Major capsid protein gene sequence analysis of the Santee-Cooper ranaviruses DFV, GV6, and LMBV

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ABSTRACT: The Santee-Cooper ranaviruses doctor fish virus (DFV), guppy virus 6 (GV6), and largemouth bass virus (LMBV) are members of the genus *Ranavirus* within the family *Iridoviridae*. The major capsid protein (MCP) is a main structural protein of iridoviruses and supports the differentiation and classification of ranaviruses. Presently the complete sequence of the *MCP* gene is known for most ranaviruses with the exception of the Santee-Cooper ranaviruses. In the present study, the complete nucleotide sequence of the *MCP* gene of DFV, GV6, and LMBV was determined. DFV and GV6 are identical within the *MCP* gene sequence. The identity compared to the corresponding sequence in LMBV amounts to 99.21 %. The *MCP* gene of DFV, GV6, and LMBV exhibits only approximately 78 % identity compared to the respective gene of other ranaviruses. Based on the sequence data obtained in the present study, a *Rana* MCP polymerase chain reaction (PCR) and subsequent restriction fragment length polymorphism (RFLP) analysis were developed to identify and differentiate ranaviruses, including DFV, GV6, and LMBV.

KEY WORDS: *Iridoviridae* · *Ranavirus* · DFV · GV6 · LMBV · EHNV · *MCP* gene · *Rana* MCP PCR

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INTRODUCTION

Ranaviruses represent a genus within the family *Iridoviridae* (Chinchar et al. 2005). Compared to the other genera of the family, *Iridovirus*, *Chloriridovirus*, *Lymphocystivirus*, and *Megalocytivirus*, which have a rather defined and small host range, ranaviruses are able to infect a broad host range including fish, amphibians, and reptiles (Marschang et al. 1999, Hyatt et al. 2002, Johnson et al. 2008, Chinchar et al. 2009, Whittington et al. 2010). Systemic infections with necrosis of kidney and spleen as well as diffuse subcutaneous and internal haemorrhages have led to high mortalities in host species in many countries worldwide (Chinchar, 2002, Williams et al. 2005). Moreover, ranaviruses are suspected of playing a decisive role in the global decline of amphibian populations (Daszak et al. 1999, Picco and Collins, 2008). *Frog virus 3* (FV3) is the first known ranavirus and represents the type species of the genus *Ranavirus*. It was isolated in 1965

from the leopard frog *Rana pipiens* in North America (Granoff et al. 2006). The first described fish-infecting ranavirus, the species *Epizootic haematopoietic necrosis virus* (EHNV), caused high mortality in wild redbfin perch *Perca fluviatilis* and high morbidity in farmed rainbow trout *Oncorhynchus mykiss* in Australia in 1986 (Langdon et al. 1986, 1988). Since then, reports about ranavirus infections of fish, amphibians, and reptiles have become more frequent worldwide. In the meantime, additional pathogenic ranaviruses have been isolated from various poikilothermic vertebrates: *Ambystoma tigrinum virus* (ATV) isolated 1996 from Sonora tiger salamanders *Ambystoma tigrinum stebbinsi* in southern Arizona; *European catfish virus* (ECV), recovered since 1990 in France and Italy; *European sheatfish virus* (ESV), first detected in 1989 in Germany; *Bohle iridovirus* (BIV), isolated in 1992 from Australian frogs *Limnodynastes ornatus*, and Santee-Cooper ranaviruses isolated from guppy *Poecilia reticulata*, doctor fish *Labroides dimidatus*, and largemouth

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bass *Micropterus salmoides* in the 1990s in the United States (Ahne et al. 1989, Pozet et al. 1992, Bovo et al. 1993, Hedrick & McDowell 1995, Jancovich et al. 1997, Plumb et al. 1996, Ariel & Owens 1997, Grizzle et al. 2002, Goldberg et al. 2003, Chinchar et al. 2005). Thus, ranaviruses have been isolated worldwide from fish, amphibians, and reptiles at an increasing frequency over the last few decades (Chinchar 2002, Duffus et al. 2008, Johnson et al. 2008, Chinchar et al. 2009, Gray et al. 2009, Mazzoni et al. 2009, Hoverman et al. 2010, Torrence et al. 2010, Whittington et al. 2010, Xu et al. 2010). Recent reports about ranavirus outbreaks in *Pseudacris clarkii* tadpoles in Texas underline the subject's topicality (Torrence et al. 2010), since the decline of amphibian populations is a severe problem on a global scale.

While EHN and ranavirus infection of amphibians were listed as notifiable diseases in 2006 (Anonymous 2006), other ranavirus infections of fish, caused by ESV/ECV, PPIV, shortfin eel ranavirus (SERV), or Santee-Cooper ranaviruses, and the reptile ranavirus soft shelled turtle iridovirus (SSTIV) have thus far not been listed by the European Union nor by the World Organisation for Animal Health (Office International des Épidémiologies, OIE). In the light of the current knowledge on the significant genetic similarity and broad host range of ranaviruses, further discussion about the declaration and listing of ranavirus infections as exotic diseases in the EU is necessary.

Ranaviruses are enveloped icosahedral virions with a diameter of 160 to 200 nm (Williams et al. 2005). The genome consists of a single linear double stranded DNA molecule of approximately 105 to 140 kbp, which is highly methylated, circularly permuted, and terminally redundant (Willis & Granoff 1980, Goorha & Murti 1982, Willis et al. 1984, Murti et al. 1985, He et al. 2002, Jancovich et al. 2003, Song et al. 2004, Tsai et al. 2005). The G+C content amounts to approximately 49 to 54 % (He et al. 2002, Jancovich et al. 2003, Song et al. 2004, Tsai et al. 2005). With the exception of grouper iridoviruses, ranaviruses share conserved group-specific antigens (Chinchar et al. 2005, Whittington et al. 2010). Therefore, a clear differentiation of the various isolates based on their antigenic profile is not possible. The major capsid protein (*MCP*) gene has been shown to be a suitable target for analysis of the genetic relatedness of various ranaviruses (Tidona et al. 1998). Most of the ranaviruses can be detected using methods for amplification of the *MCP* gene sequences (Mao et al. 1997, Hyatt et al. 2000, Marsh et al. 2002, Holopainen et al. 2009). Nevertheless, an identification of the complete *MCP* gene sequence obtained from the Santee-Cooper ranaviruses doctor fish virus (DFV), guppy virus (GV6), and largemouth bass virus (LMBV) failed (Holopainen et al. 2009). Only few sequence data cor-

responding to parts of the *MCP* gene of Santee-Cooper ranaviruses are available (LMBV: AF080250, Mao et al. 1999; DFV, GV6: U82550, AF157665, AF157671, Mao et al. 1997, Hyatt et al. 2000).

