Genomic analysis of Ranavirus and exploring alternative genes for phylogenetics

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Abstract

Ranaviruses can infect both captive and wild cold-blooded vertebrates, leading to significant economic and environmental losses. With the cases of ranavirus infection increasing, many ranavirus genomic sequences were published, but little is known about ranavirus taxonomy on a whole genome level. In this study, 44 ranaviruses core genes were identified in 32 ranaviruses genome suquences by using PanX. The Neighbor joining phylogenetic trees (NJ-tree) based on 44 ranaviruses core genes and 24 iridoviridae core genes and composition vector phylogenetic tree (CV-Tree) based on whole genome were constructed. The three of phylogenetic trees showed that 32 ranavirus isolates can be divided to 4 different subspecies including GIV-like, EHNV-like, FV3-like and CMTV-like, and subspecies taxonomic position of three phylogenetic trees were consistent. However, the phylogenetic position of ToRV could not be determined if it belongs to FV3-like or CMTV-like group. Subsequently, we carried out dot plot analysis and confirmed that ToRV should belong to CMTV-like group. Based on dot plot analysis and phylogenetic trees, taxonomic classification of ranaviruses were confirmed. Finally, 4 genes which are suitable for the construction of phylogenetic tree were selected from ranavirus core genes by recombination analysis, substitution saturation analysis and single-gene phylogenetic analysis. Phylogenetic tree based on concatenated sequences of the 4 selected genes showed that classification of subspecies was identical with 3 of the phylogenetic trees. Conclusion: our results confirmed taxonomic identification of ranaviruses, the 4 selected genes used in phylogenic analysis will make taxonomic identification more convenient and accurate.

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Abstract: Ranaviruses can infect both captive and wild cold-blooded vertebrates, leading to significant economic and environmental losses. With the cases of ranavirus infection increasing, many ranavirus genomic sequences were published, but little is known about ranavirus taxonomy on a whole genome level. In this study, 44 ranaviruses core genes were identified in 32 ranaviruses genome suquences by using PanX. The Neighbor joining phylogenetic trees (NJ-tree) based on 44 ranaviruses core genes and 24 iridoviridae core genes and composition vector phylogenetic tree (CV-Tree) based on whole genome were constructed. The three of phylogenetic trees showed that 32 ranavirus isolates can be divided to 4 different subspecies including GIV-like, EHNV-like, FV3-like and CMTV-like, and subspecies taxonomic position of three phylogenetic trees were consistent. However, the phylogenetic position of ToRV could not be determined if it belongs to FV3-like or CMTV-like group. Subsequently, we carried out dot plot analysis and confirmed that ToRV should belong to CMTV-like group. Based on dot plot analysis and phylogenetic trees, taxonomic classification of ranaviruses were confirmed. Finally, 4 genes which are suitable for the construction of phylogenetic tree were selected from ranavirus core genes by recombination analysis, substitution saturation analysis and single-gene phylogenetic analysis. Phylogenetic tree based on concatenated sequences of the 4 selected genes showed that classification of subspecies was identical with 3 of the phylogenetic trees. Conclusion: our results confirmed taxonomic identification of ranaviruses, the 4 selected genes used in phylogenic analysis will make taxonomic identification more convenient and accurate.

Keyword: Ranavirus, core genes, dot plot analysis, phylogenetics.

Introduction

Ranaviruses are nuclear cytoplasmic large DNA viruses (NCLDVs), which are one of five genera within the family Iridoviridae (other members are*Chloriridovirus* , *Iridovirus* , *Lymphocystivirus* and*Megalocytivirus*) (Jancovich, Steckler, et al., 2015). Ranaviruses have been found to infect a wide variety of cold-blooded vertebrates, including amphibians, reptiles, and fish (Chinchar, 2002; Duffus et al., 2015). Members of the genus have been isolated from at least 175 species of ectothermic vertebrates in all continents except for Antarctica, and they are recognized as significant pathogens causing die-offs in captive and wild populations across the globe (Duffus et al., 2015). Ranaviruses have become serious problems in modern aquaculture, because of their high pathogenicity and ability to cause mortality. As pathogens of wildlife, there are many evidences that ranaviruses can cause population declines in wild animal (Blaustein et al., 2012; Daszak et al., 1999; Price et al., 2014). Furthermore, outbreaks of ranaviruses could result in population extinction (Earl et al., 2014; Miller et al., 2011). Therefore, ranaviruses are known to be emerging pathogens that have potential risk to cause economic losses and serious damages to the ecological environment.

There are six species of ranaviruses recognized by the International Committee on Taxonomy of Viruses (ICTV, https://talk.ictvonline.org/taxonomy/) through 2018. These species include *Ambystoma tigrinum virus* (ATV), *Common midwife toad virus* (CMTV), *Epizootic haematopoietic necrosis virus* (EHNV),*Frog virus 3* (FV3), *Santee-cooper ranavirus* (SCRV),*Singapore grouper iridovirus* (SGIV). Among them, the CMTV and SGIV were newly recognized as ranaviruses in the 10th report. However, the taxonomy of SGIV and *Grouper iridovirus* (GIV) have long been controversial, because whole genome dot plot analyses indicated that the genomes of GIV and SGIV possess few regions of collinearity with other ranaviruses (Jancovich, Qin, et al., 2015). Traditionally, virus taxonomy was characterized by morphological features (by electron microscopy), physicochemical properties (by varying pH and temperature, adding lipid solvents and detergents, etc) and antigenic properties (by many different serologic methods) (Murphy et al., 2012). With the development of sequencing technology, more and more viral genomes have been successfully sequenced. These viral genomes and a number of virus classification tools based on viral genomes (such as CVTree3(Zuo et al., 2015), Java Dot Plot Alignments(Brodie et al., 2004) and pairwise sequence comparison (Bao et al., 2014)) have been widely applied to virus taxonomy (Eaton et al., 2007; Gao et al., 2007; Radoshitzky et al., 2015).

Members of the family Iridoviridae have linear dsDNA genomes that range from 140 to 303 kbp in size and

encode 92 to211 putative viral genes (Jancovich, Steckler, et al., 2015). With the growing number of sequenced ranavirus genomes, there are more than 30 genome sequences of ranaviruses deposited in National Center of Biotechnology Information (detailed information of isolates were summarized in Table S1). However, little is currently known about their genomic molecular biology and evolutionary taxonomy. Despite many recent advances in viral genomic sequencing, there is much more to learn about the relationship of ranavirues within the genus itself. In order to gain a greater understanding of evolutionary taxonomy within ranaviruses, we will identify core genes of ranaviruses, construct phylogenetic tree and perform dot plot analysis explore the relationship links between ranaviruses.

Method

1 Genome

A total of 32 genomic sequences of ranaviruses from various cold-blooded vertebrates (reptiles, amphibians and fishes) were obtained from NCBI (National Center of Biotechnology Information). The detailed information about host species, the country of origin and the year of detection are listed in Table S1.

