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SHORT COMMUNICATION

Comparisons between sequenced and re-sequenced genomes of historical subterranean clover mottle virus isolates

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Abstract

We report comparisons between the complete genomic sequences of five historical Western Australian isolates of subterranean clover mottle virus (SCMoV) from 1989–2000, and an infectious clone of its 1989 isolate. Sanger Sequencing (SS) and High Throughput Sequencing (HTS), or both, were used to obtain these genomes. Four of the SCMoV isolates were sequenced by SS in 1999–2002, but re-sequenced again by HTS in 2020. The pairs of sequences obtained from these four isolates differed by only 18–59 nucleotides. This small difference resulted from the different sequencing methods, the < 1–5 years each isolate was host passaged before freeze-drying prior to HTS sequencing, or a combination of both. Since SCMoV has not been reported outside Australia, this similarity suggests the population sequenced represents the progeny of either an indigenous virus that spread from a native legume to subterranean clover after its introduction or a recent seed-borne incursion from elsewhere. The ORF1 was the most variable, and the phylogenetic tree constructed with ORF1s showed the isolates grouped according to their symptom severity in subterranean clover, indicating the probability that ORF1-encoded P1 protein is a symptom determinant. A satellite RNA was associated with all SCMoV genomes obtained by HTS but none derived by SS.

Keywords Historical virus isolate collections · Sequencing methods · Evolutionary divergence · P1 protein · Suppressor of gene silencing · Incursion

Subterranean clover mottle virus (SCMoV; genus *Sobemovirus*, family *Solemoviridae*) causes a damaging disease of subterranean clover. It diminished herbage and seed yields in field experiments suggesting it depletes the pasture feed-base and causes pasture decline (= a gradual decrease in the proportion of pasture species to weeds) (Barbetti et al. 1996; Ferris and Jones 1995; Ferris et al. 1996; Jones 1996, 2012; Wroth and Jones 1992b). SCMoV is the most widespread virus found infecting subterranean clover-based pastures across southern Australia (Helms et al. 1993; Johnstone and McLean 1987; Jones 1996, 2012; Wroth and Jones 1992b). Subterranean clover is ideally suited to managed annual pastures because not only it is tolerant of defoliation by grazing but also it buries its developing seeds underground which protects them from feeding herbivores. Its seeds germinate every year producing annually self-regenerating pastures that support livestock, especially sheep and cattle (Nichols et al. 2007, 2012, 2013).

SCMoV-infected subterranean clover plants develop leaf symptoms of mottle, deformation and size reduction, and severe plant stunting (Wroth and Jones 1992a). Based on serological tests, the virus is distantly related to lucerne transient streak virus (LTSV) which also occurs in Australia, but not to other sobemoviruses (Foster and Jones 1980; Francki et al. 1983; Jones et al. 2001). SCMoV virions are isometric and have a diameter of about 25 nm. Their contents consist of a linear, positive-sense ssRNA genome of 4,258 nucleotides (nts) long, and usually 1–2 ssRNA satellite molecules. Its genome has four major overlapping open

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reading frames (ORFs). ORF1 encodes the P1 movement protein, ORF2a a polyprotein with a serine protease domain, ORF2b an RNA-dependent RNA polymerase (RdRp), and ORF3 the coat protein (CP) (Dwyer et al. 2003; Francki et al. 1983; Jones et al. 2001). Amongst the sobemoviruses, its genome sequence is most similar to that of LTSV, but its organisation most resembles that of cocksfoot mottle virus (Dwyer et al. 2003; Jones et al. 2001). Its virions are very stable, reaching high concentrations in plants, and are readily transmissible mechanically. There is no known vector but its transmission from plant-to-plant occurs readily by contact, mainly trampling by livestock and on vehicle wheels, but less readily by grazing and mowing (Francki et al. 1983; Jones et al. 2001; McKirdy et al. 1998). It is also seed-borne in subterranean clover, and persists between growing seasons in infected seeds buried in the soil, which germinate to produce infected seedlings when rainfall occurs following the hot, dry summer period (Wroth and Jones 1992a, b; Njeru et al. 1997; Jones et al. 2001). SCMoV has a narrow host range consisting of legumes (45 species, 33 infected systemically, and Chenopodiaceae (3 species, 1 infected systemically) (Francki et al. 1983; Wroth and Jones, 1992a; Fosu-Nyarko et al. 2002). Some subterranean clover cultivars have partial or complete SCMoV resistance (Wroth and Jones 1992a; Njeru et al. 1995; Ferris et al. 1996; McKirdy et al. 1998). Similar SCMoV resistances occur in other annual clovers (Wroth and Jones 1992a; Fosu-Nyarko et al. 2002), and barrel medic (*Medicago truncatula*) (Saqib et al. 2009).