For a better understanding of the variation among ranaviruses, their phylogenetic relationship, and the establishment of a comprehensive diagnostic method for the detection of ranaviruses, the *MCP* gene sequence of DFV was identified after Southern blot analyses and shotgun cloning of *Hind*III- and *Kpn*I-digested viral DNA. Using primers specific for defined sequences within the obtained DFV *MCP* gene, the *MCP* gene of GV6 and LMBV was amplified and sequenced. Phylogenetic analyses and multiple alignments of the *MCP* gene of ranaviruses were performed. Based on the resulting data a unique *Rana* MCP PCR was developed for rapid identification of different ranaviruses including the Santee-Cooper ranaviruses but excluding grouper iridoviruses. The described method was verified with a panel of 12 different ranavirus isolates including the Santee-Cooper ranaviruses. In summary, the reported PCR facilitates a rapid diagnosis of most ranavirus infections, which is an important step towards the prevention and control of the disease.

MATERIALS AND METHODS

Cells and virus strains. In the present study, the following 12 ranavirus isolates from piscine and amphibian hosts were analysed: EHN, ESV, ECV, FV3, BIV, *Rana esculenta* virus isolate Italy 282/I02 (REV), PPIV, SERV, GV6, DFV, *Rana tigrina* ranavirus (RTRV), and LMBV.

RTRV was isolated in 1998 from bullfrogs *Rana tigrina* in Asia (Kanchanakhan et al. 1998). LMBV was derived from the original type isolate obtained from the Santee-Cooper reservoir in South Carolina.

The origin and references of all other analysed virus isolates were described in Holopainen et al. (2009). All viruses were propagated on BF2 cell line CCLV Rie88 at 20°C in 2.5 % CO₂ atmosphere until a complete cytopathic effect (CPE) was observed.

Virus purification and DNA extraction. Culture supernatant from infected cells was harvested, and the cell debris was removed by centrifugation at 1100 × *g* for 15 min. PCR analyses were performed with DNA isolated from 200 µl clarified supernatant. Prior to DNA isolation applied for Southern blot analyses, virus suspension was concentrated at 187 000 × *g* for 90 min at 18°C and resuspended in phosphate buffered saline (PBS).

Cellular DNA from BF2 cells (CCLV Rie88) was extracted as negative control after sedimentation at 6200

× *g* for 2 min and entrainment in PBS. DNA extractions were performed with QIAamp DNA Mini Kit (Qiagen) according to the instruction manual.

Southern blot analyses. Southern blot analyses were carried out with DNA extracted from DFV, EHNv, and cell control. Cellular and viral DNA were digested with *Hind*III and *Kpn*I, respectively. Two µg of digested DNA were separated by agarose gel electrophoresis and transferred to nitrocellulose. The resulting DNA fragments were screened with fluorescein-dUTP-labelled probes MCP 6R, DF2-R, and RanaMCP-R (Table 1) for the presence of the MCP gene. Southern blot analyses were performed using the ECL 3'-oligolabelling and detection systems (Amersham Life Science) according to the manufacturer's instructions.

Cloning and identification of MCP gene. *Hind*III- and *Kpn*I-digested DNA from DFV and EHNv were ligated into *Hind*III- or *Kpn*I-digested and dephosphorylated pUC18 vector (shotgun cloning). DH10B cells transformed with positive clones were screened by restriction endonuclease analyses and sequenced to confirm the insertion of the MCP gene fragment. Obtained sequence data were applied for deduction of specific primers for further specific PCR analysis (gene walking).

PCR products amplified from DFV, GV6 and LMBV DNA were cloned blunt end into the pGEM-Teasy vector (Promega). After transformation of DH10B cells, plasmids from 3 independently derived clones were isolated and sequenced.

PCR amplification. Viral DNA isolated from 200 µl clarified culture supernatant of infected cells was used as template for amplification of the complete or partial MCP gene. PCR was carried out using the HotStar Taq Master Mix kit (Qiagen) according to the instruction manual. After an initial step at 95°C for 15 min, the denaturation, annealing, and extension conditions were 93°C (1 min), 55°C (2 min), and 72°C (1 min kb⁻¹), respectively, followed by a final incubation at 72°C (3 min). Oligonucleotides used in PCR reactions are listed in Table 1.

Sequence analyses. The sequence of both DNA strands was determined by cycle sequencing using the Big Dye Terminator version 1.1 Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instructions. Sequencing products were purified by Sigma Spin Post-Reaction purification columns (Sigma Aldrich), denatured with Hi-Di™ formamide, and analysed on ABI PRISM 3100-Avant Genetic Analyzer (ABI). Nucleotide sequences were evaluated with

Table 1. Oligonucleotides used in PCR reactions and in Southern blot hybridization

Oligonucleotide designation	Oligonucleotide position	Nucleotide sequence (5' to 3')
MCP 3 ^a	97774–97794 ^b	GAG GCC AAG CGC ACA GGC TAC
MCP 4R ^a	98303–98284 ^b	TTG GAG CCG ACG GAA GGG TG
MCP 6R ^a	98828–98807 ^b	AAA GAC CCG TTT TGC AGC AAA C
RanaMCP-F	98182–98201 ^b	CCA GTC CAC ATG GTC AAC CC
RanaMCP-R	98697–98676 ^b	GAT AAT GTT GTG GTT GAT GGC C
DF2-R	1584–1564 ^c	GTG TAG TTG GAA CCC ACA GAC
SCIV-F	431–451 ^c	CTG GAT CCA GCA ACA CAC TTC
SCIV-R	2056–2035 ^c	CAT ATC GCA GTT TGC GAT ATG G

^aPrimers published by Hyatt et al. (2000). The oligonucleotide position is presented relative to ^bFV3 genome, AY548484, or to ^cdetermined DFV sequence FR677324

sequence Scanner software version 1.0 (ABI). Sequence alignments and phylogenetic analyses were performed using the GCG-X-Win32 version 11.1.3 Unix (Accelrys).

RESULTS

Identification of the MCP gene of Santee-Cooper ranaviruses DFV, GV6, and LMBV

Specific primers published by Hyatt et al. (2000) were tested for amplification of the MCP gene of DFV and GV6. Applying the primer pairs MCP 1/2R and MCP 5/6R (Hyatt et al. 2000), amplification failed. Using the primer pair MCP 3 and MCP 4R (Fig. 1), the resulting weak product was 776 bp in length and differed from the positive control obtained for REV with an estimated length of 530 bp. Determined sequences of DFV and GV6 were identical. The obtained PCR products of these Santee-Cooper ranaviruses differed from the published MCP gene sequence (AY157665, AF157671) in size as well as in their coding capacity. The nucleotide identity amounts to only 81 %. The first 129 nucleotides of the obtained PCR product showed 95 % nucleotide identity to the partially published MCP sequence of LMBV (Mao et al. 1999; AF080250).