2 Core-pan analysis

In order to identify the core genes of ranavirus, the pan-genome analysis of orthologous gene clusters were carried out using PanX(Ding et al., 2017) for the 32 completed ranavirus genomes. The strictly core genes (including genes present in all viral genomes) were identified using PanX(Ding et al., 2017) with the parameters as "-dmi 0.7" and "-nsl". "-dmi 0.7" means that the sequence identity threshold to report an alignment is 0.7, and "-nsl" means to "disable long branch splitting".

3 Phylogenetic analysis of genome

Composition vector phylogenetic tree (CV-Tree)(Gao & Qi, 2007; Zuo & Hao, 2015): the amino acid sequences in FASTA format were directly summited in CVTree3 Web Server (http://tlife.fudan.edu.cn/cvtree/cvtree/) and K-tuple length was set at 6. Additionally, the genomic sequences of other Iridovirus family were included as an out-group. Visualization and annotation for phylogenetic tree were performed using Evolview v3 (Subramanian et al., 2019).

Neighbor-joining phylogenetic tree (NJ-Tree) based on ranavirus core genes: the ranavirus core genes identified by core-pan analysis were aligned using MAFFT software (Katoh et al., 2009), and the aligned sequences were concatenated in order using PhyloSuite (Zhang et al., 2018). Finally, the streamlined sequences were used to construct Neighbor-joining phylogenetic tree by using MEGA 6.0 with 1,000 bootstrap replicates(Tamura et al., 2013). NJ-Tree based on iridovirid core genes: iridovirid core genes were selected from the ranavirus core genes identified by core-pan analysis. The subsequent steps were same as Neighbor-joining phylogenetic tree (NJ-Tree) based on ranavirus core genes.

4 Dot plot analysis

Dot plot analysis of ranaviruses was performed using Java Dot Plot Alignments (JDotter) (Brodie et al., 2004). FV3 (NCBI access number is AY548484) was placed in horizontal sequence, meanwhile the other ranaviruses were placed in vertical sequence. Maximum plot size was set at 700 bases and sliding window size was set at 50. The nucleic acid sequences of FASTA format used for Dot plot analysis were obtained from NCBI.

5 Recombination analysis

The ranavirus core genes identified by core-pan analysis were concatenated using PhyloSuite (Zhang et al., 2018). The streamlined sequences were imported into the Recombination Detection Program (RDP) BETA4.67 (Darren P Martin et al., 2015). Putative recombination breakpoints were visualized in RDP 4 software by algorithms of RDP, GENECONV, Bootscan, Maxchi, Chimaera, SiSscan, PhylPro, LARD and 3Seq. UPGMA (unweighted pair group method using arithmetic average) phylogenetic tree was constructed by TREES module within RDP4.

6 Substitution saturation analysis

The ranavirus core genes were imported separately to DAMBE Version 5.3.19 (Xuhua Xia, 2013). The index of nucleotide substitution saturation was estimated using Xia method (Xuhua Xia et al., 2003).

Result

1 Core-pan analysis

The result of core-pan analysis showed that viruses within the 32 ranaviruses share 44 strictly core genes (Figure 1). The number of unique genes (gene clusters which exist in only one species) in almost all virus isolates except for ESV, SGIV and TFV is no more than 5. ESV, SGIV and TFV possess 24, 47 and 8 unique genes, respectively. The detailed information of strictly core genes and unique gene within 32 ranaviruses is listed in Table S2.

2 Phylogenetic analysis

The genomic sequences of 32 ranavirus genomes and other members of family Iridoviridae (Chloriridovirus, Iridovirus, Lymphocystivirus and Megalocytivirus) genomes were obtained from GenBank and uploaded onto CV-Tree online analysis platform to construct phylogenetic trees based on whole-genome. The result showed that ranavirus genus and other members of family Iridoviridae were clustered into their respective groups (Figure 2). In ranavirus genus, 32 ranavirus isolates can be divided to 4 different subspecies including GIV-like, EHNV-like, FV3-like and CMTV-like (Figure 2).

Based on core-pan analysis, 44 ranavirus core genes were obtained from 32 ranaviruses' complete genomes. Within 44 ranavirus core genes, 24 iridovirus core genes were identified (see Table S3). Neighbor-joining phylogenetic analyses based on the concatenated ranavirus core genes (60,721 nt characters including gaps) of genus and iridovirid core genes (37,803 nt characters including gaps) were performed (Figure 3). Neighbor-joining phylogenetic tree (NJ-Tree) based on ranavirus core genes was similar to the one constructed using iridovirus core genes (Figure 3). Both NJ-phylogenetic trees revealed that ranaviruses can be classified into four distinct lineages, and the taxonomic positions of subspecies were also consistent with CV-Tree.

The subspecies classification of 32 ranaviuses based on NJ-Tree and CV-tree were summarized in Table S4. However, the phylogenetic position of ToRV could not be clearly determined by NJ-Tree and CV-tree (Figure 2 and Figure 3).

3 Dot plot analysis

Dot plot analysis using Java Dot Plot Alignments(Brodie et al., 2004) is a visual tool used in identifying linear relationships and comparing genomic structural changes of two sequences, such as deletion, insertion and inversions. Dot plot studies clearly indicate whether gene order is conserved or not and may serve as a way to determine the classification and evolutionary relationship (Jancovich, Steckler, et al., 2015). In our study, the genomic sequence of FV3 (AY548484) was compared to other 31 completed ranavirus sequences using JDotter

software. The result indicated that the same group of ranavirus subspecies approximately showed similar linearity pattern (Figure 4). Especially in EHNV-like subspecies group, these isolates in the same subspecies showed analogous genome architecture changes. In FV3-like subspecies group, except for RCV, most isolates shared complete collinearity with FV3 (AY548484). Despite RCV not sharing complete collinearity with FV3, linearity pattern of RCV is the most similar with FV3-like subspecies group. According to previous phylogenetic analyses, evolutionary distances of GIV-like subspecies were farthest from the FV3. Likewise, GIV and SGIV display only short segments of genomic collinearity with FV3 (Figure 4). Therefore, we can use the rule of the same subspecies showing similar linearity pattern to determine the ranavirus taxonomy as a supplementary way. In general, the taxonomic positions of subspecies based on dot plot analysis were also consistent with previous phylogenetic analysis (Table S4).

Previously, the position of ToRV could not be determined clearly by phylogenetic analysis. Dot plot analysis demonstrated that linearity pattern of ToRV is more similar to CMTV-like subspecies group instead of FV3-like group (Figure 4). In order to determine the similarity degree of the ToRV genome with CMTV-like and FV3-like group, we carried out dot plot comparisons between ToRV and CMTV-like/FV3-like group ranaviruses. The result of dot plot showed that ToRV shares high collinearity with CMTV-like group (Figure S1), which indicated that ToRV should belong to CMTV-like group. In summary, taxonomic positions of 32 ranaviruses were confirmed by using phylogenetic analyses and dot plot analyses.

