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Research Article

First Insight into Genetic Variation and Population Structure of The Emerging *Citrus chlorotic dwarf-associated virus* (CCDaV, genus *Citlodavirus*)

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Abstract: *Citrus* spp. is widely planted in tropical and subtropical regions, including in Turkey and other Mediterranean countries. Due to its widespread vector and climate change, Citrus chlorotic dwarf-associated virus (CCDaV), a member of the newly formed genus *Citlodavirus*, is one of the emerging viruses that can be a serious constraint to *Citrus* crops production in the coming years. Therefore, *in-silico* analysis on all available isolates in NCBI GenBank was performed to provide the first insight into the genetic population and evolution of CCDaV, which may contribute to its control. CCDaV phylogroups based on full genome, complete movement protein, and complete coat protein sequences were found to be not associated with isolate origins or host species, and all isolates also shared a high genetic identity among them. However, neutrality tests indicated that the current populations are expanding, driven by new mutations. Low Fixation index (F_{ST}) values (0.00000-0.36207) confirmed no genetic separation among different ORFs of isolates from three countries. The constructed TimeTree suggested that CCDaV emergence was very recent compared to the other three members of the genus *Citlodavirus*. Therefore, the obtained results of this study could also expand our knowledge on other even more obscure citladovirus and even other plant DNA viruses, which are still less studied than RNA viruses.

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1. Introduction

Citrus chlorotic dwarf-associated virus (CCDaV, genus *Citlodavirus*, family *Geminiviridae*) is the causal agent of the destructive 'citrus chlorotic dwarf disease' (CCCD) affecting *Citrus* spp. and its hybrids such as lemon (*C. limon*), pomelo (*C. maxima*), orange (*C. sinensis*), mandarin orange (*C.*

reticulata), bitter orange (*C.* × *aurantium*), grapefruit (*C.* × *paradisi*), and tangelo (*C.* × *tangelo*) (Zhou et al., 2017; Karanfil and Korkmaz, 2019). CCDaV genome is a monopartite DNA of 3640 nucleotides in a full-length genome sequence, organized into five open reading frames (ORFs): ORF V2 (encodes V2-like protein), ORF V1 (coat protein - CP), and ORF V3 (movement protein - MP) are comprised in the virion-sense strand whilst ORF C1 (C1:C2-like protein) and ORF C2 (RepA-like) are in the complementary-sense strand (Loconsole et al., 2012). Phylogenetic analysis based on a limited number of full genome sequences suggested a correlation between phylogroups and geographical origins as isolates from Turkey were clustered in a group distinct from those from China (Zhou et al., 2017; Karanfil and Korkmaz, 2019).

CCDaV transmission occurs by grafting and its putative vector, the bayberry whitefly (*Parabemisia myricae*), but does not happen mechanically (Korkmaz et al., 1995). Despite the virus has only been reported in Turkey (Loconsole et al., 2012), China (Guo et al., 2015), and Thailand (Yang et al., 2020), the presence of *P. myricae* in many citrus-growing countries around the world has raised epidemiological concerns that CCDaV might spill into other regions, especially those surrounding Turkey (Loconsole et al., 2012). Thus, CCDaV is regarded as one of 11 emerging viruses that currently pose serious threats to citrus crops in the Mediterranean region (Catara et al., 2021).

Recombination and reassortment are the most frequently found evolutionary forces in populations of both RNA and DNA plant viruses, thus significantly affecting their genetic variation (Chare and Holmes, 2006; Gibbs et al., 2008). While population genetics of many emerging plant RNA viruses have been discussed in recent papers (Randa-Zelyüt and Ertunç, 2021; Tokhmechi et al., 2021; Morca et al., 2022), the genetic variability and evolutionary mechanisms of populations of DNA viruses were remained less studied (Sanz et al., 1999; Ng et al., 2011). *Citrus* spp., themselves, as commercially important fruits in Turkey, are constantly improved through breeding (Kurtuluş et al., 2021). However, our grasp on the molecular profiles of CCDaV is still at a very early stage and needs to be further advanced as the data gained in genetic diversity, and population studies could contribute to the understanding of the molecular evolution of viruses and in the management of the viral disease through the development of either more specific or universal detection methods as well as determination of resistant gene(s) in the plant (Sokhandan-Bashir and Melcher, 2012; Stobbe and Roossinck, 2016; Santosa and Ertunç, 2021). Therefore, results of the first population analysis on CCDaV were presented in this study to learn more about some evolution aspects of the virus, which might also provide vital information for the even more obscure other three *Citlodavirus* species.