The complete MCP gene sequence of DFV was cloned and sequenced after Southern blot hybridization of viral DNA with probe MCP 6R (Fig. 2, Lane 2). To verify the results, the specific oligonucleotide probes DF2-R and RanaMCP-R were generated based on the obtained sequence, and were included in the hybridization analyses (Fig. 2). DNA isolated from EHNv (Lane 3) and non-infected cells (Lane 1) were used as controls. The probe MCP 6R published by Hyatt et al. (2000) binds downstream of the MCP gene and corresponds to the nucleotides 98828–98807 of the FV3 genome (Tan et al. 2004; AY548484). The synthetic

Fig. 1. PCR analysis of *MCP* gene of DFV and GV6 DNA from non-infected (C-) and virus-infected cells (1 DFV, 2 GV6) was used for amplification with primers MCP 3 and MCP 4R (Hyatt et al. 2000). DNA from REV-infected cells was used as positive control (C+). Location of DNA 1 kb ladder (Invitrogen) separated in Lane M is indicated on the left

oligonucleotide DF2-R hybridizes approximately 800 nucleotides upstream of the primer MCP 6R binding site. The probe RanaMCP-R corresponds to the nucleotides 98697–98676 of the FV3 genome (AY548484) and binds near the 3' end of the *MCP* gene.

EHNv and DFV genomes show a different restriction pattern (Hyatt et al. 2000, 2002). Southern blot analysis confirmed the position of the *MCP* gene of EHNv and DFV on different restriction fragments. The labelled oligonucleotides MCP 6R and RanaMCP-R recognized fragments of *Hind*III- and *Kpn*I-cleaved viral DNA of DFV and EHNv specifically. Both probes hybridized with the same fragments of digested EHNv DNA (Fig. 2, Lane 3, *Hind*III: >20 kb; *Kpn*I: ~11 kb) but reacted with different fragments of *Hind*III- or *Kpn*I-digested DFV DNA (Fig. 2, Lane 2). The MCP 6R probe identified a ~3 kb *Hind*III and a 5 kb *Kpn*I fragment of digested DFV DNA, whereas the oligonucleotide RanaMCP-R bound to a >15 kb *Hind*III and a 3 kb *Kpn*I fragment. The probe MCP 6R showed only a weak reaction with DFV and EHNv DNA but recognized specifically an approximately 200 bp fragment of the 1 kb ladder (Invitrogen).

In contrast to MCP 6R and RanaMCP-R, the probe DF2-R hybridized specifically to viral DNA from DFV only (Fig. 2,

Lane 2) but not to EHNv (Fig. 2, Lane 3) or cellular DNA (Fig. 2, Lane 1). The recognized fragments exhibited a size >15 kb of *Hind*III- and 3 kb of *Kpn*I-digested DFV DNA. The reaction pattern of DF2-R correlates to results observed in a hybridization assay with RanaMCP-R.

Based on the obtained DFV sequence, primers specific for amplification of the complete *MCP* gene of Santee-Cooper ranaviruses were generated. The synthetic oligonucleotides are localized upstream at the nucleotide position –189 to –169 (SCIV-F) and downstream at +45 to +24 (SCIV-R) of the *MCP* gene from DFV (FR677324). Using these primers, the complete *MCP* gene of the Santee-Cooper ranaviruses DFV, GV6, and LMBV was amplified. Corresponding to the determined DFV *MCP* gene sequence, a 1627 bp amplicon was produced. The obtained PCR products were sequenced directly and after cloning into the pGEM-Teasy vector (Promega).

In summary, the complete *MCP* gene including the flanking regions of the Santee-Cooper ranaviruses DFV, GV6, and LMBV was identified. The *MCP* gene of LMBV starts at nucleotide position 191 and ends at nucleotide position 1582. It contains 1392 bp with a coding capacity for a 50 kDa protein consisting of 463 amino acids (Fig. 3). A putative transcription initiation site 5'-TAT AAT-3' is located at position 178–183.

Two possible start codons were identified for GV6 and DFV (Fig. 3). The first start codon is unlikely to initiate transcription and translation due to its poor

Fig. 2. Detection of DFV and EHNv *MCP* gene by DNA hybridization. *Hind*III- and *Kpn*I-digested cellular DNA (Lane 1) and viral DNA (Lane 2: DFV; Lane 3: EHNv) were separated by agarose gel electrophoresis and transferred to nitrocellulose for Southern blot analyses. DNA hybridizations were performed using fluorescein-dUTP-labelled oligonucleotides DF2-R, MCP 6R, and RanaMCP-R.

DNA 1 kb ladder (Invitrogen) separated in lane M is indicated on the left

Fig. 3. MCP sequence alignment of LMBV, DFV, GV6, FV3, and EHNV *MCP* gene and flanking regions of ranaviruses FV3 (GenBank accession no. AY548484, nt 97155-98834), EHNV (FJ433873, nt 19731-21410), DFV (AF157665, nt 1-586), GV6 (AF157671, nt 1-586), LMBV (AF080250, nt 1-495), DFV (FR677324, nt 431-2110), GV6 (FR677325, nt 1-1626), and LMBV (FR682503, nt 1-1627) were compared. Primer sequences and positions are indicated in **bold**. Arrows represent the primer direction. Identical nucleotides within the *MCP* gene and the primer sequences are shaded grey. Start and stop codons are indicated in **bold** and underlined.

