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Long-term study of an infection with ranaviruses in a group of edible frogs (*Pelophylax* kl. *esculentus*) and partial characterization of two viruses based on four genomic regions

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ABSTRACT

Several edible frogs (*Pelophylax* kl. *esculentus*) collected into a single group from various ponds in Europe died suddenly with reddening of the skin (legs, abdomen) and haemorrhages in the gastrointestinal tract. Ranavirus was detected in some of the dead frogs using PCR, and virus was also isolated in cell culture. Over the following 3 years, another two outbreaks occurred with low to high mortality in between asymptomatic periods. In the first 2 years, the same ranavirus was detected repeatedly, but a new ranavirus was isolated in association with the second mass-mortality event.

The two different ranaviruses were characterized based on nucleotide sequences from four genomic regions, namely, major capsid protein, DNA polymerase, ribonucleoside diphosphate reductase alpha and beta subunit genes. The sequences showed slight variations to each other or GenBank entries and both clustered to the Rana esculenta virus (REV-like) clade in the phylogenetic analysis. Furthermore, a quiescent infection was demonstrated in two individuals. By comparing samples taken before and after transport and caging in groups it was possible to identify the pond of origin and a ranavirus was detected for the first time in wild amphibians in Germany.

Introduction

Ranaviruses are large (150–170 nm), icosahedral, double-stranded DNA viruses that belong to the family *Iridoviridae*. Since the first isolation of a ranavirus from *Lithobates pipiens* (formerly *Rana pipiens*) in 1965 (Granoff et al., 1965), an increasing number of infections caused by ranaviruses have been detected in ectothermic vertebrates (amphibians, fish and reptiles).

Although environmental changes are most likely to be the most important threat to amphibian populations, infectious diseases are suspected to play an important role in the global amphibian decline (Daszak et al., 1999). Most current studies focus on the fungus disease chytridiomycosis which has been termed the '*worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and it's propensity to drive them to extinction*' (Gascon et al., 2007). However, disease caused by ranaviruses is also often associated with mass-mortality events and seems to occur worldwide (Gray et al., 2009). Ranaviral disease is therefore considered an emerging infectious disease in amphibians and is notifiable to the World Organisation for Animal Health (OIE).

In European amphibians, infections with ranaviruses have been detected in the UK in common frogs (*Rana temporia*) (Drury et al., 1995; Cunningham et al., 1996; Hyatt et al., 2000; Duffus and Cunningham, 2010), common toads (*Bufo bufo*) (Hyatt et al., 2000; Duffus and Cunningham, 2010), common midwife toads (*Alytes obstetricans*) (Duffus and Cunningham, 2010) and common or smooth newts (*Lissotriton vulgaris*, formerly *Triturus vulgaris*) (Duffus and Cunningham, 2010). The first proven ranavirus-associated mass-mortality event in mainland Europe occurred in Spain 2007 in common midwife toads (Balseiro et al., 2009). In connection with a second disease outbreak in the same species in the Spanish Pyrenees, a ranavirus was also detected in alpine newts (*Ichthyosaura alpestris cyreni*, formerly *Mesotriton alpestris cyreni*) (Balseiro et al., 2010). In Portugal, a ranavirus has been detected associated with mass mortality episodes affecting the newts *Triturus marmoratus* and *T. boscai* in 2003 (Alves de Matos et al., 2008).

The first report of a disease outbreak in *Pelophylax esculentus* (formerly *Rana esculenta*) in former Yugoslavia – described as '*viral haemorrhagic septicaemia of frogs*', which probably resulted from

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ranavirus infection – was reported in 1968 (Kunst and Valpotic, 1968). Mass mortality events in this species caused by ranaviruses have also been detected in Croatia (Fijan et al., 1991), Denmark (Ariel et al., 2009) and Italy (Ariel et al., 2010). In September 2010, the first ranavirus-associated mass mortality event in wild water frogs (*Pelophylax* spp.) and common newts occurred in the Netherlands (Kik et al., 2011). The virus found in that outbreak appears to be identical to the ranavirus (common midwife toad virus, CMTV) that was previously isolated in the Spanish Pyrenees (Balseiro et al., 2009, 2010).

The present study describes the detection and characterization of a ranavirus during an outbreak of fatal disease in a study group of edible frogs collected from various European ponds. Following the initial outbreak, surviving frogs were kept and sampled repeatedly for virus shedding. New animals were added to the group yearly. Screening was continued for 3 years and detected viruses were characterized based on partial sequences of four different genes.

