with the exception of Cum30 and Mat1). Similarly, most Venezuelan isolates possessed a 78 kDa polypeptide with the possible exception of Cum6. BIV and FV3, however, had different polypeptide patterns within this molecular weight range (Fig. 4), the most noticeable difference being the absence of the 90 and 98 kDa polypeptides.

Restriction endonuclease digestion and hybridization analyses

Fig. 5A, B, C shows RE digestion profiles for DNA isolated from the 7 Venezuelan viruses, BIV and FV3. Digestion of DNA from the Venezuelan viruses with *Bam*HI, *Hind*III and *Xba*I resulted in similar fragments. These profiles were different from those produced by the digestion of DNA of BIV with *Bam*HI, *Hind*III, *Xba*I and from the digestion of DNA of FV3 with *Hind*III, and *Xba*I.

Nucleic acid from the 7 Venezuelan viruses was also digested with *Hpa*II and *Msp*I. Both enzymes identify

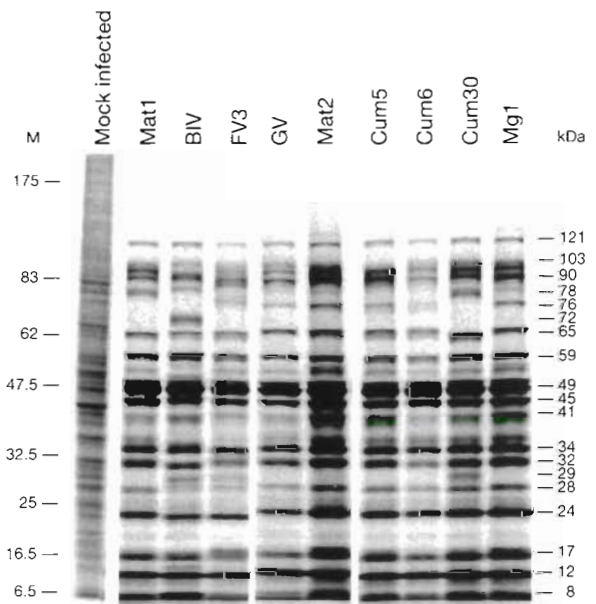
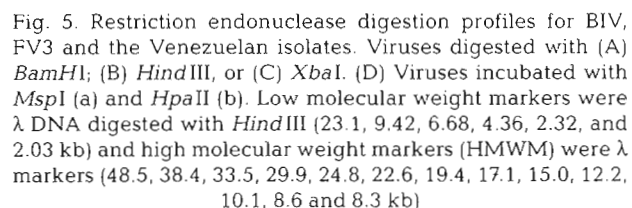
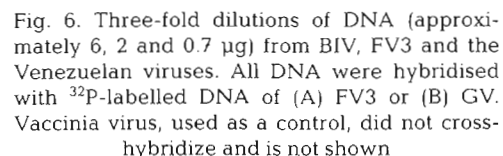


Fig. 4. Autoradiograph of a 10 to 20% SDS-PAGE of ³⁵S-methionine labelled intracellular proteins harvested from BIV, FV3 and Venezuelan virus-infected FHM cells. Molecular weights (kDa) were estimated by reference to stained markers (M)



The hybridisation of DNA of virus isolates with nick translated ^{32}P -labelled FV3 and GV DNA is shown in Fig. 6. The blots were washed under high stringency conditions and all DNA preparations, except that from vaccinia virus, hybridised, thereby showing a high level of sequence homology. As the concentrations of DNA used in this experiment were approximate, no inferences are made from the intensities of the individual spots.

HNV antigen-capture ELISA indigenous viruses described in this study share characteristics with ranaviruses. The cross-reactive epitopes with BIV are located on the surface of the structure of infected cells, such as cytoplasmic inclusions, cytoplasmic virus arrays and viruses budding from the plasma membrane, is characteristic of ranaviruses (Tripiet et al. 1974, Hengstberger et al. 1991, Hengstberger et al. 1993). The ability of the viruses to replicate in a range of amphibian cell lines was also characteristic of ranaviruses (Hengstberger et al. 1993). GV,



representative of the Venezuelan viruses, replicated at temperatures from 18 to 30°C and unlike FV3 did not show an optimum temperature (from approximately 24 to approximately 30°C) for replication. Neither FV3 or GV replicated at 37°C; this is a characteristic of the ranavirus type species FV3 (Gravell & Granoff 1970). Examination of the viral proteins indicates that the Venezuelan virus isolates were very similar. These isolates shared many proteins with BIV and FV3 but lacked the 72 kDa present in BIV. The Venezuelan isolates also differed from both BIV and FV3 in the presence of different high molecular weight proteins. The methylated genomes of the isolates were characteristic for ranaviruses (Essani & Granoff 1989). The RE restriction digestion profiles for the Venezuelan isolates were very similar but are distinct from BIV and FV3.

The above data indicate that the Venezuelan viruses belong to the genus *Ranaviruses* within the family *Iridoviridae*. This inference is supported by the hybridisation data that showed a high degree of homology between the genomes of the Venezuelan isolates and FV3. The data derived from RE digestion of the DNA of all Venezuelan isolates suggest that the Venezuelan isolates are very similar. Sequencing of the isolates will determine whether these isolates are one species.

This is the first report of a ranavirus isolated from *Bufo marinus* and the first report of a ranavirus being isolated from an aquatic animal in South America. The isolation of GV is also of importance because it indicates that ranaviruses are present on most continents, i.e. North America (Essani & Granoff 1989), Australia (Hengstberger et al. 1993), Europe (Ahne et al. 1989, Fijan et al. 1991, Mao et al. 1997), Asia (Hedrick & McDowell 1995, Mao et al. 1997) and South America. Other viruses tentatively identified as ranaviruses have been identified in the United Kingdom (Cunningham et al. 1993, Drury et al. 1995) and in Japan, Hong Kong, Singapore and Thailand (Miyata et al. 1997).

Many of the viruses tentatively assigned as ranaviruses cause systemic diseases in fish and amphibians (Ahne et al. 1997). For example, BIV can, under experimental conditions, cause disease in multiple species of fish and amphibians, including barramundi *Lates calcarifer*, the toad *Bufo marinus* (Moody & Owens 1994), the ornate burrowing frog *Limnodynastes ornatus* (Speare & Smith 1992) and *L. terraereginae* and *Litoria latopalmata* (Cullen et al. 1995). Antibodies against ranaviruses have been detected in *B. marinus* populations in Australia and Venezuela (Zupanovic et al. 1998). This information together with the knowledge that GV can, under experimental conditions, cause disease in *B. marinus* (A. D. Hyatt & H. Parkes unpubl. results) suggests that *B. marinus* may be both a host and vector for ranaviruses such as GV.

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