	641								720
LMBV (AF080250)	TACAACATGA	TTGGCAACAC	TAGCGATCTC	GTCATATCCC	CCCCCGCCAC	CGATCAAGCA	GGAGCTAGGG	TCCTGCCCGC	
LMBV (FR682503)	TACAACATGA	TTGGCAACAC	TAGCGATCTC	GTCATATCCC	CCCCCGCCAC	CGATCAAGCA	GGAGCTAGGG	TCCTGCCCGC	
DFV (FR677324)	TACAACATGA	TTGGCAACAC	TAGCGATCTC	GTCATATCCC	CCCCCGCCAC	CGGACAAGCA	GGAGCTAGGG	TCCTGCCCGC	
GV6 (FR677325)	TACAACATGA	TTGGCAACAC	TAGCGATCTC	GTCATATCCC	CCCCCGCCAC	CGGACAAGCA	GGAGCTAGGG	TCCTGCCCGC	
DFV (AF157665)									
GV6 (AF157671)									
FV3 (AY548484)	TATAACATGA	TAGGCAACAC	CAGCGATCTC	ATCAACCCCG	CCCCGGCCAC	AGGCCAGGAC	GGAGCCAGGG	TCCTCCCGGC	
EHNH (FJ433873)	TATAACATGA	TAGGCAACAC	CAGCGATCTC	ATCAACCCCG	CCCCGGCCAC	AGGCCAGAAC	GGAGCCAGGG	TCCTCCCGGC	
	721								800
LMBV (AF080250)	CAAAAACCTT	GTCCTTCCTC	TCCCC						
LMBV (FR682503)	CAAAAACCTT	GTCCTTCCTC	TCCCCCTTCT	TTTCGGCAGA	GACAGCGGCC	TGGCCCTGCC	TACAGTCACC	CTGCCTTACA	
DFV (FR677324)	CAAAAACCTT	GTCCTTCCTC	TCCCCCTTCT	TTTCGGCAGA	GACAGCGGCC	TGGCCCTGCC	TACAGTCACC	CTGCCTTACA	
GV6 (FR677325)	CAAAAACCTT	GTCCTTCCTC	TCCCCCTTCT	TTTCGGCAGA	GACAGCGGCC	TGGCCCTGCC	TACAGTCACC	CTGCCTTACA	
DFV (AF157665)									
GV6 (AF157671)									
FV3 (AY548484)	CAAGAACCTG	GTTCTTCCTC	TCCCATCTCT	CTTCTCCAGA	GACAGCGGCC	TGGCCCTGCC	AGTCGTCTCC	CTCCCTTACA	
EHNH (FJ433873)	CAAGAACCTG	GTTCTTCCTC	TCCCATCTCT	CTTCTCCAGA	GACAGCGGCC	TGGCCCTGCC	AGTCGTCTCC	CTCCCTTACA	
	801								880
LMBV (AF080250)									
LMBV (FR682503)	ACGAAATTAG	AATCACCATC	AGCCTGAGAT	CCATTCCAGGA	TCTCCTGATT	CTTCAGCACA	AGACGACCGG	AGAAGTCAAG	
DFV (FR677324)	ACGAAATTAG	AATCACCATC	AGCCTGAGAT	CCATTCCAGGA	TCTCCTGATT	CTTCAGCACA	AGACGACCGG	AGAAGTCAAG	
GV6 (FR677325)	ACGAAATTAG	AATCACCATC	AGCCTGAGAT	CCATTCCAGGA	TCTCCTGATT	CTTCAGCACA	AGACGACCGG	AGAAGTCAAG	
DFV (AF157665)									
GV6 (AF157671)									
FV3 (AY548484)	ACGAGATCAG	GATAACAGTC	AAGCTGAGGG	CCATCCACGA	CCTCCTGATC	CTCCAGCACA	ACACCACAGG	GGCAATCAGC	
EHNH (FJ433873)	ACGAGATCAG	GATAACAGTC	AAGCTGAGGG	CCATCCACGA	CCTCCTGATC	CTCCAGCACA	ACACCACAGG	GGCAATCAGC	
	881								960
LMBV (AF080250)									
LMBV (FR682503)	CCAATCGTGG	CCACAGATCT	GGAAGGAGGT	CTCCAGACACA	CGGTAGAGGC	TCACGTCTAC	ATGACTGTGG	GTCTGGTGAC	
DFV (FR677324)	CCAATCGTGG	CCACAGATCT	GGAAGGAGGT	CTCCAGACACA	CGGTAGAGGC	TCACGTCTAC	ATGACTGTGG	GTCTGGTGAC	
GV6 (FR677325)	CCAATCGTGG	CCACAGATCT	GGAAGGAGGT	CTCCAGACACA	CGGTAGAGGC	TCACGTCTAC	ATGACTGTGG	GTCTGGTGAC	
DFV (AF157665)									
GV6 (AF157671)									
FV3 (AY548484)	CCCATCGTGG	CCTCCGACCT	TGCGGGAGGT	CTCCCCGACA	CCGTGAGGCG	CAACGTCTAC	ATGACCGTCG	CCCTCATCAC	
EHNH (FJ433873)	CCCATCGTGG	CCGCCGACCT	CGAGGGAGGT	CTCCCCGACA	CCGTGAGGCG	CAACGTCTAC	ATGACCGTCG	CCCTCATCAC	
	961								1040
LMBV (AF080250)									
LMBV (FR682503)	TGCCCGCCGAG	CGTCAGGCTA	TGAGCAGCTC	AGTCAGGGAC	ATGGTGGTGG	AGCAGATGCA	GATGGCTCCA	GTCCACATGG	
DFV (FR677324)	TGCCCGCCGAG	CGTCAGGCTA	TGAGCAGCTC	AGTCAGGGAC	ATGGTGGTGG	AGCAGATGCA	GATGGCTCCA	GTCCACATGG	
GV6 (FR677325)	TGCCCGCCGAG	CGTCAGGCTA	TGAGCAGCTC	AGTCAGGGAC	ATGGTGGTGG	AGCAGATGCA	GATGGCTCCA	GTCCACATGG	
DFV (AF157665)									
GV6 (AF157671)									
FV3 (AY548484)	CGGGGACGAG	AGACAGGCCA	TGAGCAGCAC	AGTCAGGGAC	ATGGTGGTGG	AGCAGGTGCA	GGCCGCCCCA	GTCCACATGG	
EHNH (FJ433873)	CGGGGACGAG	AGGCAGGCCA	TGAGCAGCAC	AGTCAGGGAC	ATGGTGGTGG	AGCAGGTGCA	GGCCGCCCCA	GTCCACATGG	
	1041								1120
LMBV (AF080250)	TCAACCC								
LMBV (FR682503)	TCAACCCCAA	GAACGCCACC	GTCTTTTCAG	CAGACCTGCG	CTTTTCCAC	GCCGTCAAAG	CGCTCATGTT	TATGGTGCAA	
DFV (FR677324)	TCAACCCCAA	GAACGCCACC	GTCTTTTCAG	CCGACCTGCG	CTTTTCCAC	GCCGTCAAAG	CGCTCATGTT	TATGGTGCAA	
GV6 (FR677325)	TCAACCCCAA	GAACGCCACC	GTCTTTTCAG	CCGACCTGCG	CTTTTCCAC	GCCGTCAAAG	CGCTCATGTT	TATGGTGCAA	
DFV (AF157665)									
GV6 (AF157671)									
FV3 (AY548484)	TCAACCCAG	GAACGCCACC	ACCTTCCACA	CCGACATGCG	GTTCTCACAC	GCAGTCAAGG	CCTTGATGTT	TATGGTGCA	
EHNH (FJ433873)	TCAACCCAG	GAACGCCGCC	ACCTTCCACA	CCGACATGCG	GTTCTCACAC	GCAGTCAAGG	CCTTGATGTT	TATGGTGCA	
	1121								1200
LMBV (AF080250)									
LMBV (FR682503)	AACGTCACTC	ACAAGTCTGT	GGGTTCACAC	TACACTTGCG	TCACTCCTGT	TGTTGGAGCG	GGTAACACCG	TCCTGGAGCC	
DFV (FR677324)	AACGTCACTC	ACAAGTCTGT	GGGTTCACAC	TACACTTGCG	TCACTCCTGT	TGTTGGAGCG	GGTAACACCG	TCCTGGAGCC	
GV6 (FR677325)	AACGTCACTC	ACAAGTCTGT	GGGTTCACAC	TACACTTGCG	TCACTCCTGT	TGTTGGAGCG	GGTAACACCG	TCCTGGAGCC	
DFV (AF157665)	AACGTCACTC	ACCCCTCCGT	CGGCTCCAAT	TACACTTGCG	CCACTCCCGT	CGTGGGAGTC	GGCAACACGG	TCCTGGAGCC	
GV6 (AF157671)	AACGTCACTC	ACCCCTCCGT	CGGCTCCAAT	TACACTTGCG	TCACTCCCGT	CGTGGGAGTC	GGCAACACGG	TCCTGGAGCC	
FV3 (AY548484)	AACGTCACTC	ACCCCTCCGT	CGGCTCCAAT	TACACTTGCG	TCACTCCCGT	CGTGGGAGTC	GGCAACACGG	TCCTGGAGCC	
EHNH (FJ433873)	AACGTCACTC	ACCCCTCCGT	CGGCTCCAAT	TACACTTGCG	CCACTCCCGT	CGTGGGAGTC	GACAACACGG	TCCTGGAGCC	
	1201								1280
LMBV (AF080250)									
LMBV (FR682503)	CGCCCTGGCC	GTCGATCCGG	TCAAGAGCGC	CAGTCTGGTG	TACGAAAACA	CTACCAGGCT	TCCAGACATG	AGCGTAGAGT	
DFV (FR677324)	CGCCCTGGCC	GTCGATCCGG	TCAAGAGCGC	CAGTCTGGTG	TACGAAAACA	CTACCAGGCT	TCCAGACATG	AGCGTAGAGT	
GV6 (FR677325)	CGCCCTGGCC	GTCGATCCGG	TCAAGAGCGC	CAGTCTGGTG	TACGAAAACA	CTACCAGGCT	TCCAGACATG	AGCGTAGAGT	
DFV (AF157665)	AGCCCTGGCG	ATGGATCCCG	TCAAGAGTGC	CAGCCTGGTG	TACGAAAACA	CCACAAGGCT	CCCCGACATG	GGAGTCGAGT	
GV6 (AF157671)	AGCCCTGGCG	GTCGATCCCG	TCAAGAGTGC	CAGCCTGGTG	TACGAAAACA	CCACAAGGCT	CCCCGACATG	GGAGTCGAGT	
FV3 (AY548484)	AGCCCTGGCG	GTCGATCCCG	TCAAGAGCGC	CAGCCTGGTG	TACGAAAACA	CCACAAGGCT	CCCCGACATG	GGAGTCGAGT	
EHNH (FJ433873)	AGCCCTGGCG	GTCGATCCCG	TCAAGAGCGC	CAGCCTGGTG	TACGAAAACA	CCACAAGGCT	CCCCGACATG	GGAGTCGAGT	