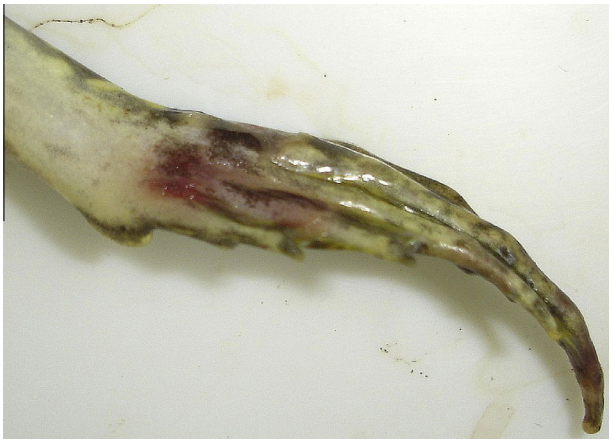

Fig. 1a. Haemorrhagic ulcerations of digits and joints in a ranavirus infected edible frog (*Pelophylax* kl. *esculentus*).

Fig. 1b. Ventral petechial haemorrhages on the lower abdomen and upper thighs of an edible frog (*Pelophylax* kl. *esculentus*) infected with a ranavirus.

Materials and methods

Outbreaks of disease

Adult frogs (4–5 years of age) of the *Pelophylax esculentus* complex were collected from wild populations for crossing experiments and behavioural studies on hybridization at the University of Zürich. In 2008, 218 frogs were collected from 17 localities (Table 1). Most frogs were exposed to handling during capture and transport, and were placed in restricted housing conditions in the laboratory during experiments for several days. After this time, the animals were kept under species-appropriate conditions in fenced outdoor enclosures (4×8 m^2) containing an artificial pond with natural pond vegetation. Frogs were fed with crickets (*Acheta domestica*) and protected against predators by strong top netting.

Some days after the first release of frogs into the enclosures in late May, dead animals were detected. Pathological examination was performed on three of the affected frogs in June 2008. Prior to death, the animals showed no signs of pre-existing chronic disease conditions. Pathohistological changes were detected in several organs, namely, necrosis of lung capillaries partially associated with bacterial foci, minor haemorrhages, heterophilic infiltration and slight leukostasis, focal interstitial kidney necrosis associated with bacterial foci, multifocal bacteria in liver sinusoids, and multifocal necrosis of single liver cells. Oedema of the lamina propria including necrotic foci associated with bacteria was detected in the small intestine. Multifocal interstitial necrosis of the testis and multifocal bacteria in the vessels of the choroidea were also detected in one animal. *Aeromonas sobria* was isolated from the liver. Based on the morphological changes and bacterial results, a bacterial sepsis ('red leg disease') was diagnosed.

To prevent the spread of infection, apparently healthy animals were isolated (1–5 animals) in large cattle tanks ($1.6\ m \times 1\ m \times 1\ m$) providing shelter and a pool of clean saline water (10 g NaCl/100 L aged water). An increase in water salinity was supposed to slow bacterial growth. Approximately 50 frogs were additionally treated with enrofloxacin corresponding to the antibiogram (bathing for 5 min in 1.5 mL/L H_2O enrofloxacin 10% oral solution (Baytril, Bayer) for 5 days). However, this treatment did not seem to affect the progress of the disease since all frogs that showed symptoms at the beginning of the enrofloxacin treatment died.

Approximately 160 animals died in 2008. Signs of the disease included: haemorrhagic ulcerations of digits and joints (Fig. 1a), abnormal body shape (bloat due to oedema, cachexia), ventral petechial haemorrhages ('red leg') (Fig. 1b) and, rarely, hairy fungal plaques growing on skin. Some animals showed none of these symptoms, but morbidity was indicated by lethargic floating and impaired movement. Most sick animals died within 1 day after showing first signs of disease. The surviving frogs overwintered in small groups in plastic boxes with aged water and dry sitting places in a cold room at 4–5 °C between November and March. Cleanness of water, room temperature and animal condition were checked on a regular basis. A very small number of the surviving frogs died during hibernation or soon after their release to the outdoor enclosures in spring 2009 (Table 1). The rest of the frogs remained healthy, and newly introduced animals did not show any signs of disease.

Table 1
Number of animals collected, original habitat, number of dead and surviving animals.

Year	Newly collected animals		Dead animals	Surviving animals
	Number	Locality		
2008	218	17 (ponds in Sweden, Czech Republic, Slovakia, Poland, Eastern Germany)	156	62
2009	97	9 (ponds in Sweden, Slovakia and Eastern Germany)	6 (collected 2008, died after hibernation); 4 (collected 2009, not clear whether or not they were infected)	56 (collected 2008); 93 (collected 2009)
2010	90	5 (ponds in Eastern Germany and Switzerland)	100 died, 108 euthanased	23 (collected 2008); 93 (collected 2009)
2011	–	–	3 (died during hibernation)	28

