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COMMUNICATION

An examination of the *Iridovirus* core genes for reconstructing *Ranavirus* phylogenies

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Abstract

Ranaviruses are globally emerging infections of poikilothermic vertebrates and belong to the viral family *Iridoviridae*. The six species of ranaviruses are responsible for unknown numbers of infections and disease and mortality events around the world in amphibians, fish, and reptiles. Genomic investigations have shown that there are 24 core genes shared by all iridoviruses. In this study, we examine the utility of each of these genes in reconstructing phylogenetic relationships across six species of *Ranavirus*. We also performed dot-plot analysis for the 17 isolates in the study. For large-scale differentiation, using the major capsid protein gene creates a tree similar to the whole genome tree. Other comparable genes include open reading frame (ORF) 19R (a serine–theonine protein kinase) and ORF 88R (Erv I/Alr Family protein). The poorest candidate for phylogenetic reconstruction, due to high homology, was ORF 1R (a putative replication factor and (or) DNA binding-packing protein). There are a plethora of genes that may be useful to examine phylogenies at smaller scales (e.g., to examine local adaptation); however, they do not necessarily belong to the set of highly conserved core genes.

Key words: emerging infectious disease, amphibians, reptiles, fish, global distribution, genomics

Introduction

Ranaviruses from the viral family *Iridoviridae* are large double-stranded DNA viruses with a large vertebrate host range (Duffus et al. 2015; Chinchar et al. 2017). They were first described in North American northern leopard frogs (*Rana pipiens* now *Lithobates pipiens*) in the mid-1960s (Granoff et al. 1965). Since then, they have been described in over a hundred other species of amphibians, reptiles, and fish from all around the globe (Duffus et al. 2015). Ranaviruses are responsible for countless morbidity and mortality events in affected species and have even caused population declines (e.g., common frogs (*Rana temporaria*) in the UK; Teacher et al. 2010) and local extirpations (e.g., multiple species in the Spanish Pyrenees; Price et al. 2014). They are also known to affect threatened and endangered species such as the Chinese giant salamander (*Andrias davidanus*, Geng et al. 2011) and pallid sturgeon (*Scaphirhynchus albus*, Waltzek et al. 2014), making them a cause for conservation concern.

Currently, there are six species of ranavirus recognized by the International Committee on Taxonomy of Viruses (ICTV; Chinchar et al. 2017); this is as a result of a deeper understanding of *Ranavirus* genomics. Initially, it was thought that the major capsid protein (MCP) was adequate for the study of viral evolution of iridoviruses, as it has both highly conserved, but also some variable domains (Tidona et al. 1998). However, some studies have used only portions of the MCP and have had trouble parsing possible local adaptations (e.g., Duffus and Andrews 2013). Other studies have used multiple genes, including the MCP, and have been able to detect genetic variation in large-scale data sets (e.g., Stöhr et al. 2015) and in genetic information from geographically isolated data sets (Ridenhour and Storfer 2008). While the MCP gene may have enough variation to place ranaviruses into their different species, a study that uses only the MCP may miss local variation or adaptation. For example, Ridenhour and Storfer (2008) only found evidence of local adaptation of *Ambystoma tigrinum* virus strains in the southeastern United States when they examined >5% of the genome and included genes such as the eIF-2α homologue, which is not present in all iridoviruses.

Eaton et al. (2007) reexamined the genomes of 12 different iridoviruses. They found that there were 26 core genes across all iridoviruses. These core genes are typically conserved, with functions that are generally associated with virulence, replication, and gene expression (Eaton et al. 2007; Jancovich et al. 2015). However, further molecular studies of newly discovered and sequenced iridovirids bring this total of shared genes down to 24 (e.g., Shrimp hemocyte iridescent virus lacks the small subunit of ribonucleotide reductase (Qiu et al. 2018) and European Chub Iridovirus lacks a deoxinucleotide reductase (GenBank accession number MK6376310)). Here we examine the utility of 24 core iridovirus genes across six species of ranavirus and perform a large scale dot-plot analysis of 17 different isolates. However, it is important to note that all ranaviruses contain the original 26 core genes and a number of other genes that are found in all ranaviruses. We hope that the results from this study will be useful for those who study ranavirus phylogenomics, genomics, and phylogenetics.