Fig. 3. (continued)

bits only 81% identical nucleotides compared to the respective coding region (nt 900–1392 of the *MCP* gene) published by Hyatt et al. (2000) (AF157665, AF157671; Fig. 3). However, comparison of the identified sequence with further known data of the *MCP* gene of DFV (nt 43–333 of *MCP* gene; Mao et al. 1997, U82550) shows 99% identity.

The identity of the *MCP* gene of LMBV described here to the published 495 bp nucleotide sequence (nt 61–555 of the *MCP* gene; Mao et al. 1999; AF080250) amounts to 100% (Fig. 3). Compared to the determined LMBV *MCP* gene sequence, DFV and GV6 had identity values of 99.21% (Table 2). From the 11 substitutions detected within the 1392 bp sequence, 9 nucleotide exchanges are localized within the first 500 bp (Fig. 3).

GIV and SGIV exhibit around 98% identity within the *MCP* gene. The highest identity compared to the *MCP* gene of the other ranaviruses was detected for LMBV with 71.26% (Table 2).

Within the main group of ranaviruses, consisting of FV3, BIV, tiger frog virus (TFV), REV, RTRV, and ATV/Ambystoma tigrinum stebbinsi virus (ATSV); SSTIV (Zhao et al. 2007) and EHN, ECV/ESV, PPIV, SERV, and REV, the nucleotide identity of the *MCP* gene varies from a maximum of 100% between ESV and ECV to a minimum of 93.89% between RTRV and SERV.

Although viruses within the family *Iridoviridae* encode for a *MCP* gene of similar but different size, no differences in length were detected within the same genera, except for the genus *Iridovirus*. Members of the genus *Megalocyttivirus* encode for a *MCP* gene of 1362 bp. *MCP* gene of viruses within the genus *Lymphocystivirus* consists of 1380 bp, and chloriridoviruses exhibit a *MCP* gene of 1401 bp. The nucleotide sequences of the *MCP* gene of viruses within the genus *Iridovirus* vary in length between 1389 bp (*Costelytra zealandica* iridescent virus, CZIV; AF025775) and 1455 bp (invertebrate iridescent virus 9, IIV-9; AF025774). The alignment of the *MCP* gene of ranaviruses with the respective nucleotide sequence of other iridoviruses shows an identity of 56.6 to 52.9% to megalocytiviruses, 50.8 to 47.6% to iridoviruses, 56.8 to 50.5% to lymphocystiviruses, and 54 to 52.4% to chloriridoviruses.

Phylogenetic analyses

To analyze the position of Santee-Cooper ranaviruses within the genus *Ranavirus* and the family *Iridoviridae*, phylogenetic studies based on the complete *MCP* gene were performed. Data published in GenBank were included. Sequence alignments and dendrogram analyses of the *MCP* gene were generated by the sequence alignment programs Bestfit and Paup-

Search (GCG-X-Win32 version 11.1.3, Accelrys). The respective results are illustrated in Figs. 3 & 4.

The family *Iridoviridae* is divided into 5 genera (Chinchar et al. 2005, 2009). *MCP* gene sequence identities do not exceed 60% between the different genera. Based on the sequence alignment of the *MCP* gene, the genus *Ranavirus* is clustered in three groups (Table 2). The main group is composed of viruses isolated from amphibian species, containing the type species FV3, BIV, TFV, REV, RTRV, and ATV/ATSV; reptile species (SSTIV); and fish species represented by EHN, ECV/ESV, PPIV, SERV, and REV.

Santee-Cooper ranaviruses cluster in a separate group. The third phylogenetic group comprises the isolates SGIV and the GIV. Santee-Cooper ranaviruses, as well as grouper iridoviruses, exhibit *MCP* gene sequence identities of less than 80% compared to the main group of ranaviruses. Based on the presented data the classification as members of the genus *Ranavirus* is reasonable, especially regarding the equal length of the gene on the one hand and the phylogenetic distance to the other genera on the other hand.

Ranavirus MCP gene amplification (Rana MCP PCR) and restriction fragment length polymorphism (RFLP)

The present study presents the identification of the complete *MCP* gene sequences of DFV, GV6, and LMBV and the phylogenetic relationship to other ranaviruses. Based on these data a unique PCR (Rana MCP) was established to identify different ranavirus isolates. Specific primers RanaMCP-F and RanaMCP-R were deduced from *MCP* coding region nt 838 to 1353. The sequence of primer binding sites is identical for all analysed ranaviruses, with the exception of SGIV and GIV. The Rana MCP PCR product is 516 bp in size. The partial *MCP* gene sequence from 12 different ranavirus isolates including the Santee-Cooper ranaviruses DFV, GV6, and LMBV was amplified. The resulting specific PCR products confirm the expected size of 516 bp. In order to differentiate various ranavirus isolates, Rana MCP PCR sequences were validated for virus-specific RFLP. Predicted restriction fragments calculated from available sequence data are presented in Table 3. The restriction pattern of the Rana MCP PCR product was confirmed after digestion with *Bam*HI, *Pst*I, *Afl*III, and *Sal*I. Based on the restriction pattern of these 4 enzymes, the studied ranaviruses can be divided into 4 groups (Table 3).

