

**Genome sequence and
variation in the Australian
native
*Velvet tobacco mottle virus***

by

Kieren Arthur

MSc (Hons), The University of Auckland, NZ

A thesis submitted for the degree of
Doctor of Philosophy

at

The University of Adelaide,
School of Agriculture, Food and Wine

February, 2011

- Table of Contents -

Thesis Summary	vii
Statement	ix
Acknowledgements	x
Dedication.....	xi
List of Abbreviations	xii
Chapter 1: General Introduction	1
1.1 Velvet tobacco mottle virus.....	1
1.1.1 VTMoV taxonomy.....	1
1.1.2 The genus <i>Sobemovirus</i>	2
1.2 Unique features of VTMoV.....	5
1.2.1 VTMoV virusoid.....	6
1.2.2 VTMoV Transmission	6
1.2.3 VTMoV RNA.....	7
1.3 Sequence variation in VTMoV	8
1.4 Genetic bottlenecks during vector transmission	8
1.5 Scope of this thesis	9
Chapter 2: General Materials & Methods	10
2.2 Materials.....	10
2.2.1 Virus.....	10
2.2.2 Plants	10
2.2.3 Solutions, buffers, media.....	10
2.2.4 Primers.....	10
2.3 Methods	10
2.3.1 Inoculation of plants	10
2.3.2 Harvest and storage of leaf	11
2.3.3 Partial purification of VTMoV	11
2.3.4 Serological testing - DIBA	11
2.3.5 Total nucleic acid extraction	12
2.3.6 Virus RNA extraction	12

2.3.7 Polyacrylamide Gel Electrophoresis.....	13
2.3.8 cDNA synthesis.....	13
2.3.9 PCR Amplification	14
2.3.10 Agarose gel electrophoresis.....	14
2.3.11 Purification of PCR products	14
2.3.12 Cloning.....	14
2.3.13 Sequence analysis	16
Chapter 3: Sequencing the VTMoV genome	17
3.1 Introduction	17
3.2 Materials and Methods.....	17
3.2.1 Sequence fragment collection	17
3.2.2 Sequence analysis	18
3.3 Results	19
3.3.1 VTMoV genome	19
3.3.2 Virusoid sequence.....	22
3.3.3 ORF analysis.....	23
3.4 Discussion	27
Chapter 4: Annotation of the VTMoV genome	29
4.1. Introduction	29
4.2. Materials and methods	29
4.3. Results	30
4.3.1 Nucleotide sequence annotation	30
4.3.2 Annotation of putative translation products	34
4.3.3. Tertiary structure and function predictions of VTMoV proteins.....	36
4.3.4 Phylogenetic analysis.....	38
4.4. Discussion	41
4.4.1 Annotation of nucleotide sequence	41
4.4.2 VTMoV proteins	42
4.4.3 Phylogenetic analysis.....	44
Chapter 5: VTMoV isolate R17	45
5.1 Introduction	45
5.2 Materials and Methods.....	45
5.2.1 Sequence fragment collection	45
5.2.2 Sequence analysis	46

5.3 Results	46
5.3.1 Nucleotide sequence.....	46
5.3.2 Amino acid sequence.....	48
5.3.3. Sequence similarities between isolates of VTMoV	50
5.3.4 Sequence differences between isolates of VTMoV	51
5.4 Discussion	52
Chapter 6: Sequence variation in the RdRp gene of various isolates of VTMoV	54
6.1 Introduction	54
6.2 Materials and Methods	54
6.2.1 Collection of sequences from isolates	54
6.2.2 Analysis of RdRp sequence	56
6.2.3 Estimation of sequence error rate	56
6.2.4 Detection of Recombination	57
6.2.5 Assessing evolutionary mechanisms	58
6.2.6 Determining relationships between VTMoV isolates	58
6.3 Results	59
6.3.1 Estimation of error rate.....	59
6.3.2 Polymorphism classification	61
6.3.3 Assessing mechanisms of evolution	63
6.3.4 Relationships between VTMoV isolates	66
6.4 Discussion	68
6.4.1 VTMoV polymorphisms	68
6.4.2 Variation across amino acid sequences	69
6.4.3 Evolutionary selective pressures	69
6.4.4 Recombination	70
6.4.5 VTMoV isolate relationships.....	70
Chapter 7: Changes in VTMoV sequence associated with mechanical and mirid transmission	71
7.1 Introduction	71
7.2 Materials and Methods	71
7.2.1 Mechanical transmission.....	71
7.2.2 Mirid transmission	71
7.2.3 Sample selection and collection	73
7.2.4 Extraction of nucleic acids.....	73
7.2.5 RT-PCR	73

