

***Citrus tristeza virus* populations in Grapefruit trees,
pre-immunized with two different cross-protecting
sources**

By

David Read

Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae (Microbiology)

In the Faculty of Natural and Agricultural Sciences

University of Pretoria

Pretoria

South Africa

June 2011

DECLARATION

I, David Alan Read, declare that the thesis which I hereby submit for the degree of Magister Scientiae at the University of Pretoria, is my own work and has not been previously submitted by me for a degree at this or any other tertiary institution.

Signature

Date

ACKNOWLEDGEMENTS

I would like to thank the following people and organisations for their invaluable contributions made to the completion of this study.

Citrus Research International (CRI), University of Pretoria (UP) and the Technology and Human Resources for Industry Program (THRIP) funded the study.

My supervisor, Prof G Pietersen for his continued expert guidance throughout all aspects of the project.

K. Scott for her friendship, guidance and support in the lab.

Dr F van Vuuren of CRI who was a constant source of knowledge, regarding CTV cross-protection.

Dr E. Steenkamp for proofreading and for her valuable input.

Students in the Department of Microbiology and Plant Pathology, particularly D. Paterson, M. Schade, E. Vermaak, O. Bihl, H. Walsh, S. MacRae, R. Viljoen and O. Zablocki for their friendship and support.

My parents for their endless support throughout my academic career.

CONTENTS

Acknowledgements	iii
List of Figures	vii
List of Tables	x
Abstract	xv
Chapter 1: Introduction	1
Chapter 2: Literature Review	5
(2.1) CTV as an agent of Citrus disease	5
(2.2) CTV structure, genomics and epidemiology	6
(2.3) Control of CTV through cross-protection and the problem of superinfection	11
(2.4) Methods for CTV characterization	14
(2.5) Studies of population dynamics	17
Chapter 3: Materials and Methods	
(3.1) Experimental block and sampling	27
(3.2) RNA extractions	27
(3.3) cDNA synthesis of the A-fragment and p23 gene region	28
(3.4) PCR amplification of the A-fragment and p23 gene region	28
(3.5) Cloning of the A-fragment and p23 gene region PCR products	29
(3.6) Recombinant plasmid extraction, verification and sequencing	30
(3.7) Phylogenetic analysis	31

Chapter 4: Results

(4.1) Visual symptom evaluation of sampled field plants.....	36
(4.2) PCR amplification of the A-region and p23 gene region of the field and greenhouse collected samples.....	36
(4.3) Incidence of strains within field samples.....	37
(4.4) Incidence of strains within the greenhouse collected reference samples.....	40
(4.5) Predicted amino acid sequence analysis of the p23 gene region.....	42
(4.6) Recombination analysis.....	44
Chapter 5: Discussion.....	56
Appendix 1.....	74
Appendix 2.....	79
Appendix 3.....	83
Appendix 4.....	87
Appendix 5.....	91
Appendix 6.....	96
Appendix 7.....	100
Appendix 8.....	104
Appendix 9.....	108
Appendix 10.....	111
Appendix 11.....	117
Appendix 12.....	122
Appendix 13.....	127
Appendix 14.....	132
Appendix 15.....	134
Appendix 16.....	137

Appendix 17.....	140
List of Abbreviations.....	152

LIST OF FIGURES

Figure number	Page
1. CTV symptoms.....	7
2. CTV genome organisation.....	10
3. Phylogenetic grouping based on 5'UTR.....	18
4. Gel photograph of purified amplicons of field samples.....	39
5. Multiple protein alignment of the p23 gene of various strains of CTV..	43
6. Summary tree for the A-region of field samples of GFMS 12 on Star Ruby.....	48
7. Summary tree for the p23 gene region of field samples of GFMS 12 on Star Ruby.....	49
8. Summary tree for the A-region of field samples of GFMS 12 on Flame.....	50
9. Summary tree for the p23 gene region of field samples of GFMS 12 on Flame.....	51
10. Summary tree for the A-region of field samples of GFMS 35 on Star Ruby.....	52
11. Summary tree for the p23 gene region of field samples of GFMS 35 on Star Ruby.....	53
12. Summary tree for the A-region of field samples of GFMS 35 on Flame.....	54
13. Summary tree for the p23 gene region of field samples of GFMS 35 on Flame.....	55

APPENDICES

Figure number	Page
1.1 Phylogenetic tree of the A-fragment of sample replicate 1 of GFMS 12 on Star Ruby.....	75
1.2 Phylogenetic tree of the A-fragment of sample replicate 2 of GFMS 12 on Star Ruby.....	76