The first group contains the isolates EHN, BIV, ECV/ESV, PPIV, REV, and ATV/ATSV (Table 3). The *Bam*HI, *Pst*I, and *Afl*III restriction patterns of the amplified Rana *MCP* gene fragment of these isolates are identical. To distinguish EHN from other rana-

		-9	+5	+20
FV3 (AY548484)	CGTG TATCT TATAA TAAAAAGGAA	ATG TCTTCTG	TAAC TGGT T	
EHN (AY187045)	 TATAA TAAAAAGGAA	ATG TCTTCTG	TAAC CGGT T	
ATV (NC_005832)	TGTG TATCT TATAA TAAAAAGGAA	ATG TCTTCTG	TAAC CGGT T	
ATSV (AY150217)	TGTG TATCT TATAA TAAAAAGGAA	ATG TCTTCTG	TAAC CGGT T	
RCV JB (AB474588)	 TATAA TAAAAAGGAA	ATG TCTTCTG	TAAC TGGT T	
SSTIV (EU627010)	TGTG TATCT TATAA TAAAAAGGAA	ATG TCTTCTG	TAAC TGGT T	
TFV (AF389451)	TGTG TATCT TATAA TAAAAAGGAA	ATG TCTTCTG	TAAC TGGT T	
SGIV (AY521625)	GTTT GTATTTAAAA AACAGCAACA	ATG ACTTGTA	CAAC GGGT G	
GIV (AY666015)	GTTT GTATTTAAAA AACAGCAACA	ATG ACTTGTA	CAAC GGGT G	
LMBV	TCTA TCGCT TATAA TAAAA.GG.A	ATG TCTTCTG	TTAC GGGT T	
DFV /GV6	TCTA TCGCT TATAA TAAAA.GG.A	ATG TCTTCTG	TTAC GGGT T	
BPIV-07 (EU847417)	ATAT ATAAAAGTAT CATTGTCATC	ATG TCTGCAA	TCTCAG GTG C	
BPIV-08 (EU847418)	ATAT ATAAAAGTAT CATTGTCATC	ATG TCTGCAA	TCTCAG GTG C	
CPIV (EU847420)	ATAT ATAAAAGTAT CATTGTCATC	ATG TCTGCAA	TCTCAG GTG C	
GSPIV (EU315313)	ATAT ATAAAAGTAT CAGTCTCATC	ATG TCTGCAA	TCTCAG GTG C	
KGIV (EU847414)	ATAT ATAAAAGTAT CATTGTCATC	ATG TCTGCAA	TCTCAG GTG C	
LYCIV (AY779031)	ATAT ATAAAAGTAT CATTGTCATC	ATG TCTGCGA	TCTCAG GTG C	
SSBIV (EU847419)	ATAT ATAAAAGTAT CATTGTCATC	ATG TCTGCGA	TCTCAG GTG C	
OIFV (EU276417)	ATAT ATAAAAGTAT CACCGTCATC	ATG TCTGCAA	TCTCAG GTG C	
TRBIV (AY590687)	ATAT ATAAAAGTAT CACCGTCATC	ATG TCTGCAA	TCTTAG GTG C	
LCDV (NC_001824)	ACTG ATACAAT.AT AAAATAGAAT	ATG ACTTCTG	TAGCAG GTG C	
LCDV (AY29774)	ACGA ACCGAATAAT AAAATAGAAT	ATG ACTTCTG	TAGCG GTG T	
CZIV (AF025775)	GCTA CTAATAAATT AAAC ATG TCT	ATG TCTTCAT	CCAATATA AC	
IIV-9 (AF025774)	GCTA CTAATAAATT AAAC ATG TCT	ATG TCTTCAT	CAAATATA AC	
IIV-22 (M32799)	GCTA CTAATAAATT AAAC ATG TCT	ATG TCTTCAT	CGAATATA AC	
TIV (M33542)	GCTA CTAATAAATT AAAC ATG TCT	ATG TCTTCAT	CGAATATA AC	
IIV-6 (AF303741)	TGGC AATGTAATAA ATAAACAAAG	ATG TCTATTT	CTTCGT CAAA	
ATIV (DQ643392)	ATAT TGCAACTAAT AAATCTAAAC	ATG TCTATCAA	GTATA ACTT C	

Fig. 4. Comparison of sequences flanking the MCP gene start codon ATG. Regions up- and downstream of the MCP gene start codon are compared. The initiation codons are shown in **bold**; potential TATA boxes are indicated in **bold** and *italics*. The first MCP start codon of tipula iridescent virus (TIV), invertebrate iridescent virus 22 (IIV-22), invertebrate iridescent virus 9 (IIV-9), and Costelytra zealandica iridescent virus (CZIV) is indicated in **bold** and underlined. Numbers indicate the position of conserved nucleotides (shaded black) within all analysed sequences. The multiple alignment includes available data from members of the family *Iridoviridae*. Representatives of the genus *Ranavirus* are FV3, *Epizootic haematopoietic necrosis virus* (EHN), *Ambystoma tigrinum virus* (ATV), *Ambystoma tigrinum stebbinsi virus* (ATSV), *Rana catesbeiana virus* JB (RCV JB), soft-shelled turtle iridovirus (SSTIV), tiger frog virus (TFV), Singapore grouper iridovirus (SGIV), grouper iridovirus (GIV), largemouth bass virus (LMBV), guppy virus 6 (GV6), and doctor fish virus (DFV). Genus *Megalocytivirus* is represented by barramundi perch iridovirus isolate BPIV-08 (BPIV-08), barramundi perch iridovirus isolate BPIV-07 (BPIV-07), common ponyfish iridovirus isolate CPIV-05 (CPIV), giant sea perch iridovirus (GSPIV), king grouper iridovirus (KGIV), large yellow croaker iridovirus (LYCIV), silver sea bream iridovirus (SSBIV), olive flounder iridovirus (OIFV), and turbot reddish body iridovirus (TRBIV). Members of genus *Iridovirus* are TIV, IIV-22, IIV-9, CZIV, and invertebrate iridescent virus 6 (IIV-6). *Lymphocystis disease virus* (LCDV) represents the genus *Lymphocystivirus* and *Aedes taeniorhynchus* iridescent virus (ATIV) the genus *Chloriridovirus*. GenBank accession numbers are indicated in brackets

viruses, a cleavage of the Rana MCP PCR product with *SalI* is recommended. The resulting restriction products have an expected size of 365 and 151 bp, and differ from the respective restriction fragments of the Rana MCP PCR product of SERV (332/184 bp). A *SalI* recognition sequence is not present within the respective region of the other analysed ranaviruses.