Materials and methods

Sequence data and initial analysis

Sequence data from 24 iridovirus core genes were obtained from GeneBank. The accession numbers of all isolates used, their abbreviations used for this study, and the isolate name as it appears in GenBank can be found in **Table 1**. Also see **Table 1** for strains that are representative of each species of ranavirus. Sequence data for each gene was aligned using the default settings of the MAFFT server (Katoh et al. 2019; mafft.cbrc.jp/alignment/server/). FASTA formatted text files from the MAFFT alignment were then converted to MEGA format and were then analyzed to find the best fit nucleotide substitution model (**Table 2**).

Phylogenetic analysis

Phylogenetic analysis was done in MEGA 6 (Tamura et al. 2013). Maximum likelihood trees were built using the best fit nucleotide substitution model (**Table 2**; **Figs. S1**–**S22**). When the full genome trees were produced, noncollinear segments were left as such and no attempts at genome reorganization were undertaken. (This does not appear to have affected our results, because the trees share similar topologies with those created with the previously described 26 iridovirid core genes by Eaton et al. (2007) (e.g., Eaton et al. 2010; Jancovich et al. 2015)). With the increasing access to full genome sequencing, the use of the concatenated core genes will become less common and using full genomes will become the norm, and adjustments for noncolinear segments of the genome will less often be corrected. Open reading frame (ORF) 8R (partially duplicated gene in Singapore grouper iridovirus (SGIV)) or ORF 27R (truncated gene in SGIV) in our analyses of ranaviruses were not included in our analysis (data not shown). This is the reason why there are 24 genes in the analysis. The trees that we created are also unrooted.

Table 1. Isolate names, abbreviations used, and accession numbers of all *Ranavirus* isolates used in this study.

Species designation	Name	Abbreviation	GenBank accession number
Ambystoma tigrinum virus (ATV)	*Ambystoma tigrinum virus*—RRV	ATV-3	KR075879.1
	Ambystoma tigrinum virus—UTAH	ATV-2	KR075877.1
	Ambystoma tigrinum virus	ATV-1	NC_005832.1
Chinese Giant Salamander Virus (CGSV)	*Andrias davidianus* ranavirus—2010SX	CGSV-1	KF033124.1
	Andrias davidianus ranavirus—1201	CGSV-2	KC865735.1
	Chinese giant salamander iridovirus isolate CGSIV-HN1104	CGSV-3	KF512820.1
Common midwife toad virus (CMTV)	Common midwife toad ranavirus—*Mesotriton alpestris*/2008/E	CMTV-1	JQ231222.1
	Common midwife toad ranavirus isolate Pelophylax kl. esculentus/2013/NL	CMTV-2	KP056312.1
	Common midwife toad ranavirus isolate Pe/2016/Netherlands/UU3160714042	CMTV-3	MF125270.1
Epizootic haematopoietic necrosis virus (EHNV)	*Epizootic haematopoietic necrosis virus*	EHNV-1	FJ433873.1
European catfish virus (ECV)	*European catfish virus*	ECV-1	KT989884.1
	European catfish virus	ECV-2	KT989885.1
European sheatfish virus (ESV)	*European sheatfish virus*	ESV-1	JQ724856.1
Frog virus 3 (FV3)	*Frog virus 3*	FV3-1	NC_005946.1
	Frog virus 3 isolate SSME	FV3-2	KJ175144.1
	Bohle iridovirus	BIV-1	KX185156.1
Singapore grouper iridovirus (SGIV)	*Singapore grouper iridovirus*	SGIV-1	NC_006549.1

Trees were compared visually using bootstrap values and distances (provided by the scale bars). To quantify the differences between the full genome tree, the MCP tree and the trees made from each ORF, CompPhy (Fiorini et al. 2014) was used, and Treedist was then used to determine the symmetrical distance between each ORF tree and the full genome or MCP (Felsenstein 2008) (**Table 2**). Trees that had a symmetrical distance of 10 and under were considered to be adequate for phylogenetic reconstruction.