2. Material and Methods

2.1. Multiple sequence alignment and phylogenetic analysis

In-silico analysis using different bioinformatic software was carried out within this study. Twenty-three CCDaV isolates from Turkey, China, and Thailand with full genome sequences were retrieved from NCBI (National Center for Biotechnology Information) GenBank on February 7, 2022, and aligned using ClustalW (1.6) performed in MEGA X software v.10.2.4 (Kumar et al., 2018), then trimmed to extract their complete genome and five gene regions (V2, coat protein (CP), movement protein (MP), C1:C2-like, and RepA-like) sequences. Complete CP sequences of the other 19 global isolates were then aligned with the constructed CP alignment to create a dataset of 42 isolates. The pairwise nucleotide (nt) and amino acid (aa) sequence's identity matrix of isolates at the complete genome, CP, and MP levels were generated using Sequence Demarcation Tool (SDT) v1.2 software (Muhire et al., 2014).

The complete genome and CP alignments were subjected to recombination analysis using RDP, GENECONV, Chimaera, MaxChi, Bootscan, Siscan, and 3Seq algorithms implemented in the Recombinant Detection Program (RDP v.4.56) software with default parameters (Martin et al., 2015). Phylogenetic anomalies identified by less than five methods and with a Bonferroni-corrected *P*-value of < 0.05 were ignored (Martin et al., 2015).

The best DNA models to study the evolutionary relationship of isolates at the complete genome, CP, and MP levels were determined using MEGA X. Phylogenetic trees for the three regions comparisons were constructed using Maximum-Likelihood (ML) statistical method based on the Kimura

2-parameter model (Kimura, 1980) with uniform rates as implemented in MEGA X software, and branches were supported by bootstrap method with 1000 replications.

2.2. Current CCDaV population structure

The complete picture of variation among different CCDAV populations in each of the complete and five genome regions (V2, coat protein (CP), movement protein (MP), C1:C2-like, and RepA-like) was estimated according to four parameters: the number of haplotypes (H), haplotype diversity (Hd), average pairwise nt diversity (π), and transcriptional constrain (ω=dN/dS) suited in DnaSP software v.6.12.03 (Rozas et al., 2017). Any tested genome region having a dN/dS ratio > 1, = 1, or < 1 was considered to have been under negative (purifying), neutral, or positive (diversifying) constrain, respectively (Rozas et al., 2017). The results of three suites of neutrality constrain tests: Tajima's D (Tajima, 1989), Fu and Li's D* and F* (Fu and Li, 1993), as well as the determination of FST KS*, Z*, and Snn metrics (Hudson et al., 1992; Hudson, 2000), were presented using DnaSP v.6.12.03 to allow the quantitative estimation of genetic variation and gene flow between CCDaV populations for each of the datasets. Infrequent gene flow and expanding genetic divergence among CCDAV populations were determined by having FST (fixation index) value > 0.33 (Rozas et al. 2017).

2.3. Molecular dating analysis

The divergence time of four *Citlodavirus* species, including CCDaV, camellia chlorotic dwarf-associated virus (CaCDaV), passion fruit chlorotic mottle virus (PCMoV), and paper mulberry leaf curl virus 2 (PMLCV-2) with two *Mulcrilevirus* species: mulberry mosaic dwarf associated virus (MMDaV) and paper mulberry leaf curl virus 1 (PMLCV-1) as outgroup sequences were estimated based on the age evaluation of internal nodes (Kumar et al., 2018). TimeTree was reconstructed using the fast-dating RelTime-ML computational method under the Tamura-Nei parameter model (Tamura and Nei, 1993) implemented in MEGA X software, with default calibration of most recent common ancestors (MRCA) (Mello, 2018).