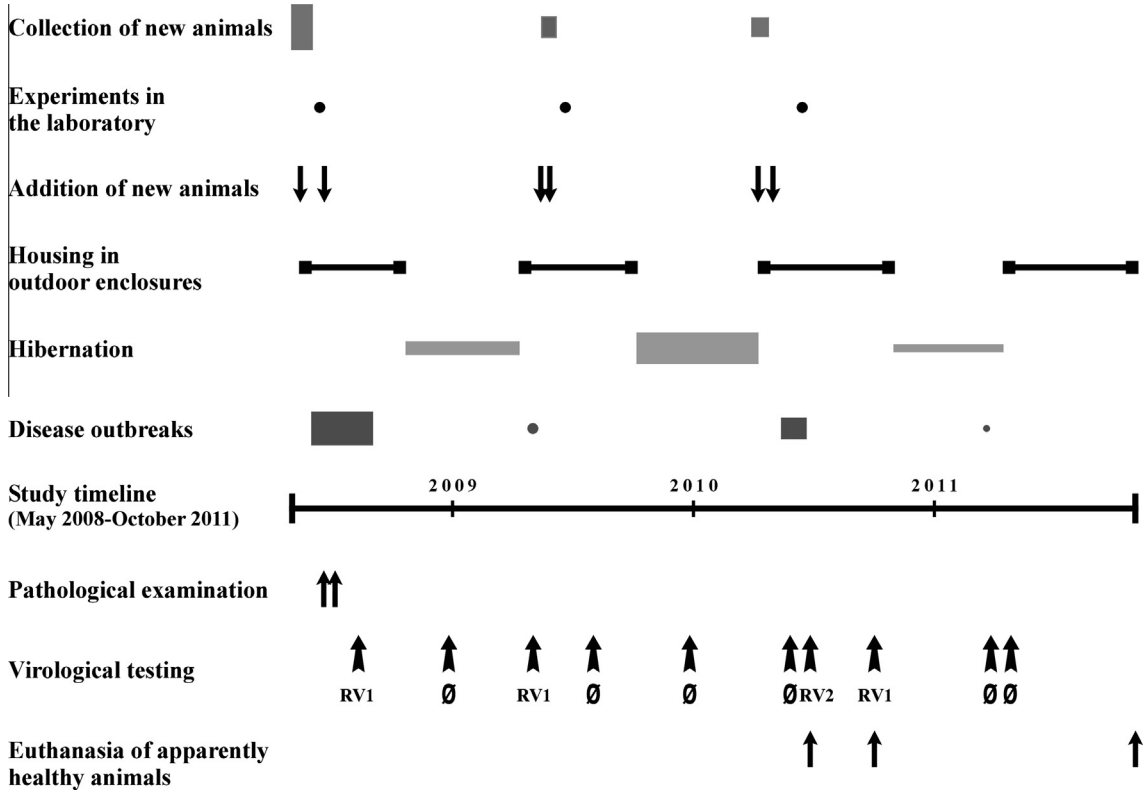

Fig. 2. Schematic timeline of outbreaks and testing of frogs. Box sizes are proportional to numbers of animals for the categories collection of animals, hibernation and disease outbreaks. ∅ = no virus detected at sampling time point; RV1, Zuerich Pelophylax collection ranavirus 1; RV2, Zuerich Pelophylax collection ranavirus 2.

In the summer of 2010, a second large disease outbreak occurred. The majority of the remaining group (108 apparently healthy animals in 2010, another 25 in 2011) were euthanased using an overdose of tricaine anaesthetic (buffered MS-222 solution 1 g/L) and stored at −20 °C for later examination. From a total of 405 frogs collected over 3 years, 277 died in connection with disease symptoms during three ranavirus outbreaks. A schematic timeline of outbreaks and testing of frogs is presented in Fig. 2. Release to the ponds of origin was not possible due to risk of infection.

Sampling

Dead frogs frozen at −20 °C (2008, found dead: $n = 8$; 2009, found dead: $n = 6$; 2010, found dead: $n = 27$; 2010, euthanased: $n = 9$; 2011, found dead: $n = 3$) were sent for virological testing. Clinical signs and gross pathological changes are listed in Table 2. Histological examination of animals found dead was not undertaken, as the tissues were autolysed. Skin and cloacal swabs from asymptomatic frogs were collected and submitted for virological testing (2008, $n = 32$; 2009 and 2010, $n = 101$; 2011, $n = 30$). In a retrospective study, toe clips from 229 frogs, which had been collected before removal from their habitat (2008–2010), and one ethanol-fixed edible frog that had died shortly after collection in 2008, were tested for the presence of ranaviral DNA.

Virus isolation

When it was possible to identify all organs of an animal (and depending on pathological findings) small tissue samples of the kidney, liver, intestine, spleen, heart and the skin were collected separately in cell culture medium; swabs and toe clips were individually collected in 3 mL Dulbecco's modified Eagle Medium (DMEM) (Biochrom) supplemented with antibiotics (penicillin-G solution 200 U/mL; streptomycin sulfate solution, 380 U/mL; gentamicin sulfate solution 6.4 U/mL; amphotericin B solution 0.5 μg/mL) (Biochrom).