Dot-plot analysis was performed in R (R Core Team 2019) in the package DICIPHER v 2.6 (Wright 2016).

Results

Most of the phylogenetic trees for the 24 core genes of the 17 isolates of ranavirus that were examined were able to group at the species level (six species). SGIV, however, is problematic as it appears as an individual group. It is important to note that at the time of this analysis only one sequence of SGIV was available; however, Grouper Iridovirus (not included in this analysis) may be a strain of SGIV. The tree constructed with full genome sequences (**Fig. 1**) shows the usual groupings of the six ranavirus species used in this study. Visually, the best trees, besides those made by the major capsid protein

Table 2. Reference open reading frame (ORF) in *Frog virus 3* (FV3) and the best-fit model for nucleotide substitution as determined in MEGA 6.

FV3 ORF	Gene name[a]	MEGA 6 best fit model	Distance between MCP tree[b]	Distance between full genome tree[c]
ORF 1R	Putative replication factor and (or) DNA binding or packing protein	Kimura Two Parameter and Invariability	12	14
ORF 2L	Myristilated membrane protein	Kimura Two Parameter and Invariability	16	14
ORF 9L	Putative NTPase I	Kimura Two Parameter and Gamma Distributed	14	16
ORF 12L	Unknown	Kimura Two Parameter and Invariability	10	14
ORF 15R	ATPase-like Protein	Kimura Two Parameter	14	18
ORF 19R	Serine–threonine protein kinase	Kimura Two Parameter	10	12
ORF 21L	Helicase family	Kimura Two Parameter	16	16
ORF 22R	D5 family NTPase involved in DNA replication	Kimura Two Parameter	14	18
ORF 37R	NIF–NLI interacting factor	Kimura Two Parameter	12	16
ORF 41R	Unknown	Kimura Two Parameter	22	22
ORF 53R	Myristilated membrane protein	Kimura Two Parameter	22	22
ORF 57R	Serine–threonine protein kinase	Kimura Two Parameter	14	18
ORF 60R	DNA polymerase family B exonucleases	Kimura Two Parameter	10	12
ORF 62L	DNA-dependent RNA polymerase II second largest subunit	Kimura Two Parameter	10	14
ORF 67L	Ribonucleotide reductase small subunit	Kimura Two Parameter	6	14
ORF 80L	Ribonuclease III	Kimura Two Parameter	4	14
ORF 81R	Transcription elongation factor TFIIS	Kimura Two Parameter	8	20
ORF 84R	Proliferating cell nuclear antigen	Kimura Two Parameter	14	14
ORF 85R	Deoxynucleoside kinase	Kimura Two Parameter	10	14
ORF 88R	Ervl/Alr family	Kimura Two Parameter	12	14
ORF 90R	Major capsid protein	Kimura Two Parameter	0	12
ORF 91R	Immediate early protein infected-cell protein-46	Kimura Two Parameter	10	10
ORF 94L	Hypothetical protein—*Clostridium tetani*	Kimura Two Parameter	6	10
ORF 95R	Putative XPPG-RAD2-type nuclease	Kimura Two Parameter	14	8

[a]Gene name as defined by Eaton et al. (2007).
[b]Distance between the major capsid protein (MCP) tree and the stated tree in CompPhy (Fiorini et al. 2014) as calculated by Treedist based on the symmetric distance (Felsenstein 2008).
[c]Distance between the full genome sequence tree and the stated tree in CompPhy (Fiorini et al. 2014) as calculated by Treedist based on the symmetric distance (Felsenstein 2008).

(ORF 90R in *Frog virus 3* (FV3); Fig. 2), are ORF 19R in FV3 (Fig. 3), and ORF 88R in FV3 (Fig. 4). One of the worst ORFs for reconstruction was ORF 1R in FV3 (Fig. S1) because the branch lengths were extremely short between the different species with low bootstrap support. (All other trees can be found in the Figs. S2–S22.)