The first complete genomic sequence of SCMoV was obtained using Sanger Sequencing (SS) (Dwyer et al. 2003). Here we report (i) the complete genomic sequences of three other SCMoV isolates, and an infectious clone of one, all obtained through SS, and (ii) the re-sequencing of these four isolates and a previously unsequenced historical isolate by High Throughput Sequencing (HTS). The resequencing of four isolates 18–21 years apart allowed genomic sequences obtained by SS and HTS to be compared and conclusions drawn from the association between nt sequence identities and their origins, or those of viral proteins and the symptom severities they elicited in subterranean clover.

The first SCMoV isolate sequenced was P23 which produced severe foliage symptoms in subterranean clover. In 1999, this sequence was obtained from overlapping cDNA clones spanning the entire genome and the 5' and 3' untranslated regions (UTR) generated using the Random Amplification of cDNA Ends (RACE) technology (Dwyer et al. 2003). The genomic sequences of three other SCMoV isolates causing symptoms of varying severities in subterranean clover (AL, MB, MJ), and that of infectious clone pFL were obtained in 2002 but never published outside a PhD Thesis (Fosu-Nyarko 2005). They were determined from total RNA isolated from infected subterranean clover leaves using overlapping cDNA fragments spanning the virus genome. The first strand cDNAs used as templates for the overlapping amplicons were generated using MoMLV reverse transcriptase, followed by PCRs with the proof-reading enzyme rT*th* DNA polymerase XL (Applied Biosystems, Australia). The amplicons were cloned using standard molecular cloning techniques. The pFL infectious clone was based on isolate P23 and constructed as a gene silencing vector (Fosu-Nyarko 2005). Both strands of all clones of isolates AL, MB and MJ and the DNA of pFL were sequenced using an Applied Biosystems Model 373 automated sequencer using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The raw sequence data were analysed using SeqEd Version 1.0.3 software and multiple sequence comparison tools from the Australian National Genomic Information Service (ANGIS).

The HTS of previously sequenced SCMoV isolates P23, AL, MB and MJ and unsequenced historical isolate NN was done in York, UK in 2020. First, total RNA was extracted from each freeze-dried leaf sample using a Total RNA kit (Qiagen, U.K.), including the optional DNAse I treatment. The HTS reads were assembled as described by Fowkes et al. (2021). An indexed sequencing library was produced from this RNA using a Scriptseq complete plant leaf kit (Illumina, USA.) and sequenced on a MiSeq instrument (Illumina), using a 600-cycle V3 kit. The complete genome sequences of each isolate were obtained from assembly of reads generated from HTS. The exception to this was at the 5' and 3' UTRs of some of them when compared to the refseq SCMoV sequence with GenBank accession number NC_004346.1. The HTS sequences lacked between 0–5 nt in the 5'UTR and between 0–10 nt in the 3'UTR.