4 Filtering of ranavirus core genes

The phylogenetic analyses and dot plot analyses based on whole viral genome analysis can precisely determine taxonomic positions of ranavirus subspecies. However, these methods need to perform high throughput sequencing, which is costly and time-consuming. Generally, single-gene or the concatenated several sequences taxonomic analysis is cost-effective and convenient. To determine which genes are suitable for phylogenetic analysis, recombination analysis and substitution saturation analysis were performed to screen out qualified genes.

4.1 Recombination analysis of ranavirus core genes

If the sequences used in phylogenetic analysis have recombinant fragments, recombination events can seriously decrease the accuracy of phylogenetic trees(Posada et al., 2002). In order to avoid the problems caused by recombination events, recombination analysis using RDP4 was performed to remove recombinant sequences (Darren P. Martin et al., 2015). The analysis performed with RDP4 showed 38 recombination events within ranavirus core genes (Figure S2 and Table S5), involving 21 ranavirus core genes. These core genes that have recombinant fragments are not suitable for phylogenetic trees. For example, No.1 recombination event, fragment (nt 18929-23747) of RCV-Z-MF187209 had a recombination with FV3-AY548484 (Table S5). The sequences of this interval were used for phylogenetic analysis, the result showed that RCV-Z was closest to FV3 (Figure 5A). However, RCV-Z and FV3 belong to different subspecies based on our previous analysis (Figure 3 and Figure 4) and UPGMA analysis (Figure 5B). Therefore, ranavirus core genes involved with recombination events should be eliminated to make sure of the accuracy of phylogenetic trees.

4.2 Substitution saturation analysis of ranavirus core genes

The accuracy of phylogenetic tree depends on sequence divergence, which means qualified sequences are neither too conserved (contain few substitutions) nor too diverged (experience substantial substitution saturation). In this study, we used DAMBE7 software to evaluate the substitution saturation of ranavirus core genes (Xuhua Xia, 2018). The results showed that cluster 5, cluster 8, cluster 13, cluster 15, cluster 20, cluster 22 and cluster 38 are not suitable for phylogenetic analysis (Table S5), because their values of I_{ss}(index of substitution saturation) are not significantly smaller than the respective values of $I_{ss.c}$ (critical Iss). IfIss is not smaller than $Iss.c$, then we can conclude that the sequences have experienced severe substitution saturation and should not be used for phylogenetic reconstruction. To determine these sequences are not qualified to phylogenetic analysis, NJ- phylogenetic trees were constructed by using these genes (Figure S3).

The classification result based on sequences with substantial substitution saturation are inconsistent with the genomic phylogenetic trees and dot plot analysis (Figure S3). In addition, most of the bootstrap values were very low.

5 Phylogenetic analysis of single core gene

The 16 ranavirus core genes were filtered out based on recombination analysis and substitution saturation analysis (Table S6). These genes do not contain any recombination sites and experience little substitution saturation, hence they are qualified to be used in the phylogenetic analysis. In order to find out single-gene taxonomic analysis which are consistent with whole genome analysis, single-gene phylogenetic analysis was performed. The 16 Neighbor-joining phylogenetic trees revealed that only clusters 2, 9, 12 and 21 of taxonomic analysis were consistent with whole genome analysis (Figure S4). Then, a phylogenetic tree was constructed based on the concatenated 4 nucleotide sequences (Figure 6), tree structures based on the concatenated 4 sequences is very similar to the tree based on the 44 ranaviruses core genes (Figure 3 and Figure 6), and the taxonomic positions of subspecies were also consistent with phylogenetic analysis based on core genes (Table S4).

Discussion

Ranaviruses have been recognized as agents of emerging infectious disease that are now globally distributed (Duffus et al., 2015). Establishing a rapid and effective taxonomic method plays an important role in virus identification and epidemiological investigation. Multiple molecular biology methods, such as RFLP (Restriction endonuclease fragment length polymorphism) profiles and cross-hybridization, are used for virus classification (King et al., 2011). But, with the development of DNA sequencing technology, genomic DNA sequencing and analysis as demarcation criteria are becoming more common. An increasing number of ranavirus genome sequences have been published in the public databases, which can contribute to the investigation of the evolutionary history and taxonomy study. In this study, we employed genomic phylogenic analysis and dot plot comparison to gain a better understanding of classification within *ranavirus* subspecies. Dot plot analysis can provide insight into genome architecture changes (containing deletions, inversions and duplications) among ranaviruses (Jancovich, Steckler, et al., 2015). These genome architecture changes can be visualized in a picture using JDotter software (Brodie et al., 2004). In previous research, dot plot analysis was widely used in the investigation of the evolutionary history by comparison of genome architecture changes (Chen et al., 2013; Jancovich et al., 2003). In this study, we found that the same group of ranavirus subspecies approximately showed similar linearity pattern (Figure 4) according to dot plot analysis maps of the FV3-AY548484 genome versus other ranavirues genomes. Neither NJ-Tree nor CV-tree can clearly determine taxonomic position of ToRV (Figure 2 and Figure 3), while ToRV should belong to CMTV-like group by Dot plot analysis (Figure 4 and Figure S1). Therefore, dot plot analysis as an important supplementary method can assist phylogenic analysis to confirm taxonomic position of ranaviruses (Table S4).

Presently, the most commonly used classification of newly isolated ranaviruses is phylogenetic analysis based on a single gene (e.g, MCP gene, NF-H1 gene and DNA polymerase gene (Allender et al., 2013; George et al., 2015; Jancovich, Steckler, et al., 2015; Zhou et al., 2013). While phylogenic analysis of a single gene is convenient, it is unlikely to be as robust as whole genome analysis and even causes mistakes. For example, MCP gene is a highly conserved gene and is most widely applied in phylogenic analysis (Jancovich, Steckler, et al., 2015). The Neighbor-Joining phylogenic tree using MCP gene showed that ToRV and CMTV-E was closely related to the FV3-like group, which is not consistent with taxonomic identification based on genome analysis (Figure S5, Cluster10). As a structural protein, MCP gene is too highly conserved sequence to distinguish among various virus strains (Duffus et al., 2013). In addition, Anke C. Stöhr *et* (Stöhr et al., 2015) constructed a phylogenic tree using concatenated sequences (3223 bp) of MCP, DNApol, RNR-α and RNR-β genes. The tree showed that ToRV was most closely to the FV3-like group, but otherwise shows CMTV-like characteristics in regard to the global arrangement of its genome (Figure S1) (Stöhr et al., 2015). The reason

for the difference observed is that the genes used in phylogenic analysis experienced substantial substitution saturation or contained recombinant fragments. For example, the RNR-β gene (Cluster 28) contained recombinant fragments (Table S5). In this study, recombination analysis and substitution saturation analysis were performed to select qualified genes, and the phylogenetic positions of the concatenated 4 selected sequences were also consistent with phylogenetic analysis based on core genes. These results can further improve the accuracy of single-gene or multiple-genes phylogenetic trees in ranavirus taxonomy.