After sonication and centrifugation at low speed (2000 *g*, 10 min), 200 μL of the supernatant was inoculated onto approximately 70% confluent iguana heart cell monolayers (IgH-2, ATCC: CCL-108) in 30 mm diameter tissue culture dishes (Cellstar, Greiner Bio-One). After incubating for 2 h at 28 °C, each dish was cultured with 2 mL nutrient medium (DMEM supplemented with 2% fetal calf serum (FCS) (Biochrom) and 1% non-essential amino acids (NEA; Biochrom). Tissue cultures were observed twice a week for cytopathic effects (CPE). When a CPE appeared, the cultures were frozen at −20 °C, thawed and reinoculated onto IgH-2 for a second passage. Dishes showing no CPE were frozen after 2 weeks incubation for a second passage.

Polymerase chain reaction, sequence analysis

DNA was extracted from the original sample or from cell culture supernatant using the DNeasy Kit (Qiagen), and PCR was undertaken for the detection of ranaviruses in 25 μL reaction tubes as described previously (Mao et al., 1997; Marschang et al., 1999) using primers OL T1 and OL T2R targeting a 500 bp portion of the ranavirus major capsid protein (MCP) gene.

For positive tested samples, additional PCRs targeting the major part (1402 bp) of the MCP gene in overlapping fragments, partial sequences of the DNA polymerase (DNApol), ribonucleoside diphosphate reductase beta subunit-like protein (RNR-α) and alpha subunit-like protein (RNR-β) genes were performed using different primer pairs for each gene (Table 3). Primers and reaction conditions have been published before (Ariel et al., 2010; Hyatt et al., 2000; Holopainen et al., 2009). Oligonucleotides were purchased from MWG Biotech. The obtained PCR products were separated by agarose gel electrophoresis (1.5% agarose gel (Bioenzym) in TAE buffer containing 0.5 μg/mL ethidium-bromide and evaluated under 320 nm UV light. PCR amplicons were gel purified using peqGOLD gel extraction kit (Peqlab Biotechnologie) and sent for sequencing from both directions to MWG Biotech.

The sequences were edited, assembled and compared using STADEN Package version 2003.0 Pregap4 and Gap4 programmes (Bonfield et al., 1995). The edited original sequences were compared to those in GenBank online[1] using BLASTX and BLASTN. Multiple alignments of nucleotide sequences were performed with the ClustalW algorithm of the BioEdit Sequence Alignment Editor program (Hall, 1999). This alignment was further used for phylogenetic calculations in the PHYLIP program Package version 3.6. (Felsenstein, 1989) trying distance based, maximum-likelihood and parsimony methods to obtain an optimal tree. Bootstrap analysis of 100 replicates was carried out. GTR + G (general time reversible assuming gamma distribution) substitution model for MrBayes (with 1 million generations, sample frequency: 10 and burnin ratio: 40%) was also used to reconstruct phylogenies (Huelsenbeck and Ronquist, 2001) as an application of the TOPALi v2.5 programme.

Results

During suddenly increased mortality in the summer of 2008, ranavirus was detected by MCP gene PCR in 7/8 tested edible frogs (Table 2) and isolated in cell culture from the same seven animals. Twenty-eight per cent of the animals from the infected group in

[1] See: http://www.ncbi.nih.gov/blast/.

Table 2
Samples from a group of *Pelophylax* kl. *esculentus* analyzed over the course of 3 years with short case histories, the results of virus isolation on cell culture, PCR and sequencing.