7.2.6 Cloning and sequencing	74
7.2.7 Sequence analysis	74
7.2.8 Protein analysis	75
7.3 Results	75
7.3.1 Mechanical transmission	75
7.3.2 Mirid transmission	81
7.3.3 Analysis of selective pressures	88
7.4 Discussion	89
7.4.1 Mutations in nucleotide sequence	89
7.4.2 Mutations in amino acid sequence	90
7.4.3 VTMoV rate of mutation	91
7.4.4 Trends in mutations	91
Chapter 8: General Discussion	93
8.1 VTMoV genome sequence	93
8.2 VTMoV sequence variation	95
8.3 VTMoV transmission	97
References	99
APPENDICES	111
Appendix 1: Published manuscript	112
Appendix 2: List of VTMoV isolates in WINC	140
Appendix 3: Solutions, buffers and media	142
Virion purification solutions	142
Nucleic acid extraction buffers	142
Protein digestion solutions	142
Polyacrylamide gel electrophoresis solutions	142
Agarose gel electrophoresis solutions	143
DIBA buffers	143
Suppliers	144
Appendix 4: Primers	145
Table A4.1 Degenerate primers used	145
Table A4.2 VTMoV sequence specific primers	145
Table A4.3 Primers from external sources	146
Appendix 5: Sequences of fragments 1-12 from VTMoV isolate K1	147
Appendix 6: I-TASSER output of VTMoV-P1 analysis	150

Appendix 7: Multiple sequence alignments for Figure 4.10A-C.....	151
Figure 4.10A	151
Figure 4.10B	157
Figure 4.10C	163
Appendix 8: Phylogenetic analysis of ORF1 sobemovirus sequences.....	167
Appendix 9: Sequences of fragments 1-4 from VTMoV isolate R17	168
Appendix 10: Amino acid groupings.....	170
Appendix 11: Nucleotide diversity figures including recombinant isolates	171
Appendix 12: Sequence alignments from the transmission experiment	172
Region 1 from nucleotides 8-990	172
Region 2 from nucleotides 3560-4115	175

- Thesis Summary -

Velvet tobacco mottle virus (VTMoV; genus Sobemovirus) infects *Nicotiana velutina* (Velvet tobacco), a native of the arid region of central Australia. In the field, the virus, mirid vector and native host plant together comprise a unique plant virus pathosystem which is well adapted to its ecological niche, and independent of anthropogenic influences. The purpose of this research was to describe the sequence variation amongst VTMoV isolates and relate this to ecological factors.

The full genome sequence of VTMoV was obtained using a genome walking strategy with both degenerate and specific primers. Sequence and genome organisation confirm that VTMoV is a unique sobemovirus and phylogenetic analysis groups it separately from other sequenced Australian sobemoviruses. This is consistent with the hypothesis that VTMoV is not a recently introduced sobemovirus, but rather a product of evolution within a unique Australian ecosystem, representing a novel plant virus lineage. The genome sequences of two isolates of VTMoV, K1 and R17 were compared and a limited amount of variation was observed between these isolates.

Sequence diversity was observed in the RNA dependent RNA polymerase (RdRp) gene from 15 isolates of the virus. Analysis determined mutations were limited to maintain protein function, which is indicative of purifying selection. In addition, the first evidence of recombination in the RdRp of a sobemovirus was detected in three VTMoV sequences.