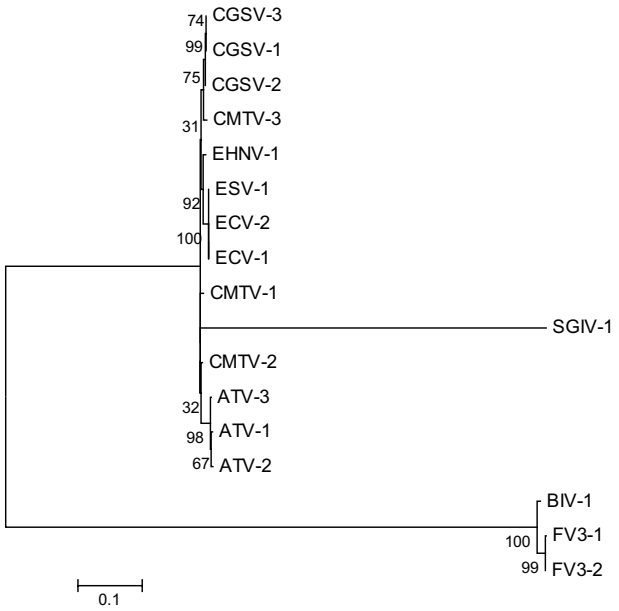

Fig. 1. A maximum-likelihood tree made from the whole genomes of the 17 *Ranavirus* isolates using the general time reversible model of nucleotide substitution. Bootstrap values are for 1000 replicates. Tree is unrooted. See **Table 1** for the abbreviations used.

The trees that had the shortest symmetrical distance (i.e., 10 or below) to the MCP were made from ORFs 12L, 19R, 60R, 62L, 67L, 80L, 81R, 88R, 91R, and 94L. However, trees that had the shortest symmetrical distance to the full genomes were ORF 91R, ORF 94L, and ORF 95R (Table 2).

The best trees over all were made from ORF 19R, ORF 88R, ORF 91R (**Fig. S19**), and ORF 94L (**Fig. S20**) when all three types of analyses were considered together.

Dot-plot analysis of all 17 isolates of ranavirus used in the current study showed collinearity at the species level (e.g., Common midwife toad virus and Chinese Giant Salamander Virus, FV3, and Bohle Iridovirus) and there tends to only be smaller genomic rearrangements between most of the different species of *Ranavirus*. However, SGIV is not collinear with any other ranaviruses analyzed in this study (see **Fig. 5**).

Discussion

While most of the 24 core iridovirus genes used in this study will sort out the different ranavirus isolates to the species level, their utility is quite limited for finer-scale differentiation. There are only two genes that we feel make good approximations to phylogenetic trees constructed with the whole genome or the full sequence of the major capsid protein gene (ORF 90R in FV3), these are ORFs 19R and 88R when visually compared. ORF 19R is hypothesized to be a serine–threonine protein kinase (Eaton et al. 2007). This group of enzymes typically catalyze the transfer of phosphate groups from adenosine triphosphate (ATP) to proteins (Jacob et al. 2011). Our results here are surprising because viral serine–threonine protein kinases are usually highly conserved (Jacob et al. 2011). However, iridoviruses are hypothesized to have two serine–threonine protein kinases (ORF57R is a second FV3 serine–threonine kinase) in their core gene set (Eaton et al. 2007). It is common for genes that are duplicated in the genome to have one that is more variable than the other because of differences in