Date of sampling	Case history	Clinical signs	Sample type	Number of samples	Virus isolation	PCR from original sample	Sequencing, type of virus
06–07/2008	High mortality, abnormal body shape (bloat due to oedema, anorexia), lethargic floating and impaired movement	Reddening of the skin (legs, abdomen); rarely: hairy fungal plaques growing on skin, haemorrhages in the gastrointestinal tract, fragile intestine, brown aqueous ascites	Frozen animals (kidney, liver, intestine, spleen, heart)	8	7/8 positive (kidney: 6/8, liver: 6/8, intestine: 5/8, spleen: 5/8, heart: 7/8)	7/8 positive (kidney: 7/8, liver: 6/8, intestine: 6/8, spleen: 7/8, heart: 5/8)	Isolates from three animals sequenced (ZPRV1)
12/2008			Skin swabs	32	–	–	–
04/2009	Several animals died during artificial winter or shortly after removal to outdoor enclosures	Most animals: reddening of the skin (legs, abdomen); one animal: ascites, partially dark red coloured intestine, renomegaly	Animals (kidney, liver)	6	–	3/6 positive (kidney: 3/6, liver 3/6)	n.d.
04/2009			Skin + cloacal swabs	19	4/19 positive (skin swabs: 3/19, cloacal swabs: 2/14)	14/19 positive (skin swabs: 14/19, cloacal swabs: 10/19)	Isolates from two animals sequenced, partial sequences (MCP, DNApol) from two animals (ZPRV1)
07/2009			Skin + cloacal swabs	30	–	–	–
12/2009			Skin + cloacal swabs	28	–	–	–
05/2010			Skin + cloacal swabs	24	–	–	–
05–06/2010	High mortality or euthanasia of apparently healthy animals	Some dead animals: reddening of the skin, locomotive troubles	Animals (kidney, liver)	32	23/32 positive (kidney + liver: 21/32, only kidney: 1, only liver:1)	22/32 positive (kidney: 19/32, liver: 21/32)	Isolates from two animals sequenced (ZPRV2)
09/2010	Euthanasia	Reddening of the skin; one animal: hepatomegaly, splenomegaly	Animals (kidney, liver, spleen)	4	2/4 positive (kidney: 1/4, spleen: 1/1)	–	Isolates from two animals sequenced (ZPRV1)
03/2011	Animals found dead in their cages during artificial winter	Red fluid ascites, haemorrhages in the kidneys, fragile yellow liver with small dark dots	Animals (kidney, liver, skin)	3	–	–	
04/2011			Skin swabs	30	–	–	

n.d., not done.

Table 3
Primers used in PCR reactions.

Target gene	Primer	Primer position	Amplicon size	Nucleotide sequence (5′–3′)	Reference
MCP	OL-T1	97,387–97,404	531	GACTTGGCCACTTATGAC	Mao et al. (1997); Marschang et al. (1999)
	OL-T2R	97,917–97,899		GTCTCTGGAGAAGAAGAAT	
	MCP-BF	97,813–97,830	548	ACCAGCGATCTCATCAAC	Ariel et al. (2010)
	MCP-BR	98,360–98,341		AGCGCTGGCTCCAGGACCGT	
	MCP-6	98,244–98,263	585	CGCAGTCAAGGCCTTGATGT	Hyatt et al. (2000)
	MCP-6R	98,828–98,807		AAAGACCCGTTTTGCAGCAAAC	
DNApol	DNApol-F	67,188–67,208	560	GTGTAYCAGTGGTTTTGCGAC	Holopainen et al. (2009)
	DNApol-R	67,747–67,728		TCGTCTCCGGGYCTGTCTTT	
RNR-α	RNR-AF	43,729–43,748	806	CTGCCCATCTCKTGCTTTCT	Ariel et al. (2010)
	RNR-AR	44,534–44,513		CTGGCCCASCCCATKGCGCCCA	
RNR-β	RNR-BF	78,029–78,012	646	AGGTGTRCCRGGGYCGTA	Ariel et al. (2010)
	RNR-BR	77,384–77,403		GACGCTCCAYTCGACCACTT	

The primer position is presented relative to the FV3 genome (AY548484).
Y = C/T, K = G/T, S = C/G, R = A/G.

Zürich survived the outbreak and did not show any symptoms during the following months. Before hibernation of the surviving animals, no virus was detected in skin swabs (n = 32), but six animals died during or shortly after artificial winter, three of which were tested positive for ranavirus in liver and kidneys via PCR. Several days after this second outbreak, skin and cloacal swabs from 19 apparently healthy animals were taken and ranavirus was detected in 14 frogs via PCR, virus was isolated in cell culture from four animals. Until May 2010, none of the remaining animals showed any signs of disease and virological testing from a total of 83 skin and cloacal swabs was negative.

Some days after new edible frogs were added, a third disease outbreak with high mortality occurred in May 2010. A total of 22/32 examined frogs tested positive for ranavirus by PCR (Table 2) and virus was isolated from 23 animals. No virus was detected in the apparently healthy animals which were euthanased during this outbreak whereas all examined animals which died naturally were tested positive.

For further characterization additional gene sequences of the obtained isolates from each outbreak were analyzed as described previously (Ariel et al., 2010; Hyatt et al., 2000; Holopainen et al., 2009) (Table 3). These studies showed the presence of two distinct ranaviruses in this group of animals – one from the first

Table 4
Ranavirus (RV) sequence identity of the four analyzed parts of the genome. The two different ranaviruses (ZPRV1 and 2) detected in this study are presented in comparison to FV3. For each gene sequence, the upper diagonal shows the values for the nucleotide sequence identity, the amino acid identity values are provided in the lower diagonal.