3. Results

3.1. Multiple sequence alignment and phylogenetic analysis

The phylogenetic trees showed that there was no clear consensus on the isolates clustering in comparisons based on complete genome, CP, and MP regions (Figure 1). Although the three trees showed different topologies, no significant recombinant signal indicative of recombinant isolates was detected by RDP software.

Pairwise identity analysis indicated that all analyzed CCDaV isolates shared high identities in both their nt and aa sequences, but they had a greater percentage of nt than aa identities in all compared regions; as an example, sequences of CCDaV isolates were 97.3-99.9% aa and 98.8-99.9% nt identical at the complete genome level (Table 1). Since CP was the most conserved region at both nt and aa levels, and more likely to be targeted in future identification studies of CCDaV, group naming was then done based on the result of the CP region comparison in which isolates were clustered into two distinct major groups (1 and 2) (Figure 1).

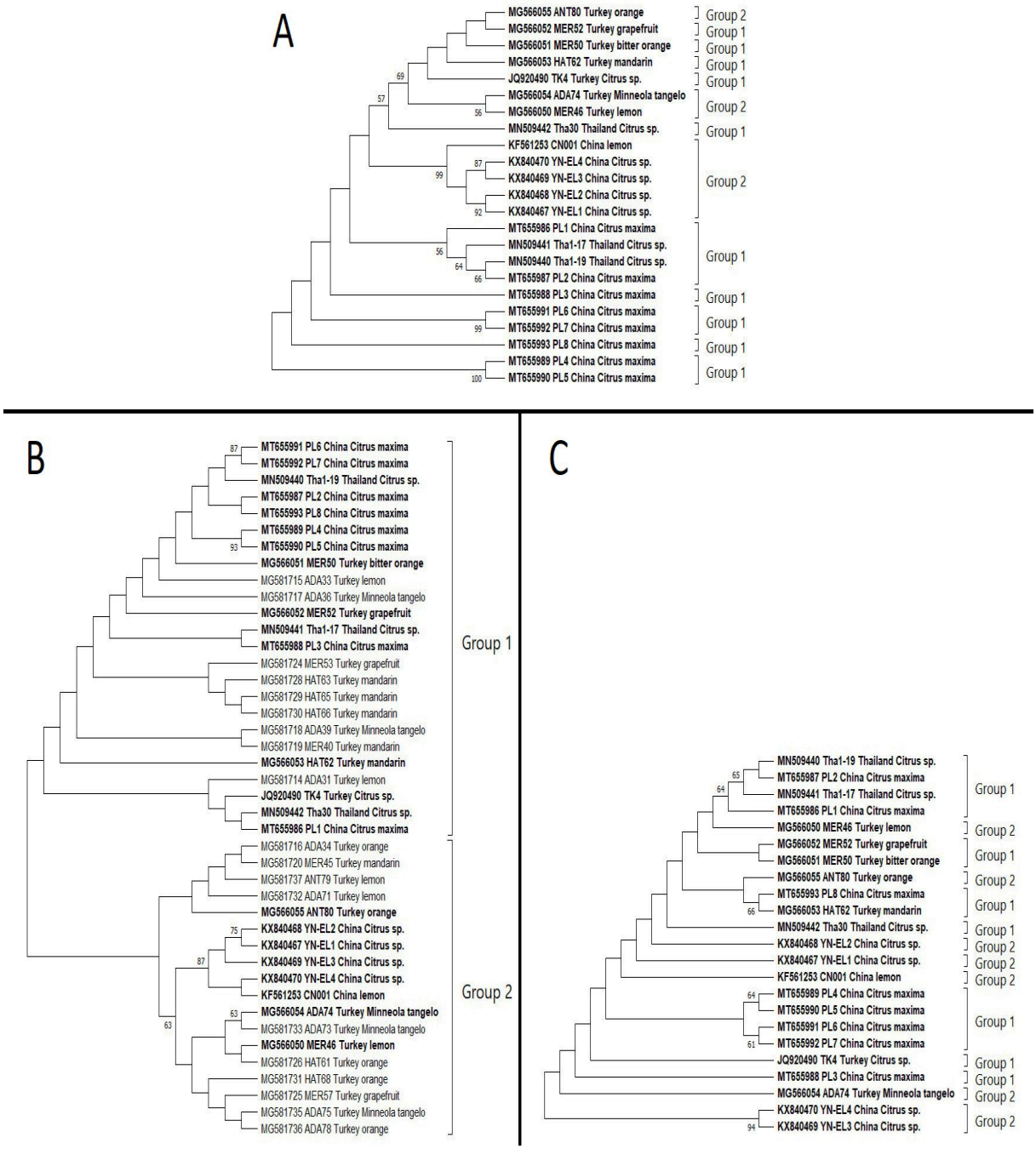

Figure 1. Maximum-Likelihood phylogenetic analysis of the nucleotide sequences of three different CCDaV genomic regions using Kimura 2-parameter model (K2) with Uniform Rates among Sites by MEGA X software, branches were supported by 1000 bootstrap replicates (only >50% values were shown). A. Phylogenetic analysis of the complete genome of 23 isolates, B. complete CP of 42 isolates, C. complete MP of 23 isolates. Group naming was based on the results of the complete CP analysis. Names of isolates that have complete genome sequences were printed in bold.

Table 1. Identity percentage of nucleotide and amino acid sequences of different CCDaV genomic regions and phylogroups

Comparison	Identity (%)	
	Nucleotide	Amino acid
Complete genome	98.8-99.9	97.3-99.9
V2	98.8-100	97.8-100
CP all	98.8-100	98.4-100
CP group 1	99.2-100	98.4-100
CP group 2	99.2-100	98.8-100
CP group 1 x group 2	98.8-100	98.8-100
MP	98.7-100	96.4-100
C1:C2-like	98.3-100	95.3-100
RepA-like	98.6-100	96.1-100

3.2. Population structure