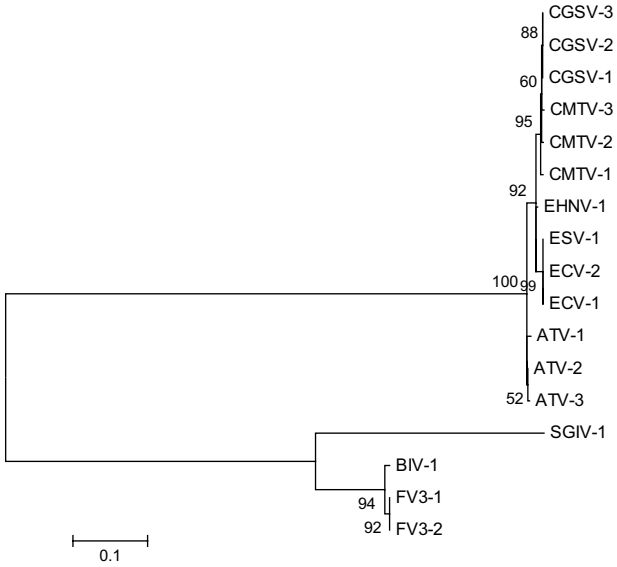

Fig. 2. Maximum likelihood tree using open reading frame 90R, the major capsid protein, sequence data from 17 different *Ranavirus* isolates created in MEGA 6. Bootstrap values represent 1000 replications and the best fit nucleotide substitution model was the Kimura 2 parameter. Tree is unrooted. See **Table 1** for the abbreviations used.

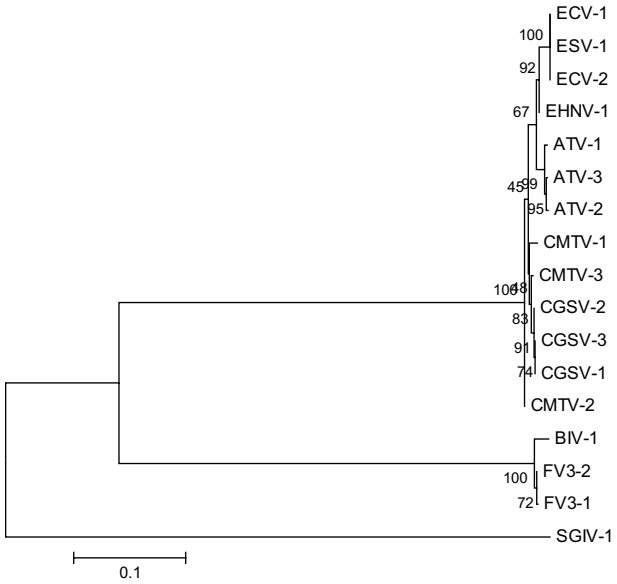

Fig. 3. Maximum likelihood tree using open reading frame 19R sequence data from 17 different *Ranavirus* isolates created in MEGA 6. Bootstrap values represent 1000 replications and the best fit nucleotide substitution model was the Kimura 2 parameter. Tree is unrooted. See **Table 1** for the abbreviations used.

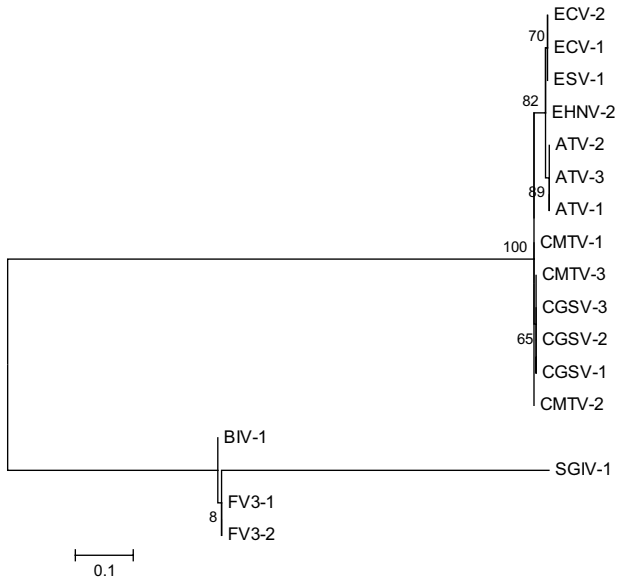

Fig. 4. Maximum likelihood tree using open reading frame 88R sequence data from 17 different *Ranavirus* isolates created in MEGA 6. Bootstrap values represent 1000 replications and the best fit nucleotide substitution model was the Kimura 2 parameter. Tree is unrooted. See **Table 1** for the abbreviations used.

selective pressures and mutation (see Shackelton and Holmes (2004) for a review of large DNA virus evolution). This is likely what is occurring in the case of ORFs 19R and 57R.