	ZPRV1 (%)	ZPRV2 (%)	FV3 (%)
MCP			
ZPRV1		99.79	98.07
ZPRV2	100		98.15
FV3	97.74	97.74	
DNApol			
ZPRV1		99.81	98.84
ZPRV2	100		99.04
FV3	98.27	98.27	
RNR-α			
ZPRV1		99.74	98.82
ZPRV2	99.21		98.82
FV3	98.82	98.82	
RNR-β			
ZPRV1		100	98.68
ZPRV2	100		98.68
FV3	98.51	98.51	

ZPRV1, Zuerich Pelophylax collection ranavirus 1; ZPRV2, Zuerich Pelophylax collection ranavirus 2.
GenBank accession number for FV3: see Table 3.

outbreak to the end of the study (Zuerich Pelophylax collection ranavirus 1, ZPRV1), the other in association with the third outbreak in May 2010 (ZPRV2) (Fig. 2). Sequences from multiple isolates of each of the two viruses were always identical (Table 2). While the two different viruses showed high similarity to each other in the nucleotide sequences of the partial sequences from the MCP, DNApol and RNR-α subunit genes, the partial sequences from the RNR-β subunit gene were 100% identical to one another. Comparison of the amino acid sequences showed that all differences except those on the RNR-α subunit were silent mutations. Comparison of the sequences of the viruses detected in this group of frogs with corresponding sequences from the FV3 genome showed identities of 98–99% (Table 4).

In the phylogenetic analysis, the gene sequences of each of the two viruses (ZPRV1, ZPRV2) were concatenated (3223 bp) and studied in comparison to previously published ranavirus sequences available in GenBank from amphibians, fish, and a reptile. Both viruses (ZPRV1 and 2) clustered closely to each other and to the Rana esculenta virus (REV-like) clade (Fig. 3). The obtained gene sequences of each virus (ZPRV1 and 2) were submitted to GenBank with accession numbers KC440841, KC440842 (MCP), KC440843, KC440844 (RNR-α), KC440845 (RNR-β), KC440846, KC440847 (DNApol).

In September 2010, the previously detected ranavirus (ZPRV1) was discovered in 2/4 euthanased individuals. In one animal, splenomegaly was observed and virus was isolated only from the