In recent years, there has been a suggestion that GIV/SGIV should be considered as a new genus (Jancovich, Steckler, et al., 2015). We tend to agree with the view based on our study. Firstly, GIV/SGIV share little collinearity with FV3 (Figure 4) and other ranaviruses, which indicated that GIV/SGIV genome architecture were significant different with those of ranaviruses. Secondly, branch length of GIV/SGIV in phylogenetic tree based on ranavirus core genes are 0.27 (Figure 3A). In contrast, branch length among FV3- like, CMTV-like and EHNV-like group are about 0.01˜0.03. The branch lengths represent the evolutionary distances. The phylogenetic tree revealed that the evolutionary distances between GIV/SGIV and other ranaviruses (FV3- like, CMTV-like and EHNV-like gourp) are far greater than the evolutionary distances among FV3-like, CMTV-like and EHNV-like group. In addition, in the process of core pan analysis, it is interesting that only 2 genes within GIV and SGIV genomes can be strictly classified into the same cluster when compared without "-nsl" parameter. Meanwhile, 30 ranaviruses (remove GIV and SGIV) share 50 strictly core genes (Figure S6). The default program of PanX is without "-nsl" parameter, which means that long branches gene will be removed from cluster. The branch lengths reflect evolutionary distances among genes within one cluster (Ding et al., 2017). Therefore, the results of core pan analysis indicated that significant portions of strictly core genes within GIV/SGIV were far from the others of 30 ranaviruses in evolutionary distances. In conclusion, genomic characterizations of GIV/SGIV are significantly different from other ranaviruses, and need to be considered as a new genus.

Conflict of interest statement

No potential conflicts of interest were disclosed.

Ethical Statement

We declare that Ethical Statement is not applicable.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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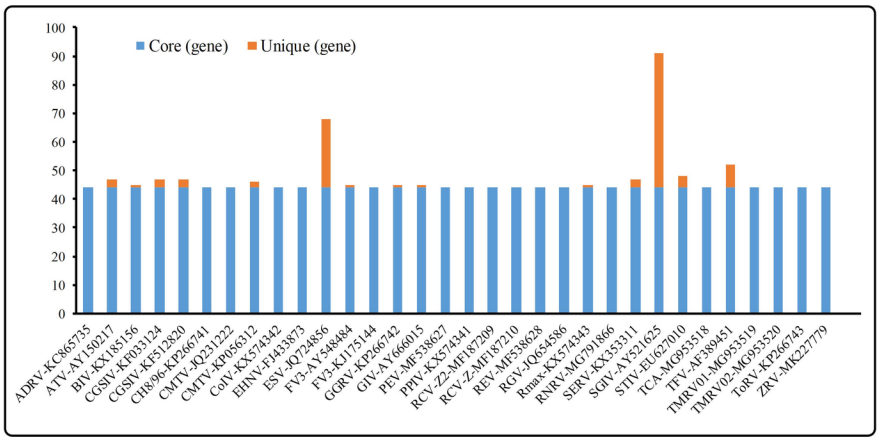


Figure 1 Genomic Core/Pan genes analysis of 32 ranaviruses

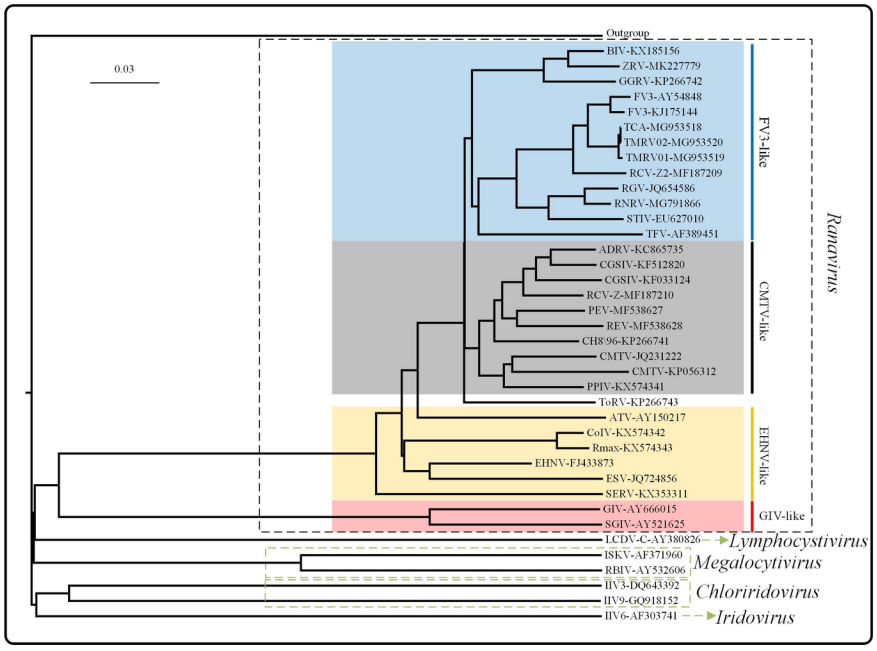


Figure 2 Phylogenetic tree based on the whole-genome by CV method (Gao & Qi, 2007). The branch lengths represent the evolutionary distances as indicated by the scale.

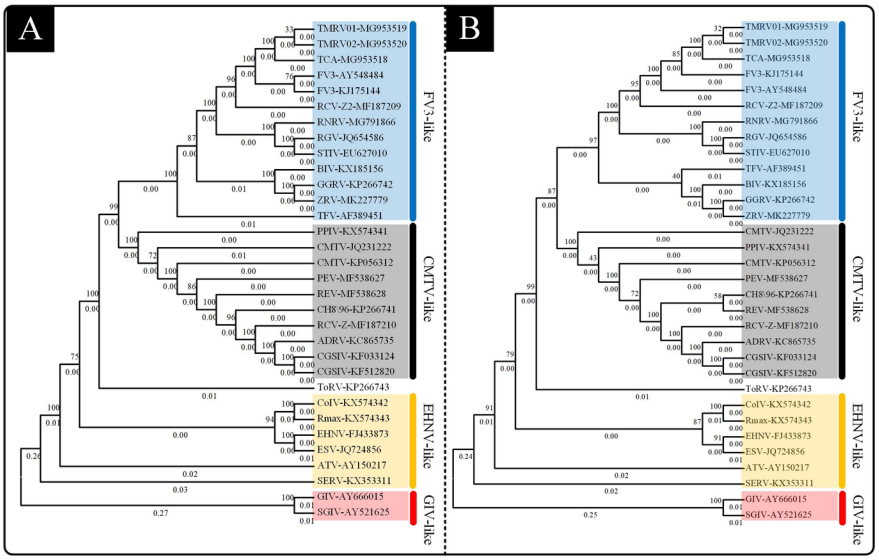


Figure 3 Phylogenetic tree based on the concatenated nucleotide (nt) sequences of the 44 ranavirus core genes (A) and iridovirid core genes (B) by neighbor-joining method. The numbers on the branch points represented bootstrap values (1 000 replicates), the numbers below branch represented genetic distance.