The second group comprises FV3, SSTIV, SERV, and RCV-JP (Table 3). Whereas for FV3, SSTIV, and RCV-JP DNA a *SalI* recognition site is missing within the

analysed region, the Rana MCP PCR product of SERV is cleavable by this enzyme (Table 3). RCV-JP can be distinguished from other ranaviruses after cleavage of the Rana MCP PCR product with *BamHI* and *AatI*. FV3, originated from amphibian and SSTIV with reptile origin exhibit identical restriction patterns for 268 different DNA endonucleases. The complete MCP gene sequences of these 2 isolates differ in only 5 nucleotides. Two nucleotide exchanges are localized within the amplified Rana MCP region. Both isolates can only be distinguished based on their origin and after sequencing analyses.

The third group is generated by the Santee-Cooper ranaviruses (Table 3). The restriction pattern of these 3 isolates is identical for 268 different restriction endonucleases. Therefore, these isolates can only be distinguished after sequencing analyses.

The fourth group lists the amphibian isolates RTRV and TFV (Table 3). Both isolates are identical within their MCP gene sequence.

Using additional restriction endonucleases, a differentiation of isolates within the first group is feasible. BIV is marked by one *AatI* recognition site within the Rana MCP PCR product (Table 3). The resulting fragments are 443 and 73 bp in length. Using *AatI*, the ranavirus BIV can be clearly identified and differentiated from other isolates.

ATV and ATSV can be distinguished from other ranaviruses after *NotI* cleavage of the Rana MCP PCR product. The resulting fragments have an expected size of 428 and 88 bp. The *NotI* restriction site is missing in this region in other ranaviruses. In addition to *SalI* enzyme analyses, a restriction with *XcmI* facilitates the differentiation of ESV/ECV from the other isolates. The *XcmI* restriction pattern is identical for EHN, PPIV, and SERV, and the resulting fragments are 375 and 141 bp in length.

In comparison to the other ranaviruses, PPIV exhibits an additional *Hpy99I* restriction site within the Rana MCP PCR product. The described Rana MCP PCR RFLP provides a practical option for rapid identification of a suspected ranavirus including the Santee-Cooper ranaviruses GV6, DFV, and LMBV. However, in light of sinking costs, sequence analyses of

Table 2. Complete *MCP* gene nucleotide sequence comparison of ranaviruses. Determined nucleotide sequences of ranaviruses largemouth bass virus (LMBV), doctor fish virus (DFV), guppy virus 6 (GV6), shortfin eel virus (SERV, FJ358612), European sheatfish virus (ESV, FJ358609), *European catfish virus* (ECV, FJ358608), pike-perch iridovirus (PPIV, FJ358610), *Bohle iridovirus* (BIV, FJ358613), *Frog virus 3* (FV3, FJ459783), and *Rana esculenta virus isolate Italy 282/I02* (REV, FJ358611) were compared with GenBank data from Singapore grouper iridovirus (SGIV, AY521625), grouper iridovirus (GIV, AY666015), *Ambystoma tigrinum virus* (ATV, NC005832), *Ambystoma tigrinum stebbinsi virus* (ATSV, AY150217), and tiger frog virus (TFV, AF389451). The original host fish, amphibian (Am) and reptile (Rept) of virus isolates (SGIV = fish host) and the percentages of identical nucleotides are given. Closely related isolates (>90 % identity) are in **bold**

Host	Virus	SGIV	GIV	LMBV	DFV/ GV6	SERV	ESV/ ECV	EHNV	PPIV	ATV/ ATSV	RTRV/ TFV	BIV	FV3	REV
Fish	GIV	98.35												
Fish	LMBV	70.69	71.26											
Fish	DFV/GV6	70.33	70.91	99.21										
Fish	SERV	70.40	70.69	78.45	78.38									
Fish	ESV/ECV	69.97	70.33	78.52	78.45	95.69								
Fish	EHNV	69.54	69.90	77.95	78.02	95.26	98.92							
Fish	PPIV	69.76	70.12	78.38	78.45	94.40	97.99	98.64						
Am	ATV/ATSV	69.90	70.26	77.80	77.80	93.68	97.49	98.13	97.20					
Am	RTRV/TFV	69.61	70.12	78.23	78.23	93.89	97.13	97.77	98.28	96.48				
Am	BIV	69.47	70.04	78.02	78.09	93.97	97.06	97.85	98.20	96.41	98.49			
Am	FV3	69.40	69.97	78.02	78.09	94.11	97.27	97.77	98.28	96.34	98.56	98.78		
Am	REV	69.54	69.90	78.23	78.31	94.54	98.20	98.85	99.35	97.41	98.35	98.42	98.35	
Rept	SSTIV	69.40	69.97	77.95	78.02	94.04	97.20	97.70	98.20	96.26	98.49	98.71	99.64	98.28

Table 3. Restriction fragment length polymorphism (RFLP) of *Rana MCP* PCR. Restriction endonuclease patterns of *Bam*HI, *Pst*I, *Afl*III, *Sal*I, *Aat*I, *Not*I, *Hae*II, and *Hpy*99I are given. The analysis is based on published *MCP* gene sequences of EHNV (GenBank accession no. AY187045), BIV (FJ358613), ESV (FJ358609), ECV (FJ358608), PPIV (FJ358610), REV 282/I02 (FJ358611), ATV (NC005832), ATSV (AY150217), FV3 (FJ459783), SSTIV (DQ335253), SERV (FJ358612), RCV-JP (AB474588), RTRV (AY033630) and TFV (AF389451). Determined *MCP* gene sequences of GV6, DFV, and LMBV (FR677325, FR677324, FR682503) are included. Viruses with similar restriction pattern were summarized in separate groups (I–IV). Isolate-specific restriction patterns are indicated in **bold**

Group	Virus	<i>Bam</i> HI	<i>Pst</i> I	<i>Afl</i> III	<i>Sal</i> I	<i>Aat</i> I	<i>Not</i> I	<i>Hae</i> II	<i>Hpy</i> 99I
I	EHNV	186/172/158	341/175	516	365/151	516	516	313/203	371/115/30
	BIV	186/172/158	341/175	516	516	443/73	516	313/203	371/115/30
	ESV/ECV	186/172/158	341/175	516	516	516	516	516	371/115/30
	PPIV	186/172/158	341/175	516	516	516	516	313/203	345/115/30/26
	REV 282/I02	186/172/158	341/175	516	516	516	516	313/203	371/115/30
	ATV/ATSV	186/172/158	341/175	516	516	516	428/88	313/203	371/145
II	FV3	358/158	341/175	516	516	516	516	313/203	371/115/30
	SSTIV	358/158	341/175	516	516	516	516	313/203	371/115/30
	SERV	358/158	341/175	516	332/184	516	516	313/203	371/115/30
	RCV-JP	358/158	341/175	516	516	443/73	516	313/203	371/115/30
III	DFV/GV6/LMBV	516	516	398/118	516	516	516	313/127/76	329/187
IV	RTRV/TFV	330/186	516	516	516	443/73	516	313/203	371/115/30

the *Rana MCP* PCR product are useful tools for identification and differentiation of unknown ranaviruses.