Genetic variation and polymorphism on the full genome and five genes of CCDaV populations from three countries were determined by four genetic diversity parameters. At the full genome level, all three countries and world populations obtained the maximum Hd value of 1.000. The nucleotide diversity of all analyzed populations in different genome regions was observed to be low values (π between 0.00106 - 0.00741), with China population obtaining the highest π values in comparisons based on V2, CP, C1:C2-like, and RepA-like genes (Table 2). In general, CP and MP genes experienced more vigorous negative constraints than other genes. The analysis also found that the RepA-like region and several countries' populations of various gene regions are undergoing diversification pressure, as shown by their dN/dS ratio of > 1 (Table 2).

Table 2. Results of analysis of genetic diversity and polymorphism of partial five ORFs of CCDaV from different countries

ORF (Protein)	nt position (no of aa)	Group/geography	H	Hd	π	dN/dS
Full genome	1-3642 (1103)	All (n=23)	23	1.000	0.00582	0.7378
		Turkey (n=7)	7	1.000	0.00327	0.6118
		China (n=13)	13	1.000	0.00642	0.8645
		Thailand (n=3)	3	1.000	0.00439	1.1084
ORF1 (V2 protein)	194-613 (139)	All (n=23)	11	0.838	0.00427	0.5566
		Turkey (n=7)	5	0.857	0.00340	nd
		China (n=13)	6	0.859	0.00464	0.3433
		Thailand (n=3)	2	0.667	0.00317	0.2640
ORF2 (Coat protein)	417-1181 (254)	All (n=42)	29	0.959	0.00458	0.1257
		Turkey (n=26)	16	0.902	0.00258	0.0584
		China (n=13)	11	0.974	0.00741	0.0986
		Thailand (n=3)	3	1.000	0.00523	0.2983
		Group 1 (n= 24)	17	0.938	0.00387	0.1957
		Group 2 (n= 18)	13	0.961	0.00432	0.0876
ORF3 (Movement protein)	1211-2131 (306)	All (n=23)	17	0.953	0.00412	0.3353
		Turkey (n=7)	7	1.000	0.00434	1.6831
		China (n=13)	9	0.936	0.00384	0.2369
		Thailand (n=3)	3	1.000	0.00362	0.0702
ORF4 (C1:C2-like protein)	2300-2707 (135)	All (n=23)	14	0.929	0.00680	0.7074
		Turkey (n=7)	6	0.952	0.00630	1.1009
		China (n=13)	7	0.846	0.00647	0.5623
		Thailand (n=3)	6	0.952	0.00630	0.9429
ORF5 (RepA-like protein)	2611-3420 (269)	All (n=23)	15	0.945	0.00513	1.1572
		Turkey (n=7)	3	0.524	0.00106	0.5759
		China (n=13)	9	0.949	0.00630	0.8831
		Thailand (n=3)	3	1.000	0.00329	nd