Table 1 contains details of the five SCMoV isolates and the infectious clone sequenced, their original isolation hosts, collection locations, years of isolation, when sequenced/re-sequenced and last freeze-dried, accession numbers, average HTS nt coverages and original references. The percent nt identities were calculated using pairwise differences obtained from a MEGA X alignment (Kumar et al. 2018; Stecher et al. 2020) and average coverage of the HTS sequencing was calculated using BBmap (https://jgi.doe.gov/data-and-tools/software-tools/bbtools/). The genomes were very similar having overall average percentage nt identities of 99.98% (Table 2) despite being derived from isolates collected up to 327 km apart (North Dandalup to Mt Barker) (Table 1). When comparing the four pairs of isolate sequences obtained by the original SS or recent HTS methodologies, there were 99.98–99.99% nt identities between each pair. Isolate P23 had the smallest number of nt differences over the whole genome (18 compared to 51—59 for isolates AL, MB and MJ). It was also the one which had been maintained by serial subculture in subterranean clover for the shortest period (< 1 year) between when it was first

Table 1 Origins and accession numbers of the five Western Australian historical subterranean clover mottle virus isolates sequenced

Isolate	Original host common name	Species	Where collected	Isolation year	Isolate severity[a]	Date last freeze-dried	Year sequenced (first/re-sequenced)	Accession Number		Average nt coverage of HTS sequencing	First isolate reference
								Sanger	HTS		
P23	Subterranean clover	*Trifolium subterraneum*	Catterick	1989	Severe	16/03/2000	1999/2020	AY208001	OM818393	6883	Wroth and Jones (1992b)
pFL (full length P23 clone)	N/A	*T. subterraneum*	N/A	N/A	N/A	N/A	2002/-	AY376454	-	-	Fosu-Nyarko (2005)
AL	Arrowleaf clover	*T. vesiculosum*	North Dandalup	1996	Very severe	17/01/2004	2002/2020	AY376451	OM818392	19,522	Fosu-Nyarko et al. (2002)
MB	Subterranean clover	*T. subterraneum*	Mount Barker	2000	Moderate	17/01/2004	2002/2020	AY376452	OM818396	12,153	Fosu-Nyarko et al. (2002)
MJ	Subterranean clover	*T. subterraneum*	Manjimup	1992	Very severe	12/06/2007	2002/2020	AY376453	OM818394	10,914	Ferris and Jones (1995)
NN	Subterranean clover	*T. subterraneum*	Nannup	1992	Unknown	16/03/2000	-/2020	-	OM818395	23,848	This study

N/A = Not applicable;— = not sequenced

[a]Severity based on symptom expression in subterranean clover plants

Table 2 Percent nucleotide identities between the complete genome sequences of an infectious clone, four re-sequenced isolates and a newly sequenced isolate of subterranean clover mottle virus

SCMoV isolate	AF208001.1 Isolate P23 (SS)	AY376451.1 Isolate AL (SS)	AY376452.1 Isolate MB (SS)	AY376453.1 Isolate MJ (SS)	AY376454.1 Isolate pFL (SS)	OM818392 Isolate AL (HTS)	OM818393 Isolate P23 (HTS)	OM818394 Isolate MJ (HTS)	OM818395 Isolate NN (HTS)	OM818396 Isolate MB (HTS)
AF208001.1 Isolate P23 (SS)										
AY376451.1 Isolate AL (SS)	99.9910									
AY376452.1 Isolate MB (SS)	99.9879	99.9876								
AY376453.1 Isolate MJ (SS)	99.9893	99.9974	99.9893							
AY376454.1 Isolate pFL (SS)	99.9969	99.9922	99.9881	99.9910						
OM818392 Isolate AL (HTS)	99.9830	99.9898	99.9791	99.9881	99.9842					
OM818393 Isolate P23 (HTS)	99.9962	99.9919	99.9874	99.9903	99.9979	99.9856				
OM818394 Isolate MJ (HTS)	99.9805	99.9883	99.9786	99.9891	99.9818	99.9922	99.9825			
OM818395 Isolate NN (HTS)	99.9832	99.9900	99.9803	99.9888	99.9844	99.9945	99.9854	99.9924		
OM818396 Isolate MB (HTS)	99.9788	99.9781	99.9883	99.9798	99.9786	99.9756	99.9793	99.9763	99.9769	

The nucleotide identity values represent a conversion of the nucleotide distances to percentages. The distance is the number of substitutions per site

SS Sanger Sequencing, *HTS* High Throughput Sequencing

sequenced and subsequently freeze-dried (Table1). The other re-sequenced isolates were maintained by serial subculture in subterranean clover plants for between 2 (AL, MB) and 5 (MJ) years prior to freeze drying. Therefore, the limited nt sequence variation between originally sequenced and re-sequenced genomes of the same isolate might be due to the different sequencing methodologies used, have occurred during routine serial subculture in plants, or both.