Figure 4 Dot plot analysis of the FV3-AY548484 genome (horizontal axis) versus other ranavirues genomes (vertical axis). GIV and SGIV display only short segments of genomic collinearity with FV3, therefore the red outlines highlighted homologous fragments.

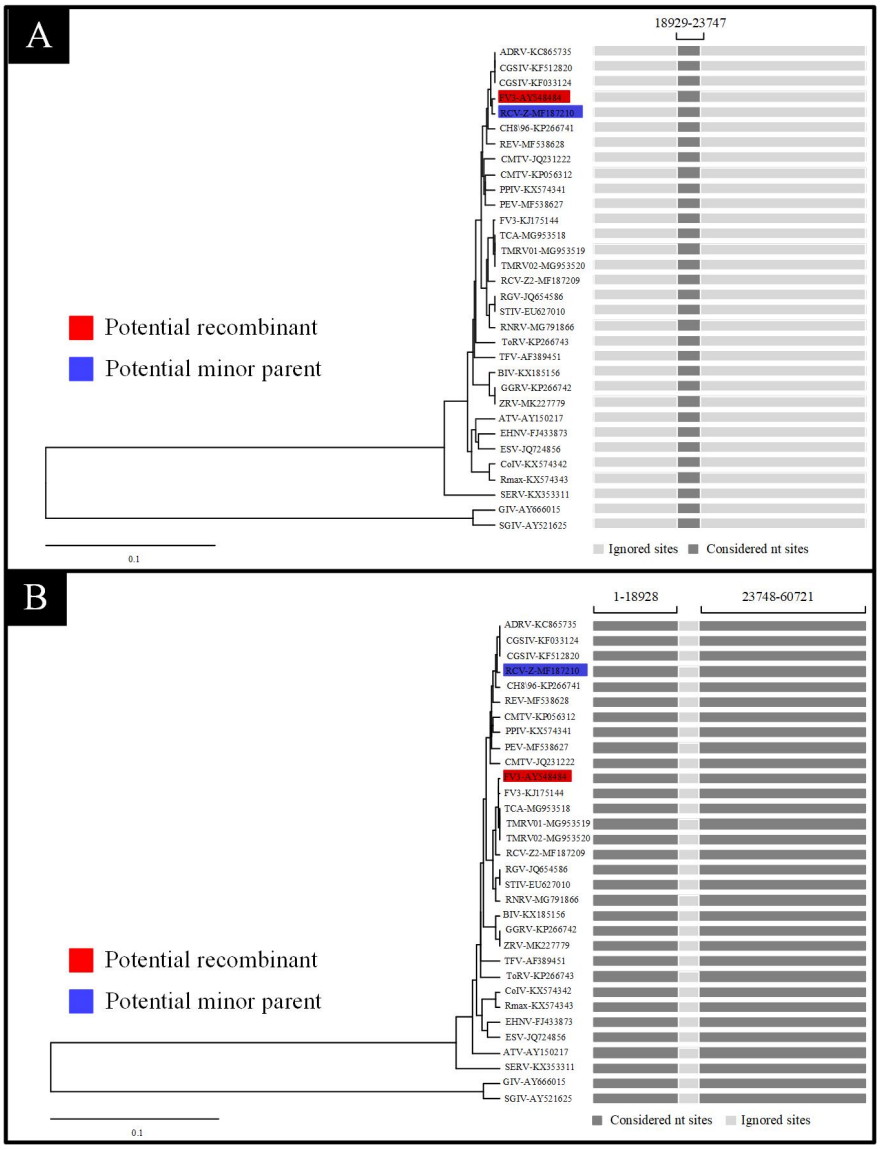


Figure 5 UPGMA (unweighted pair group method using arithmetic average) of regions derived from major parent (A,1-18928 and 23748-60721) and minor parent (B, considered nt sites: 18929-23747).