Open reading frame 88R is hypothesized to be an Erv I/Alr family protein (Eaton et al. 2007). These are sulfhydryl oxidase and are they are known to be far more divergent in viruses than in other organisms (Fass 2008). This sequence flexibility is likely what permits for a finer-scale differentiation between ranavirus species than other genes that are highly conserved based on functional needs. There is only a single gene that codes for Erv I/Alr family proteins in the core genes (Eaton et al. 2007), leaving out the potential for a gene duplication event and subsequent divergent evolution of the two genes within the iridovirus core genome.

Open reading frame 91R is hypothesized to be an immediate early protein infected-cell protein —46 homologue (Eaton et al. 2007). The function of ORF 91R has been partially characterized by Penny and Brunetti (2019). During infection, its protein product can be found in the nucleus and it is hypothesized that it is involved with viral DNA transcription as well as involved in one or more early viral replication processes (Penny and Brunetti 2019). Open reading frame 94L is hypothesized to be a homologue of a protein found in *Clostridium tetani* (Eaton et al. 2007). Its protein product localizes in the endoplasmic reticulum and are hypothesized to have a role in the viral modulation of cellular secretory pathways based on its structure (Penny and Brunetti 2019).

Our dot-plot analysis shows similar genomic rearrangements within ranaviral species. However, markedly different genomic arrangements can be seen between different species of ranavirus. Interestingly, SGIV shows minimal collinearity with the other ranavirus species examined (**Fig. 5**). The genome is very different in its arrangement, despite having the same core, and perhaps should be considered a different iridoviral genera instead of a species within the genus *Ranavirus*. In our phylogenetic analyses, SGIV tends also to be divergent, which supports this claim and previous reports of SGIV being the lowest percent similarity of all ranaviruses (see Stöhr et al. 2015).