N, number of isolates; Hd, haplotype diversity; π, nucleotide diversity; ω, dN/dS; nd, not determined.

The result of molecular variation patterns analysis suggested that Tajima's *D*, Fu and Li's *D** and *F** evaluated significantly and non-significantly negative values for CCDaV from three countries as well as worldwide populations in the full genome and all five genes, except for Turkey and world populations in C1:C2-like gene which were assigned positive values by Fu and Li's *F** test (Table 3).

Low K_S*, *Z**, and *Snn* values and the low F_{ST} values of < 0.33 were distributed among three countries in the full-length genome sequence and five gene comparisons, except Turkey vs. Thailand in RepA-like gene comparison (F_{ST} = 0.36207). Likewise, Group 1 vs. Group 2 in CP gene comparison revealed a low F_{ST} value (0.19991) (Table 4). Therefore, it can be suggested that the currently characterized global CCDaV isolates share low genetic variation.

Table 3. Results obtained from demography test statistics on five ORFs of CCDaV from different countries

ORF (Protein)	Group/geography	Tajima's *D*	Fu and Li's *D**	Fu and Li's *F**
Full genome	All (n=23)	-1.94372*	-2.38068 ns	-2.63291*
	Turkey (n=7)	-1.66129*	-1.74916*	-1.91293*
	China (n=13)	-1.04083 ns	-0.72544 ns	-0.92816 ns
	Thailand (n=3)	nd	nd	nd
ORF1 (V2 protein)	All (n=23)	-1.86429*	-1.91374 ns	-2.21294 ns
	Turkey (n=7)	-1.48614 ns	-1.56696 ns	-1.68344 ns
	China (n=13)	-0.51618 ns	0.24334 ns	0.05126 ns
	Thailand (n=3)	nd	nd	nd
ORF2 (Coat protein)	All (n=42)	-2.27745**	-3.65182**	-3.76783**
	Turkey (n=26)	-2.23397**	-3.71053**	-3.81047**
	China (n=13)	-0.51656 ns	-0.22630 ns	-0.34689 ns
	Thailand (n=3)	nd	nd	nd
	Group 1 (n= 24)	-2,90983*	-3,12995*	-2,14468*
	Group 2 (n= 18)	-1,40286 ns	-1,58014 ns	-1,27100 ns
ORF3 (Movement protein)	All (n=23)	-2.25491**	-2.89175*	-3.15463**
	Turkey (n=7)	-1.65112*	-1.74976*	-1.89968*
	China (n=13)	-1.65473 ns	-1.39773 ns	-1.67472 ns
	Thailand (n=3)	nd	nd	nd
ORF4 (C1:C2-like protein)	All (n=23)	-1.79576 ns	-2.63241*	2.77779*
	Turkey (n=7)	-1.59446 ns	-1.68667*	1.82427 ns
	China (n=13)	-0.72083 ns	-0.61664 ns	-0.73434 ns
	Thailand (n=3)	nd	nd	nd
ORF5 (RepA-like protein)	All (n=23)	-1.62927 ns	-1.37921 ns	-1.70176 ns
	Turkey (n=7)	-1.35841 ns	-1.42725 ns	-1.52246 ns
	China (n=13)	-0.89097 ns	-0.44861 ns	-0.64717 ns
	Thailand (n=3)	nd	nd	nd

Statistical significance: *, P < 0.05; **, 0.10 > P > 0.05; ns, not significant; nd, not determined.