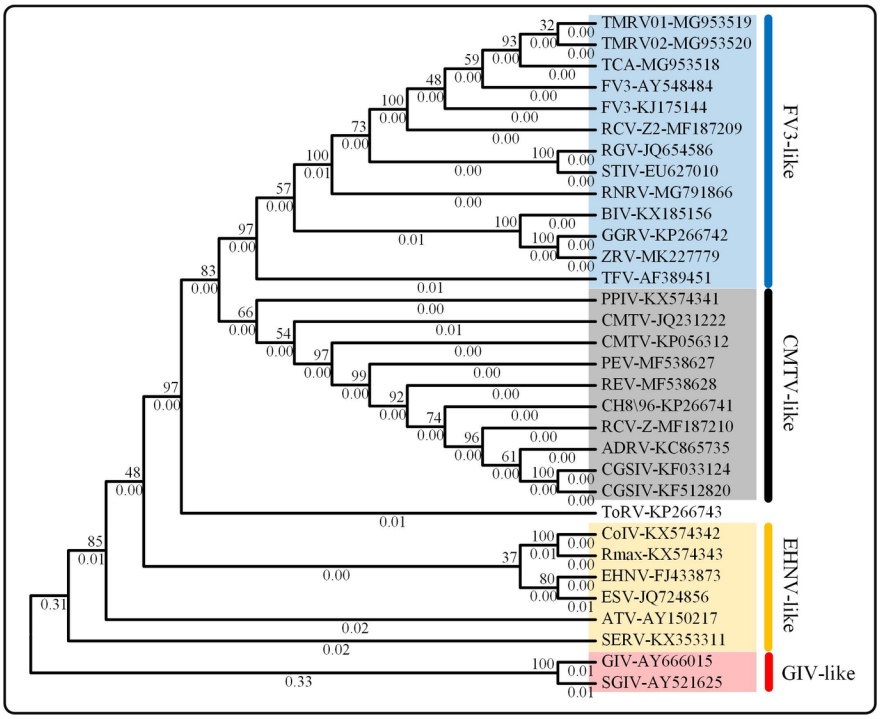
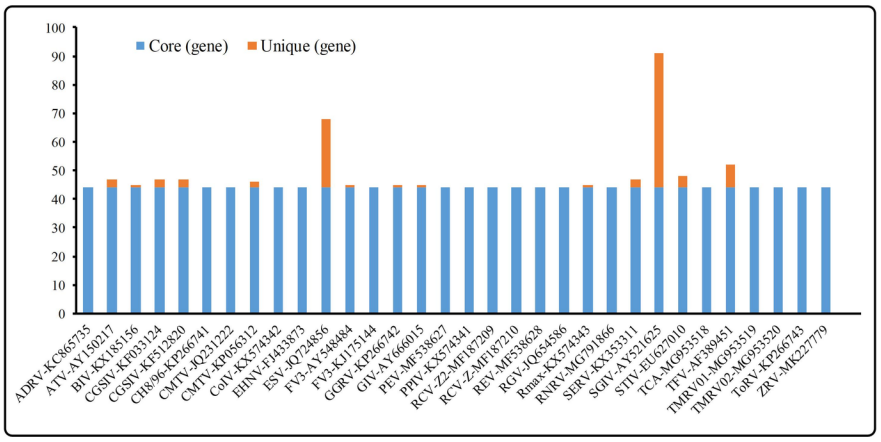
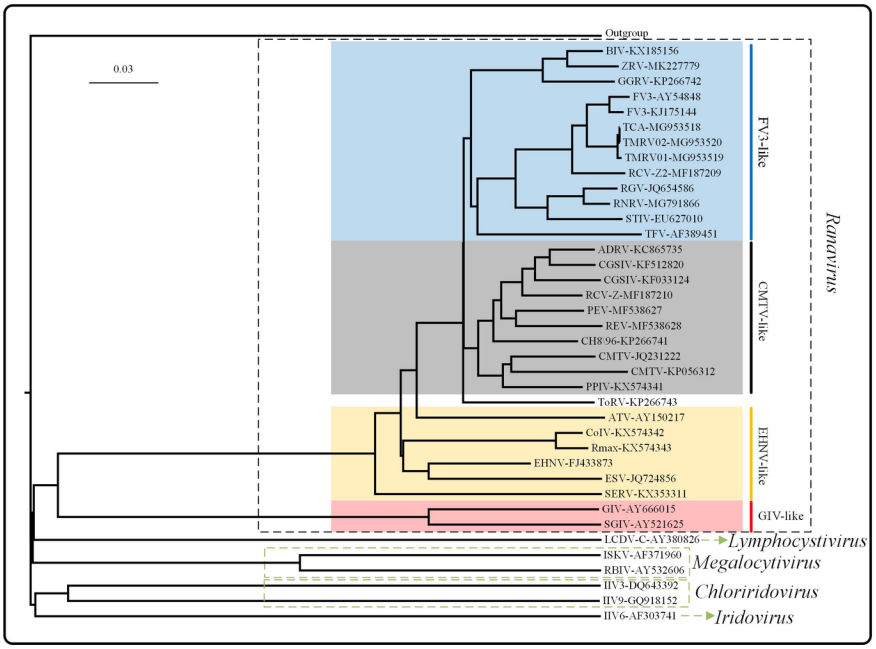
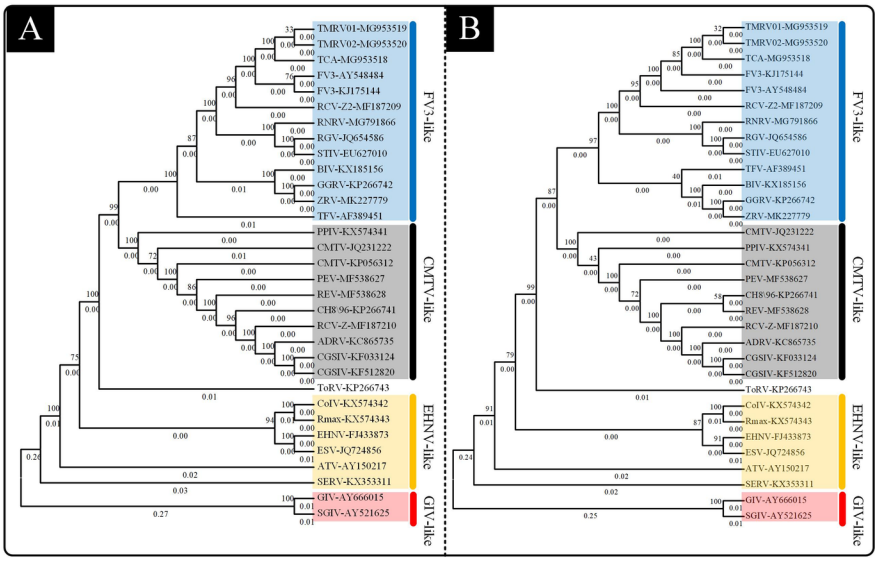
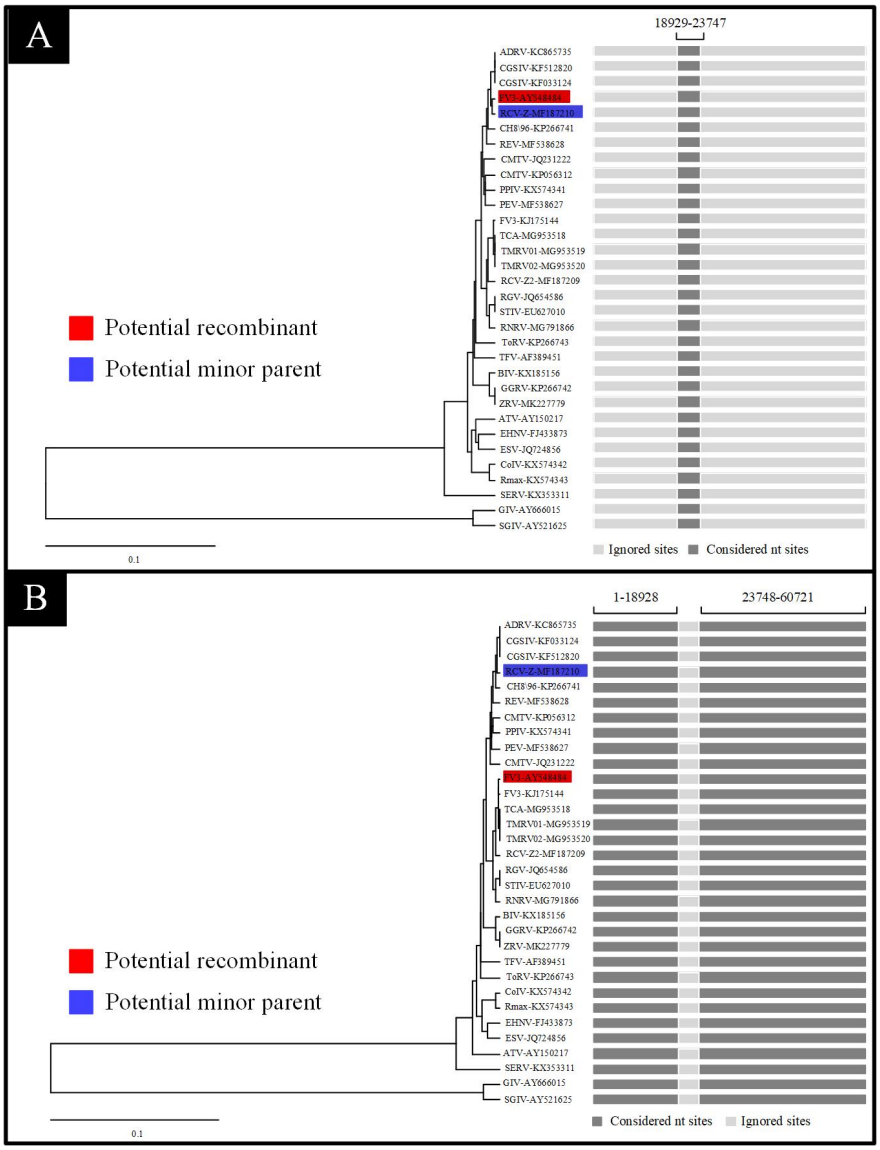