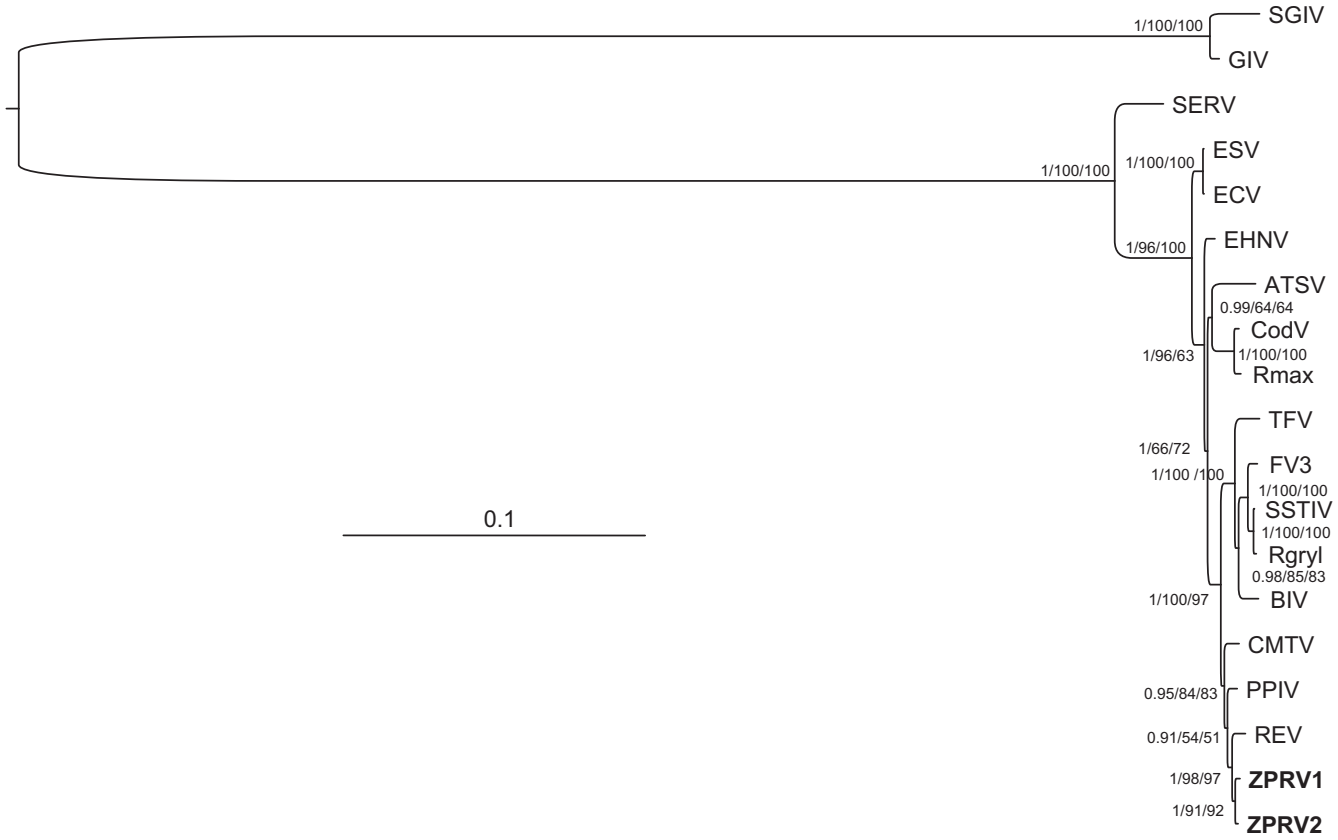

Fig. 3. Midpoint rooted MrBayes tree of the concatenated nucleotide sequences (3223 bp) of MCP, DNApol, RNR-α and RNR-β genes of the two different ranaviruses detected in this study (ZPRV1, ZPRV2) and ranavirus sequences available in GenBank. GenBank accession numbers of the sequences used in the analysis: Ambystoma tigrinum stebbensi virus (ATSV) (AY150217), Bohle iridovirus (BIV) (AY187046, FJ374280, GU391286 GU391264), CMTV (JQ231222), Cod ranavirus (CodV) (GU391284, GU391282, GU391287, GU391265), European catfish virus (ECV) (FJ358608, FJ374277, GU391288, GU391266), epizootic haematopoietic necrosis virus (EHNV) (FJ433873, FJ374274, GU391289, GU391267), European sheatfish virus (ESV) (FJ358609, FJ374278, GU391290, GU391268), FV3 (AY548484), pike-perch iridovirus (PPIV) (FJ358610, FJ374276, GU391292, GU391269), Rana esculenta virus Italy 282/I02 (REV) (FJ358611, FJ374275, GU391293, GU391271), Rana grylio iridovirus (Rgryl) (JQ654586), Ranavirus maxima (Rmax) (GU391285, GU391283, GU391291, GU391270), short-finned eel ranavirus (SERV) (FJ358612, FJ374279, GU391294, GU391272), soft-shelled turtle iridovirus (SSTIV) (EU627010), tiger frog virus (TFV) (AF389451), Singapore grouper iridovirus (SGIV) (AY521625), grouper iridovirus (GIV) (AY666015). Numbers at the nodes of the tree indicate MrBayes posterior probabilities and bootstrap values of 100 replicates in DNAdist-Fitch and maximum likelihood calculations. All calculated trees showed identical topologies.

spleen, the other animal showed no pathological changes and virus was isolated from the kidneys. Testing of tissues from these animals (liver, kidneys and spleen) via PCR was negative. Interestingly, both animals were collected in 2008 and tested positive only by PCR in April 2009 (skin and cloacal swabs) but never developed clinical disease. Sequencing of a part of the MCP and the DNA-pol gene from the previously tested swabs (from 2009) demonstrated that this ranavirus was 100% identical to the isolates obtained in September 2010 (ZPRV1). No ranavirus could be detected in three animals which died during hibernation 2011 and skin swabs from 30 frogs tested negative in April 2011.

In order to determine from which pond the infection was originally introduced, a total of 229 available pre-transport DNA samples were screened in 2008 (all ponds), 2009 (four ponds) and 2010 (one pond). Ranavirus was detected in a single sample from 2008. This animal died shortly after removal from its habitat (Untermassfeld, Germany) and was fixed in ethanol. By repeating testing using skin from the fixed animal, we were able to verify the infection. The virus detected was identical to the obtained isolates from 2008 and 2009 (ZPRV1). The origin of ZPRV2 could not be identified.

Discussion

Two different manifestations of ranaviral disease have been described in European amphibians, namely, an acute, systemic haemorrhagic disease and a cutaneous form (ulcerative syndrome) which seems to be more chronic (reviewed in Duffus and Cunningham, 2010). In our cases, the symptoms of the diseased animals varied, so they could not be clearly correlated to one of the proposed forms of disease. The documented bacterial co-infection in combination with the stress of transport are likely to have influenced the course of disease as described, for example, in an American bullfrog (*Rana catesbeiana*) ranaculture facility where >50% mortality and related pathological findings occurred due to a co-infection with a ranavirus and *Aeromonas hydrophila* (Miller et al., 2007).

The second disease outbreak with low mortality in spring 2009 was associated with relatively low environmental temperatures. Previous investigations have demonstrated the dependency of ranavirus replication on temperature (Rojas et al., 2005). Several authors have suggested that the amphibian host immune function decreases at lower temperatures (Maniero and Carey, 1997; Carey et al., 1999; Forbes et al., 2004; Rojas et al., 2005; Raffel et al., 2006) and pathogen infectivity can therefore increase. Translocation after hibernation may also have influenced the susceptibility of the immunocompromised animals to disease.