Table 4. Genetic difference, gene flow and migration rate results of five ORFs of CCDaV from different countries

Genomic region	Comparisons	K_s* (*P* value)	*Z** (*P* value)	*Snn* (*P* value)	F_{st}
Full genome	Turkey(n=7)/Thailand(n=3)	2.58027 (0.0100*)	2.50957 (0.0310*)	0.83333 (0.0560 ns)	0.18384
	Turkey(n=7)/China(n=13)	2.89176 (0.0010**)	3.93619 (0.0010**)	0.97500 (0.0000***)	0.21155
	Thailand(n=3)/China(n=13)	3.03646 (0.2700 ns)	3.77678 (0.2480 ns)	0.75000 (0.3600 ns)	0.09064
ORF1 (V2 protein)	Turkey(n=7)/Thailand(n=3)	0.79623 (0.6720 ns)	2.98372 (0.5770 ns	0.48000 (0.9180 ns)	0.00000
	Turkey(n=7)/China(n=13)	1.76667 (0.0680 ns)	4.27803 (0.1580 ns)	0.64286 (0.1030 ns)	0.09608
	Thailand(n=3)/China(n=13)	0.93537 (0.4500 ns)	3.88041 (0.4520 ns)	0.71667 (0.2650 ns)	0.07246
ORF2 (Coat protein)	Turkey(n=2)/Thailand(n=3)	1.01896 (0.0530 ns)	4.99596 (0.0700 ns)	0.89655 (0.1310 ns)	0.02900
	Turkey(n=26)/China(n=13)	1.24881 (0.0000***)	5.40646 (0.0000***)	0.91209 (0.0000***)	0.12500
	Thailand(n=3)/China(n=13)	1.77963 (0.5680 ns)	3.83233 (0.5040 ns)	0.69167 (0.4340 ns)	0.07143
	Group 1(n= 24)/ Group 2 (n= 18)	3.10888 (0.0000***)	5.65866 (0.0000***)	0.79932 (0.0000***)	0.19991
ORF3 (Movement protein)	Turkey(n=7)/Thailand(n=3)	1.53634 (0.3470 ns)	2.87517 (0.3570 ns)	0.46667 (0.7260 ns)	0.15385
	Turkey(n=7)/China(n=13)	1.40241 (0.1840 ns)	4.26661 (0.1740 ns)	0.61500 (0.1250 ns)	0.01437
	Thailand(n=3)/China(n=13)	1.34516 (0.3120 ns)	3.81914 (0.3530 ns)	0.62813 (0.6720 ns)	0.11258
ORF4 (C1:C2-like protein)	Turkey(n=7)/Thailand(n=3)	1.17124 (0.1750 ns)	2.75185 (0.1450 ns)	0.73333 (0.0470*)	0.05172
	Turkey(n=7)/China(n=13)	1.16112 (0.0980 ns)	4.20756 (0.0920 ns)	0.64667 (0.0880 ns)	0.08782
	Thailand(n=3)/China(n=13)	1.16748 (0.0980 ns)	3.76973 (0.0920 ns)	0.75000 (0.3060 ns)	0.10776
ORF5 (RepA-like protein)	Turkey(n=7)/Thailand(n=3)	0.62559 (0.0090**)	2.52964 (00090**)	0.76190 (0.0090**)	0.36207
	Turkey(n=7)/China(n=13)	1.26787 (0.0000***)	3.93528 (0.0000***)	0.96875 (0.0000***)	0.32713
	Thailand(n=3)/China(n=13)	1.59277 (0.4300 ns)	3.79660 (0.2440 ns)	0.78750 (0.1340 ns)	0.09281

PM test: Probability obtained by the permutation test with 1000 replicates); ns, not significant; **, 0.001<P<0.01; ***, P<0.001.