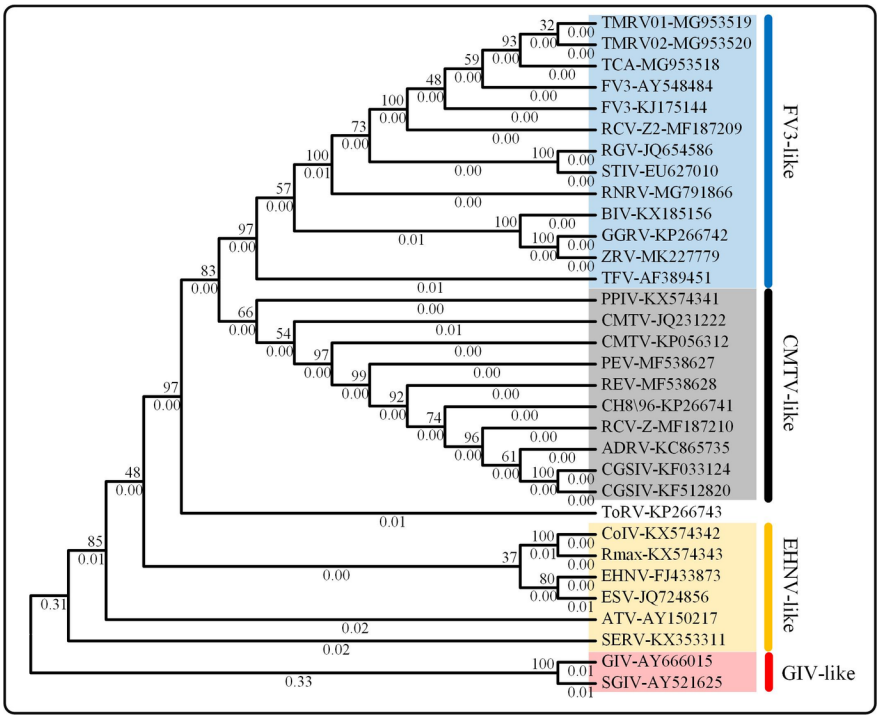
Figure 6 Phylogenetic tree based on the concatenated nucleotide (nt) sequences of the cluster 2, 9, 12 and 21 (4,267 nt characters including gaps).