The first time SCMoV was found in Australia was in 1979 infecting subterranean clover plants growing in pastures in Western Australia (WA) (McLean 1983; Price and McLean 1984). Later, it was found commonly in subterranean clover-based pastures not only in WA (Wroth and Jones 1992b) but also across southern Australia and Tasmania (Helms et al. 1993; Jones 1996, 2012). It was apparently absent from North American pastures (McLaughlin et al. 1996), and has never been searched for elsewhere including amongst subterranean clover plants growing in pastures in the Mediterranean region, subterranean clover's main centre of origin. The SCMoV isolates sequenced from WA show low sequence variability suggesting its recent arrival. Isolate sequences from other Australian states are lacking despite its widespread occurrence in their subterranean clover-based pastures. It may have been introduced to WA via SCMoV-infected subterranean clover seed from the Mediterranean region (Jones et al. 2001) afterwards being spread inadvertently to other Australian states via infected seed. However, an alternative explanation of its appearance in WA might be that a single introduction occurred from another part of Australia, either as a virus emerging from a native legume species, or from an original seed-borne introduction into Australia before its spread to WA. Although LTSV was also first reported infecting a pasture plant, alfalfa (= lucerne), in Australasia (Foster and Jones 1980) and is the virus most closely related to SCMoV (Jones et al. 2001), this does not necessarily indicate both emerged within Australasia. LTSV also infects alfalfa in North America (Canada, USA), the Middle East (Saudi Arabia) and East Asia (China) (Abou-Haidar and Paliwal 1988; Raza et al. 2017; Guo et al. 2021; Nemchinov et al. 2022). Moreover, although not yet demonstrated for LTSV, sobemoviruses are characteristically seed-borne (Tamm and Truve 2000) and so readily distributed via infected seed. Therefore, LTSV could have originated elsewhere, most likely in the Middle East where alfalfa was first domesticated before being introduced to the Mediterranean region and likely later to other continents via contaminated alfalfa seed (Prosperi et al. 2014). Indeed, we consider an original SCMoV introduction from overseas to be more likely than its emergence from Australian native flora because different types of SCMoV resistance are present in germplasm of the annual pasture legumes subterranean clover and barrel medic collected from their main centre of origin and diversity in the Mediterranean region (Jones et al. 2001; Saqib et al. 2009) where pastures containing these species have existed for thousands of years (Morley 1961; Cocks et al. 1980; Gladstones and Collins 1983; Zohary and Heller 1984; Nichols and Francis 1993; Cocks and Bennett 1999; Nichols et al. 2007, 2012, 2013). Thus, the presence of such resistances suggests a long association during which SCMoV co-evolved with annual clovers and medics within pastures in the Mediterranean region before being introduced to Australia.