Sequence variation associated with transmission of VTMoV by the mirid *Cyrtopeltis nicotianae* [Hemiptera; Miridae] was also investigated. Isolates K1 and R17 were serially passaged monthly either through obligatory mirid transmission or via mechanical inoculation. After two years, sequences were compared from two regions of the VTMoV genome associated with movement (open reading frames of protein P1 and the coat protein). Several different trends were observed in the sequences, but only one difference could be associated with the mode of transmission. The coat protein region sequence from mirid transmission had a higher mutation rate than sequence from the mechanical mode of transmission.

This thesis contains the first complete genome sequence of VTMoV. It describes natural variation amongst a range of VTMoV isolates, and assesses sequence variation in parts of the genome after long term mirid transmission. The sequence of VTMoV is discussed in the context of the unique nature of the virus and the evolutionary mechanisms that may have played a role the evolution of the virus.

- Statement -

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Kieren Arthur and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis when deposited in the University library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published works contained within this thesis (as listed below) resides with the copyright holder(s) of those works.

List of published works:

Arthur, K., S. Dogra and J. W. Randles (2010). "Complete nucleotide sequence of *Velvet tobacco mottle virus isolate K1*." *Archives of Virology* 155 (11): 1893-1896.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Kieren Arthur

- Acknowledgements -

The process of scientific discovery is, in effect, a continual flight from wonder.

Albert Einstein

Firstly, thank you to my supervisors Prof. John Randles, Dr Nick Collins and Dr Satish Dogra for all of your advice, mentorship and support during this project.

A gigantic thank you to all the morning tea, coffee and lunch buddies that I had, especially Steve, Ellena, Robin, Christine, Hayley, Dale, Cathy, Amanda, as well as the other numerous people on Waite campus. Your support through the tough times and the celebrations of the small successes was what kept me going some days.

Thanks also to me friends and family back home in New Zealand, your love and support was felt across an ocean.

Thank you to Dr Allan Gould for the supply of some primers for the sequencing work and finally thank you to the C. J Everard scholarship for their funding support.

**Dedicated to my grandfather,
Whose adventurous spirit encouraged me to dream**

- List of Abbreviations -

Abbreviation guidelines of the *Journal of General Virology* were followed for this thesis.

CP- Coat protein

DIBA- Dot immunobinding assay

d.p.i – days post inoculation

gRNA- genomic RNA

LB- Luria broth

mg- milligrams

MP- Movement protein

nm-nanometres

sgRNA – subgenomic RNA

VPg- viral genome-linked protein

WINC – Waite Institute culture collection

Virus abbreviations

Names not in italics signify species yet to be officially recognised by the International Committee on Taxonomy of Viruses (ICTV)

BBDV- *Banana bunchy top virus*

BSSV- *Blueberry shoestring virus*

BYDV- *Barley yellow dwarf virus*

CMV- *Cucumber mosaic virus*

CoMV- *Cocksfoot mottle virus*

IYMV- *Imperata yellow mottle virus*

LTSV – *Lucerne transient streak virus*

PLRV- *Potato leaf roll virus*

PLYV- Papaya lethal yellowing virus
PVY – *Potato virus Y*
RGMoV- *Ryegrass mottle virus*
RuCMoV – *Rubus chlorotic mottle virus*
RYMV- *Rice yellow mottle virus*
SBMV- *Southern bean mosaic virus*
SCMoV- *Subterranean clover mottle virus*
SCPMV – *Southern cowpea mosaic virus*
SeMV- *Sesbania mosaic virus*
SNMoV- *Solanum nodiflorum mottle virus*
SoMV- *Sowbane mosaic virus*
TMV- *Tobacco mosaic virus*
TuMV- *Turnip mosaic virus*
TRoV- *Turnip rosette virus*
VTMoV- *Velvet tobacco mottle virus*
WSMV- *Wheat streak mosaic virus*