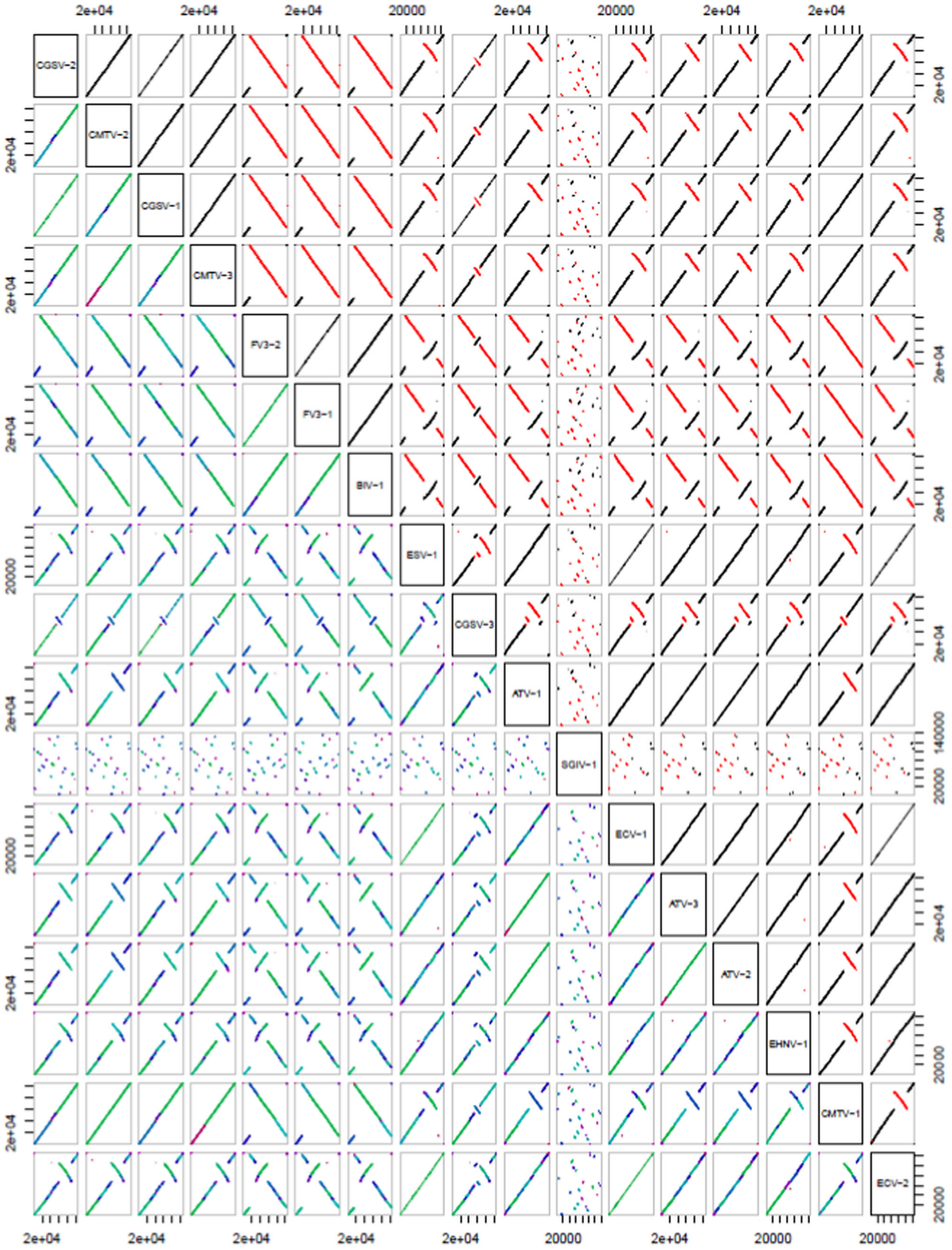

Fig. 5. A dot plot analysis of the 17 *Ranavirus* whole genomes that were used in this study. Created in R using (R Core Team 2019) the package DECIPHER v2.0 (Wright 2016). See **Table 1** for the abbreviations used.

Most of the 24 core iridovirus genes are able to more or less sort different ranavirus strains to the species level; however, it is highly likely that they are not able to show fine-scale differentiation (e.g., geographical or local adaptations), because they are not sufficiently polymorphic between species. Only two genes, ORFs 19R and 88R in FV3, make comparatively supported trees to the major capsid protein and full genomes visually. However, when the symmetrical distances are analyzed ORF 91R and 94L may be added to potentially informative sequences for phylogenetic analysis. We do recommend that single gene sequences alone not be used as there is evidence that multiple targeted concatenated genes are more useful in reconstructing the true phylogenies because of nucleotide shifts at the third position of the codon (e.g., yeasts, Collins et al. 2005).

Our dot-plot analysis suggests that each species of ranavirus has undergone unique genome rearrangements that are consistent at the species level. However, SGIV is highly divergent and may need to be considered a separate genera within the *Iridoviridae* instead of a species of ranavirus. Future directions should include determining the phylogenetic signal for each of the core genes and examining the utility of the more divergent core genes in sorting out local adaptations.

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Author contributions

ALJD conceived and designed the study. DRB, AJD, RBF, ARG, ADM, JDS, DEB, CMH, CDD, WC, BP, SER, and ALJD performed the experiments/collected the data. DRB, AJD, RBF, ARG, ADM, JDS, DEB, CMH, CDD, WC, BP, SER, and ALJD analyzed and interpreted the data. ALJD drafted or revised the manuscript.

Competing interests

The authors have declared that no competing interests exist.

Data availability statement

All relevant data are within the paper and Supplementary Material.

Supplementary material

The following Supplementary Material is available with the article through the journal website at doi:10.1139/facets-2020-0009.

Supplementary Material 1

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