The evolutionary divergence, D_N, between the ten different SCMoV genomic sequences was estimated with MEGA X (Kumar et al. 2018; Stecher et al. 2020). The overall D_N for the isolates was 0.014, which, in contrast to indigenous Australian viruses that evolved locally (Gibbs et al. 2008, 2020), suggests that little evolutionary change had occurred in Australia, and hence that the Australian population of SCMoV is young. The D_N between the SS and HTS sequences of isolate P23 was only 0.004 compared to a slightly higher D_N between the other isolate pairs, which was 0.010 for AL and 0.011 for MB and MJ. The NN isolate only sequenced with HTS was closest to the earlier-sequenced AL isolate with a D_N of 0.005. Again, the high sequence identity of these isolates points to a recent common SCMoV ancestor with little subsequent change in their genomes. The ratio of the number of nonsynonymous substitutions per nonsynonymous site (*Ka*) to the number of synonymous substitutions per synonymous site (*Ks*), *Ka/Ks*, was calculated using the Ka/Ks calculator at http://services.cbu.uib.no/tools/kaks, and used to estimate the selection pressure on the major ORFs or the associated proteins of the virus. The *Ka/Ks* for the ORF1, ORF2a, ORF2a/b and the ORF3 of all the isolates were 1.60, 0.46, 0.38 and 0.19, respectively indicating the ORF1 is more variable, with the most accumulated amino acid-altering mutations per site and under less negative selection compared to the other ORFs. The ORF1 of sobemoviruses encodes the P1 protein, required for virus movement and systemic infection in a suitable host (Bonneau et al. 1998; Meier et al. 2006; Sivakumaran and Hacker 1998), and for suppression of host gene silencing (Sarmiento et al. 2007; Siré et al. 2008). Although none of these functions has been demonstrated for the SCMoV P1 experimentally, it likely plays similar roles with this virus. Positive selection pressure on ORF1 could also make the SCMoV P1 protein a symptom severity determinant as occurs with the P1 of other sobemoviruses. Interestingly, phylogenetic trees constructed with the ORF1, ORF2a, ORF2a/2b and ORF3 sequences using Maximum Likelihood, Neighbor-Joining and Minimum evolution approaches of MEGA X with 1,000 bootstraps, grouped isolates with similar severities together only in trees constructed with ORF1 sequences (Fig. 1). Based on these analyses, isolate NN is predicted to be a very severe isolate.