3.3. Divergence time analysis

The phylogenetic Timetree showed that four *Citlodavirus* species can be clustered into three groups (Figure 2). CCDaV has a closer genetic relationship with camellia chlorotic dwarf-associated virus (CaCDaV) than passion fruit chlorotic mottle virus (PCMoV) and paper mulberry leaf curl virus 2 (PMLCV-2), in accordance with Zhang et al. (2018) finding. The molecular clock estimation between CCDaV and CaCDaV was 0.43 Mya (0.5 - 1.00 in Timescale).

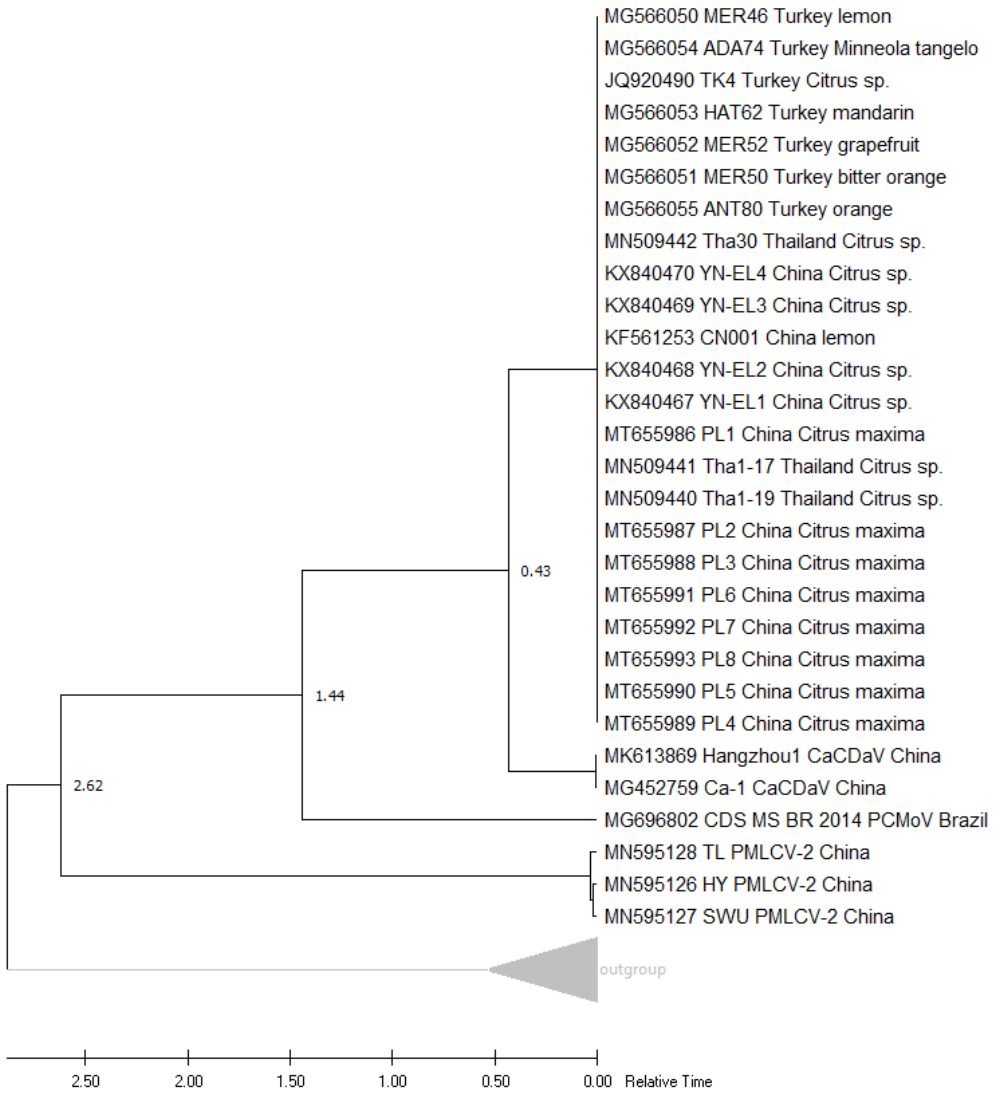

Figure 2. Divergence time estimation of four *Citlodavirus* species: citrus chlorotic dwarf associated virus (CCDaV), camellia chlorotic dwarf-associated virus (CaCDaV), passion fruit chlorotic mottle virus (PCMoV), and paper mulberry leaf curl virus 2 (PMLCV-2) by RelTime-ML in MEGA X. Two *Mulcrilevirus* species: *mulberry mosaic dwarf associated virus* (MMDaV, 4 isolates) and *paper mulberry leaf curl virus* 1 (PMLCV-1, 2 isolates) were used as out-group.

4. Discussion

The study on the population structure of CCDaV presented in this paper did not only enrich our understanding of the evolution of plant DNA viruses but also gave early insight into members of *Citlodavirus*, a newly established genus within the family *Geminiviridae* (Roumagnac et al., 2021). Even though CCDaV has been better studied than other citlodaviruses, the research on the virus is still in need of further advancement than the phylogenetic stage.

Phylogenetic trees constructed using complete genome, complete CP, and complete MP alignments were shown to have different topographies. However, no solid recombinant signals were detected by RDP analysis. These results suggested that the evolution of different genome regions did not occur simultaneously. It also provides evidence that reassortment might have a greater contribution than recombination in the direction of CCDaV evolution. Analysis using MEGA X software on the three observed regions indicated that there is no clear association between phylogroups with host or origin, contrasting a previous report by Karanfil and Korkmaz (2019), which clustered Turkish and Chinese isolates into two distinct groups.

CCDaV isolates from three different countries compared here shared high nucleotide (nt) and amino acid (aa) homology, in line with previous reports (Zhou et al., 2017; Karanfil and Korkmaz, 2019). The main transmission mode of the virus via grafting could be contributed to the high identity of isolates from around the world, as infected plants were probably grafted from the same infected source(s). The percentage identities on all studied genomic regions were higher at nt than aa level, suggesting most nt changes produced nonsynonymous aa substitutions.