DISCUSSION

In the present study, the complete *MCP* gene sequences of DFV, GV6, and LMBV were identified (FR677324, FR677325, and FR682503). DFV and GV6 are not only identical in the *MCP* gene sequence, but

also in the DNA polymerase sequence (Mao et al. 1997, Holopainen et al. 2009). Therefore, it is supposed that DFV and GV6 are isolates of the same virus. Nevertheless, further analyses are required in order to prove this assumption. *MCP* gene sequence variation compared to LMBV is negligible, as the identity amounts to 99.21 %. The *MCP* gene sequences of DFV and GV6 deviate partially from published data. The identity to a partial *MCP* gene sequence of DFV (U82550; Mao et al. 1997) is 98.97 %, whereas only 81.35 % to DFV

(AF157665; Hyatt et al. 2000) and 81.67% to GV6 (AF157671, Hyatt et al. 2000) were calculated. On the other hand, the mentioned sequences (Hyatt et al. 2000) exhibit very high identity values compared to the respective C-terminal sequences of ESV (AF157679) and ECV (AF157659; DFV vs. ESV/ECV 99.32%; GV6 vs. ESV/ECV/DFV 98.81%), which is contrary to the antigenicity as well as the hybridization assay results described in the same study. Hyatt et al. (2000) already discussed this discrepancy, and suggested that the complete MCP gene of DFV and GV6 should be determined for further analysis. Thus, the GV6 and DFV sequences published by Hyatt et al. (2000) are in dispute.

In the current OIE Manual of Diagnostic Tests for Aquatic Animals (Anonymous 2009) the recommended method for ranavirus differentiation is based on restriction enzyme analysis (REA) of the partial PCR-amplified MCP gene, but the method was developed for 6 ranavirus isolates only (ESV, ECV, EHN, Gutapo virus, Wamena virus, and BIV; Marsh et al. 2002). The recommended alternative method is a PCR and subsequent sequence analysis of the partial MCP gene (Hyatt et al. 2000). Neither of these methods has been developed for detection of grouper iridoviruses, and the REA method does not include the differentiation of the Santee-Cooper ranaviruses. PCR primers published by Hyatt et al. (2000) were designed for detection of DFV and GV6 among other ranaviruses, but results from the present study and Holopainen et al. (2009) suggest that these primers are not optimal for detection of DFV and GV6. To ensure a rapid and reliable diagnosis of EHN and amphibian ranaviruses such as FV3, BIV, REV, ATV/ATSV, and RTRV/TFV, a new method (Rana MCP PCR) was established to detect all studied ranaviruses with the exception of the marine iridoviruses SGIV and GIV. After *SalI* restriction analyses, EHN can be clearly differentiated from all other ranavirus isolates. Use of other restriction enzymes and/or sequencing analysis facilitates the further identification of all studied virus isolates. The methods presented here provide new means for detection and differentiation of Santee-Cooper and other ranaviruses, with the exception of grouper iridoviruses. The Rana MCP PCR with RFLP is a very useful diagnostic tool for rapid detection of different ranavirus isolates and facilitates prevention and control of ranavirus infection propagation. In addition, a new Santee-Cooper iridovirus (SCIV) PCR has been developed for identification of the complete MCP gene of DFV, GV6 and LMBV.

Based on Fauquet et al. (2005), the genus *Ranavirus* encompasses 6 defined (ATV, *Bohle iridovirus*, *Epi-zootic haematopoietic necrosis virus*, *European catfish virus*, *Frog virus 3*, and *Santee-Cooper ranavirus*) and

3 tentative species (*Rana esculenta iridovirus*, Singapore grouper iridovirus, and *Testudo iridovirus*). Demarcation criteria for strains within the same species of the genus *Ranavirus* are similar RFLP profiles (>70%), host range and identity of more than 95% within the MCP or other viral proteins. DNA and protein profile, host specificity, and gene sequences differentiate viral species from each other. Until now, definitive quantitative criteria have not yet been established to delineate different viral species (Chinchar et al. 2005).

Regarding the results of the present study and published data (Hyatt et al. 2000, Chinchar et al. 2009, Holopainen et al. 2009, Whittington et al. 2010), the classification within the genus *Ranavirus* should be reconsidered. The MCP gene of all ranaviruses is identical in length. Homologies in MCP gene sequences range between 69.4% (FV3 vs. SGIV) and 100% (ESV vs. ECV, ATV vs. ATSV, RTRV vs. TFV, DFV vs. GV6). In comparison to other genera within the family *Iridoviridae*, ranaviruses are more closely related to each other. The identity between the ranavirus MCP genes and published sequences of megalocytiviruses, lymphocystiviruses, iridoviruses, and chloriridoviruses varies between 47.7% (RTRV vs. IIV 6, M99395) and 56.8% (SGIV vs. Lymphocystis disease virus [LCDV], AY521625, AY297741).

Based on all available data, including morphological features, RFLP profiles, and gene sequences of MCP, DNA polymerase and neurofilament triplet H1-like protein (Mao et al. 1997, 1999, Hyatt et al. 2000, Qin et al. 2001, Goldberg et al. 2003, Holopainen et al. 2009), ranaviruses are divided into 3 groups. Isolates within the same group exhibit more than 95% identity regarding the complete MCP gene sequence.

Phylogenetic analyses indicate a classification of ranaviruses in 3 groups. The major group encompasses isolates from fish (SERV, ESV/ECV, EHN, PPIV, and MCIV), reptiles (SSTIV), and amphibians (ATV/ATSV, RTRV/TFV, BIV, FV3, and REV). Deviations within the MCP gene sequences of FV3, EHN, BIV, ESV, ECV, PPIV, REV, and RTRV average less than 3%. SERV takes an exceptional position within this group. Identity of MCP gene ranges from 93.89% (RTRV) to 95.69% (ESV/ECV).

The second group includes the Santee-Cooper ranaviruses DFV, GV6, and LMBV, isolated from ornamental and wild fish. It is obvious that these isolates are genetically distinct from all other analysed ranaviruses, since sequence homology amounts to approximately 70 to 78% compared to grouper iridovirus isolates and other ranaviruses, respectively. The same clustering of ranaviruses has been reported earlier based on DNA polymerase gene sequences (Holopainen et al. 2009).

The third group represents marine ranaviruses with grouper iridoviruses GIV and SGIV. With an MCP

gene identity of 98.35 %, both viruses are quite closely related. All published primers for detection of the ranavirus *MCP* gene are in discrepancy to the SGIV and GIV *MCP* gene. The identity of the *MCP* gene sequence of the marine ranaviruses SGIV and GIV (AY521625, AY666015) compared to the respective sequence of megalocytiviruses, iridoviruses, lymphocystiviruses, and chloriridoviruses ranges from 49.24 % (SGIV; AY521625 vs. invertebrate iridescent virus 22, IIV-22; M32799) to 56.79 % (SGIV vs. lymphocystis disease virus, LCDV; AY297741). The highest homology of 71.26 % was found for the *MCP* gene of other ranaviruses (GIV vs. LMBV). Concerning the given results, the demarcation criteria for the genus *Ranavirus* should be reconsidered.

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