Interestingly, PCR seemed to be more sensitive than cell culture in detecting ranavirus in skin or cloacal swabs in April 2009. Previous studies demonstrated that non-lethal sampling techniques are useful for ranavirus diagnostics, but the prevalence of infection may be underestimated in comparison to liver samples (Gray et al., 2012). It is questionable whether PCR was able to detect very low amounts of replication competent virus, or if viral DNA instead of active virus was detected. It is also possible that the virus was only on the surface of the skin or the cloaca without infecting the animal. By infecting *Xenopus laevis* with FV3 via water, Robert et al. (2011) demonstrated that FV3 was transcribed in the skin of only a few frogs and suggested that despite the presence of virus on the skin surface, little or no virus replication was initiated at an early stage of infection. Nevertheless, our findings could be interesting in defining the best time to screen live animals for an infection by PCR as the animals seemed to release detectable amounts of virus into the environment after hibernation and in the breeding season.

A number of studies have been carried out to understand the amphibian adaptive and innate immune response to ranavirus infections, mostly using the *Xenopus* model (see, for example, Gantress et al., 2003; Robert et al., 2005; Maniero et al., 2006; Morales et al., 2010). It has been shown that animals are able to clear ranavirus infection and that after reinfection with the same virus viral clearance was markedly accelerated and animals did not show any symptoms of illness (Gantress et al., 2003). As our two isolated viruses showed only slight variations to each other on the characterized genes, it is remarkable that not only newly added animals died in 2010, but also that animals which had been in contact with ranavirus previously were not able to resist the infection. It is possible that the new virus strain was more virulent or that the immune response was not able to clear the infection as the antibodies may have weak affinity (Maniero et al., 2006). Another factor might be an immunocompromised state of the new co-housed animals.

We hypothesize that the two frogs in which the first ranavirus (ZPRV1) was detected following euthanasia in September 2010 were infected with the ZPRV1 in 2008 but did not develop disease due to an effective antiviral immune response. Nevertheless, the infection was not eliminated completely and the animals appear to have harboured quiescent virus over a period of at least 1 year. Quiescent infections have been shown to occur in *X. laevis*, in which ranaviruses can remain in peritoneal leukocytes (Robert et al., 2007; Morales et al., 2010). It is possible that the frogs were shedding ranavirus at the time of sampling in April 2009. On the other hand, no virus was detected in two other euthanased frogs which were collected in 2008 and also tested positive in April 2009. These animals seem to have successfully cleared the infection.

Due to the retrospective nature of our study, we were able to detect ranavirus in a wild amphibian in Germany for the first time. It is possible that the positive tested animal or another animal from the same habitat first infected the group; the second type of ranavirus was probably introduced with newly collected animals in 2010, potentially from one of the ponds that we were not able to screen for ranaviral DNA in original toeclips. No mass mortality event was reported in any of the ponds in 2009 and 2010. As only one frog of those examined from their original habitat tested positive, it is possible that several samples tested were false negatives. It is also possible that the methods we used were not sensitive enough to detect small amounts of ranavirus in fixed toe clips or that storage of the DNA over a long period may have influenced the results.

Previous studies have demonstrated variations among different amphibian species to disease and variations in virulence between different virus strains (see, for example, Schock et al., 2008; Hoverman et al., 2011). The global trade in amphibians, such as the translocation of larval tiger salamanders (*Ambystoma tigrinum*) as fishing bait or the commercial exploitation of *Xenopus* for research and as pets, is an important source of pathogen pollution (Robert et al., 2007; Picco and Collins, 2008). Our report underlines the risks not only of introducing animals into new habitats but also of mixing amphibians from different origins, even when the animals appear clinically healthy.

Virus characterization based on partial genome sequencing is an important tool in understanding the course of ranaviral disease. Results of sequencing also allowed us to identify at least one source of infection. As the MCP gene is highly conserved, a part of it is very useful for diagnostics. To differentiate between various virus strains, sequence information from more genes is necessary. In the phylogenetic analysis, both ranavirus isolates detected during this study (ZPRV1, ZPRV2) were most closely related to each other. Interestingly, they also clustered close to other European isolates from amphibians and fish (REV, CMTV and PPIV) (Fig. 3).

Additional analyses are necessary to help understand the capacity of ranaviruses to adapt to new hosts, their phylogenetic relationships, variations in virulence among species and between different ranavirus strains.

Conclusions

Two different ranaviruses have been identified as causative agents for recurring disease outbreaks with low to high mortality in edible frogs collected from multiple ponds in Europe to form a single group. It has been shown that animals can be sublethally infected and harbour quiescent virus over a period of at least 1 year. Co-housing of apparently healthy animals after capture and translocation should therefore be avoided. In addition, a ranavirus was detected in a wild amphibian from Germany for the first time. In the phylogenetic analysis, both ranaviruses detected in this study were most closely related to each other and to other European ranavirus isolates from amphibians and fish.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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