DnaSP software estimated that different genome regions were under negative selection pressures, except ORF 5, which was supposedly under positive pressure. The negative constraints on the full genome, ORF1 and ORF4, were less than ORF 2 and ORF3, which are experiencing strong negative pressures (Table 2). These data further suggest that the evolution of different genome regions of CCDaV did not occur at the same rate and direction.

Three neutrality tests assigned negative numbers to the observed populations, indicating the recent expansion of CCDaV populations through new mutations. Vector transmission may have a greater influence on the shaping of future CCDaV evolution as the virus populations were shown to survive bottleneck selections which could be caused by graft transmission from the same source(s) (Roossinck and Ali, 2017). The F_{ST} values among different populations in comparisons of genomic regions were all < 0.33, except in Turkey vs Thailand at ORF5 (0.36), demonstrating once again that CCDaV populations are highly similar and almost no genetic isolation among currently known isolates from different countries.

CCDaV shared a common basal node with CaCDaV in the phylogenetic TimeTree of *Citlodavirus,* which indicated that CCDaV is related closer to CaCDaV than to PCMoV and PMLCV-2. These could add data to the ongoing discussion on citlodaviruses evolution as previously Fontenele et al. (2018) showed that CCDaV is phylogenetically closer to PCMoV while Qiu et al. (2020) determined that CCDaV is closer to PMLCV-2. The results of gene flow and time tree analyses of this current study also showed that many more isolates from other countries are needed to resolve the origin and ancestor of CCDaV.

Conclusion

The global population of CCDaV currently only consists of isolates from three countries: China, Thailand, and Turkey, which showed high genomic identity among each other. The constructed TimeTree also suggested that CCDaV separation as a distinct species was recent in the context of the evolution course of the genus *Citlodavirus*. However, different CCDaV populations were all estimated to be expanding, likely by the means of low-frequency polymorphism. This report presented an early insight, and more isolates from different regions are called in the future to further understand the evolution of the emerging CCDaV.

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