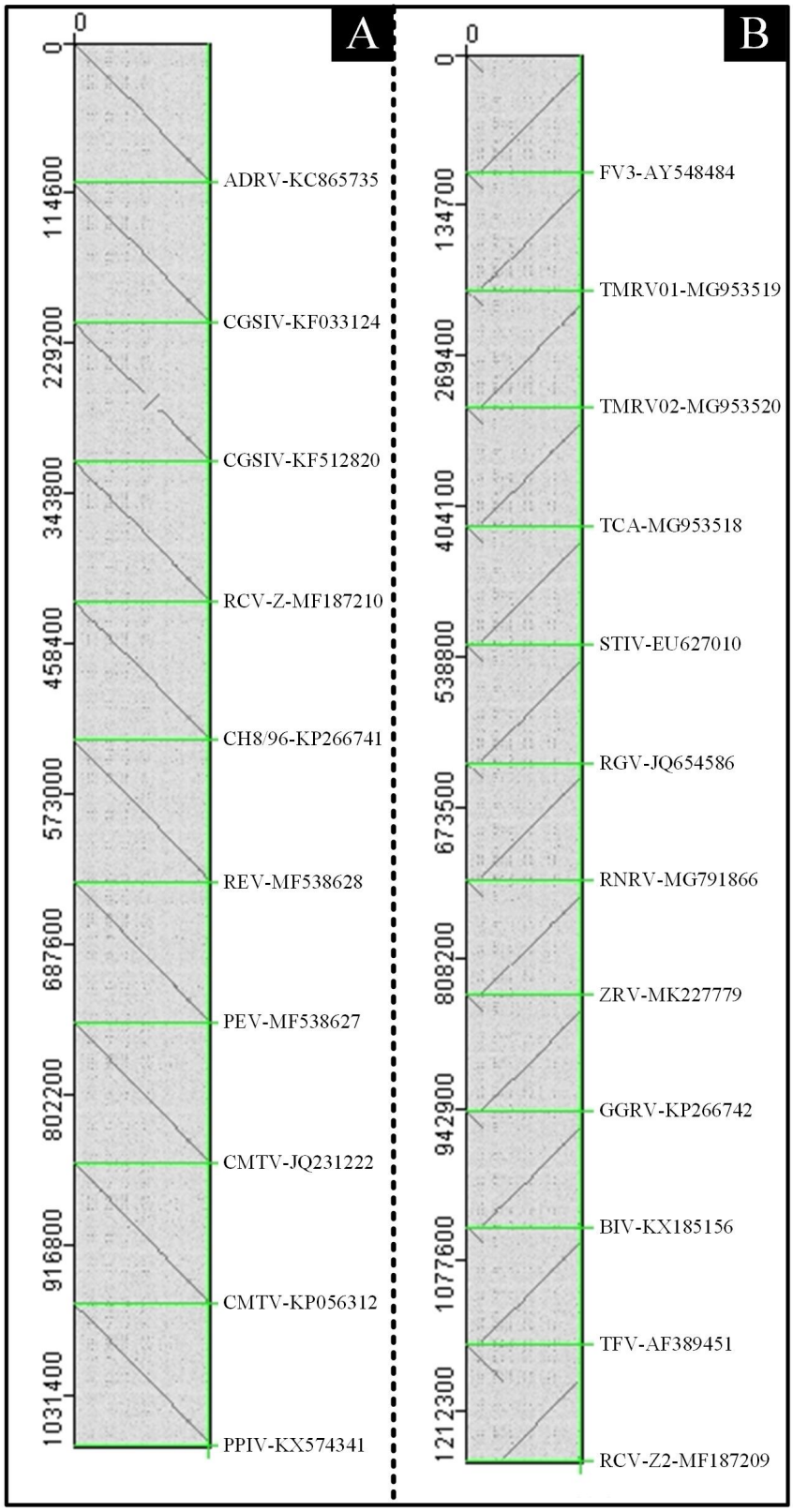


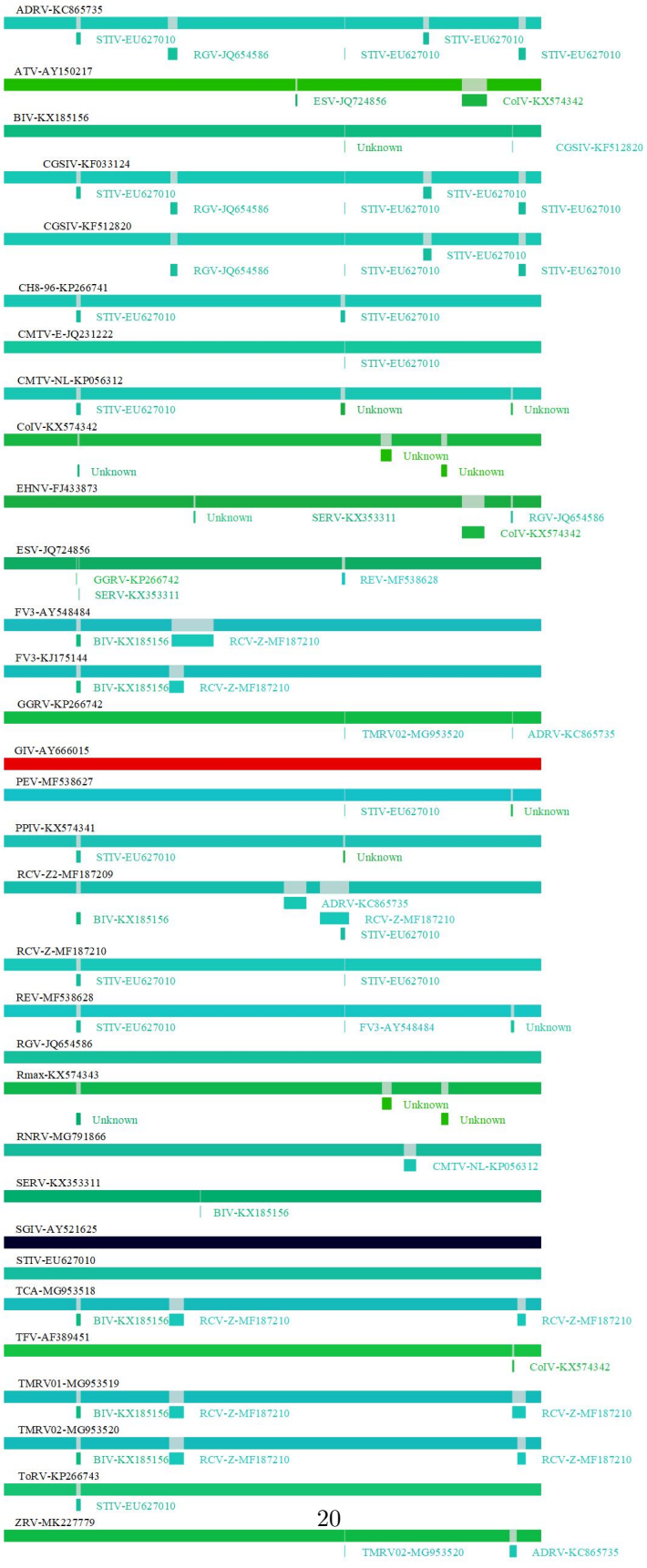


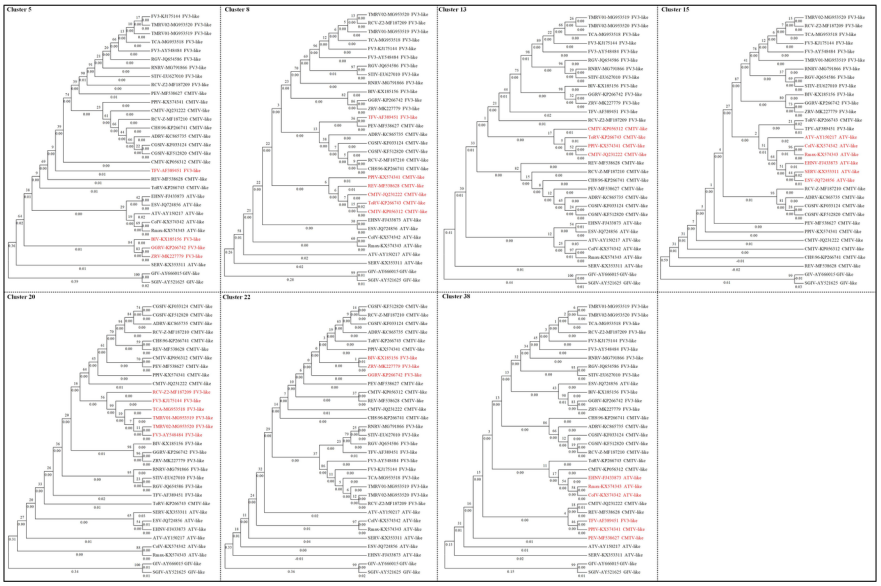


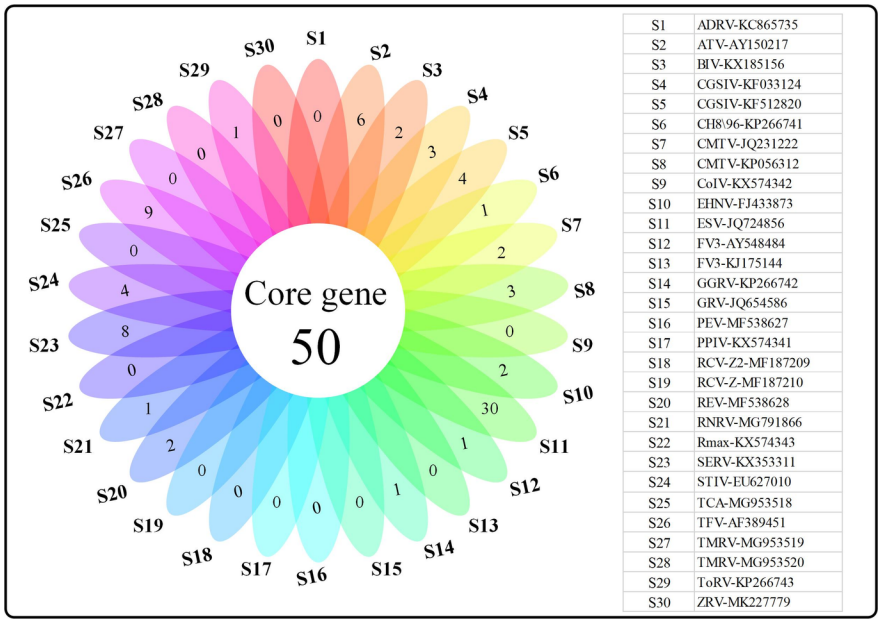












S1	ADRV-KC865735
S2	ATV-AY150217
S3	BIV-KX185156
S4	CGSIV-KF033124
S5	CGSIV-KF512820
S6	CH8\96-KP266741
S7	CMTV-JQ231222
S8	CMTV-KP056312
S9	CoIV-KX574342
S10	EHNV-FJ433873
S11	ESV-JQ724856
S12	FV3-AY548484
S13	FV3-KJ175144
S14	GGRV-KP266742
S15	GRV-JQ654586
S16	PEV-MF538627
S17	PPIV-KX574341
S18	RCV-Z2-MF187209
S19	RCV-Z-MF187210
S20	REV-MF538628
S21	RNRV-MG791866
S22	Rmax-KX574343
S23	SERV-KX353311
S24	STIV-EU627010
S25	TCA-MG953518
S26	TFV-AF389451
S27	TMRV-MG953519
S28	TMRV-MG953520
S29	ToRV-KP266743
S30	ZRV-MK227779

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