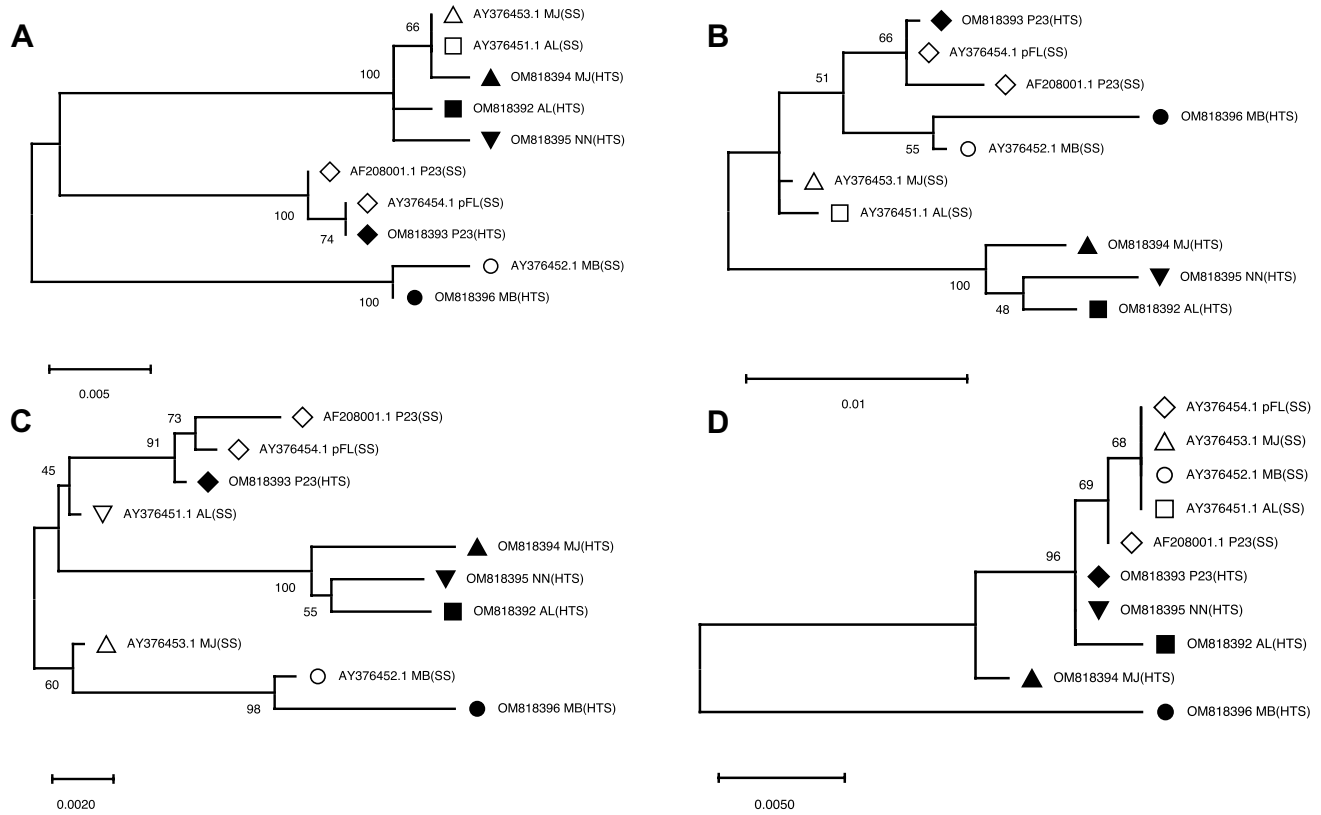

Fig. 1 Phylogenetic relationships between the Sanger-sequenced (SS) and High Throughput-sequenced (HTS) isolates of SCMoV based on the sequences of the ORF1 (**A**), ORF2a (**B**), ORF2a/2b (**C**) and ORF3 (**D**). An isolate is represented by its NCBI GenBank accession number followed by the isolate designation with the method of sequencing in parenthesis, SS or HTS. Sequences obtained by SS are further designated with unfilled symbols, whereas those obtained by HTS are designated with filled shapes as follows: isolate AL = squares; isolate MB = circles; isolates P23 and the derivative pFL clone = diamonds; isolate MJ = upright triangles; isolate NN = inverted triangle

Finally, although not identified during the de novo assembly and mapping of the HTS reads of the five historical SCMoV isolates against previously identified satellites (Davies et al. 1990), BWA (Li and Durbin 2009) and SAMtools (Li et al. 2009) revealed single satellite RNAs associated with each isolate (satellite accession numbers OM818397- OM818401) with between 96.4—98.2% nt identities to SCMoV satellite with accession number M33001.1 (Davies et al. 1990). Presence of a turnip yellows virus (TuYV) sequence within samples containing isolates AL, MB and NN was confirmed by real time PCR (Buxton-Kirk et al. 2021). By contrast, SS did not identify satellite RNA or TuYV within any samples presumably because SS employs virus-specific or group-specific degenerate primers, whereas HTS can potentially sequence any RNA present in a sample. The advent of the HTS technology is responsible for identification of many viruses from infected plant hosts and environmental samples (Adams et al. 2018; Bernardo et al. 2018; Jones et al. 2021; Villamor et al. 2019). Application of such technology has not only increased our understanding of the existence of additional viruses, including those in historical isolate collections and asymptomatic hosts, but also, as in this study, can be used to confirm the genomic sequences of previously sequenced viruses in a less laborious manner.

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Data availability In 2003, the sequences of four isolates of subterranean clover mottle virus (SCMoV) reported below were deposited in the National Center for Biotechnology (NCBI) Genbank database under the accession numbers AY376451—AY376454, and in 2021 those of five SCMoV isolates under the accession numbers (OM818392 -OM818396). The High Throughput Sequencing data can be found in Bioproject PRJNA808246 as Biosamples SAMN26038424-SAMN26038428 in the Sequence read archive (https://trace.ncbi.nlm.nih.gov/Traces/sra/).

Code availability All software used in this analysis is referenced in the report.

Declarations

Financial interests The authors declare they have no financial interests.

Conflicts of interest The authors declare no conflicts or competing interests that are relevant to the content of this article.

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