1.3	Phylogenetic tree of the A-fragment of sample replicate 3 of GFMS 12 on Star Ruby.....	77
1.4	Phylogenetic tree of the A-fragment of sample replicate 4 of GFMS 12 on Star Ruby.....	78
2.1	Phylogenetic tree of the A-fragment of sample replicate 1 of GFMS 12 on Flame.....	80
2.2	Phylogenetic tree of the A-fragment of sample replicate 2 of GFMS 12 on Flame.....	81
2.3	Phylogenetic tree of the A-fragment of sample replicate 3 of GFMS 12 on Flame.....	82
3.1	Phylogenetic tree of the A-fragment of sample replicate 1 of GFMS 35 on Star Ruby.....	84
3.2	Phylogenetic tree of the A-fragment of sample replicate 2 of GFMS 35 on Star Ruby.....	85
3.3	Phylogenetic tree of the A-fragment of sample replicate 3 of GFMS 35 on Star Ruby.....	86
4.1	Phylogenetic tree of the A-fragment of sample replicate 1 of GFMS 35 on Flame.....	88
4.2	Phylogenetic tree of the A-fragment of sample replicate 2 of GFMS 35 on Flame.....	89
4.3	Phylogenetic tree of the A-fragment of sample replicate 3 of GFMS 35 on Flame.....	90
5.1	Phylogenetic tree of the p23 gene region of sample replicate 1 of GFMS 12 on Star Ruby.....	92
5.2	Phylogenetic tree of the p23 gene region of sample replicate 2 of GFMS 12 on Star Ruby.....	93
5.3	Phylogenetic tree of the p23 gene region of sample replicate 3 of GFMS 12 on Star Ruby.....	94
5.4	Phylogenetic tree of the p23 gene region of sample replicate 4 of GFMS 12 on Star Ruby.....	96
6.1	Phylogenetic tree of the p23 gene region of sample replicate 1 of GFMS 12 on Flame.....	97

6.2	Phylogenetic tree of the p23 gene region of sample replicate 2 of GFMS 12 on Flame.....	98
6.3	Phylogenetic tree of the p23 gene region of sample replicate 3 of GFMS 12 on Flame.....	99
7.1	Phylogenetic tree of the p23 gene region of sample replicate 1 of GFMS 35 on Star Ruby.....	101
7.2	Phylogenetic tree of the p23 gene region of sample replicate 2 of GFMS 35 on Star Ruby.....	102
7.3	Phylogenetic tree of the p23 gene region of sample replicate 3 of GFMS 35 on Star Ruby.....	103
8.1	Phylogenetic tree of the p23 gene region of sample replicate 1 of GFMS 35 on Flame.....	105
8.2	Phylogenetic tree of the p23 gene region of sample replicate 2 of GFMS 35 on Flame.....	106
8.3	Phylogenetic tree of the p23 gene region of sample replicate 3 of GFMS 35 on Flame.....	107
12.1	Phylogenetic tree of the A-fragment of sample MS12.ML.A from the greenhouse.....	123
12.2	Phylogenetic tree of the A-fragment of sample MS12.SR.A from the greenhouse.....	124
12.3	Phylogenetic tree of the A-fragment of sample MS35.ML.A from the greenhouse.....	125
12.4	Phylogenetic tree of the A-fragment of sample MS35.SR.A from the greenhouse.....	126
13.1	Phylogenetic tree of the p23 gene region of sample MS12.ML.p23 from the greenhouse.....	128
13.2	Phylogenetic tree of the p23 gene region of sample MS12.SR.p23 from the greenhouse.....	129
13.3	Phylogenetic tree of the p23 gene region of sample MS35.ML.p23 from the greenhouse.....	130
13.4	Phylogenetic tree of the p23 gene region of sample MS35.SR.p23 from the greenhouse.....	131

LIST OF TABLES

Table number	Page
1	List of replicate samples collected from the ARC experimental block Malelane H7.....27
2	Primer sequences used for the amplification of the p23 gene and A-fragment.....29
3	Number of clones of the A-region analysis within each field Sample.....39
4	Number of clones of the p23 gene region analysis within each field sample.....40
5	Number of clones from the A-region analysis within each source population sample.....42
6	Number of clones from the p23 gene region analysis within each source population sample.....42
7	Field collected samples with clones showing evidence of recombination events.....45
8	Reference samples with clones showing evidence of recombination events.....46
9	Nucleotide diversities within groups for the A-region of the field samples.....109
10	Nucleotide diversities within groups for the p23 gene region of the field samples.....110
11	Nucleotide diversities between groups for the A-region of the field sample 301.....112
12	Nucleotide diversities between groups for the A-region of the field sample 302.....112
13	Nucleotide diversities between groups for the A-region of the field sample 303.....112
14	Nucleotide diversities between groups for the A-region of the field sample 304.....113
15	Nucleotide diversities between groups for the A-region of the field sample 305.....113

16	Nucleotide diversities between groups for the A-region of the field sample 306.....	113
17	Nucleotide diversities between groups for the A-region of the field sample 307.....	114
18	Nucleotide diversities between groups for the A-region of the field sample 308.....	114
19	Nucleotide diversities between groups for the A-region of the field sample 309.....	114
20	Nucleotide diversities between groups for the A-region of the field sample 310.....	115
21	Nucleotide diversities between groups for the A-region of the field sample 311.....	115
22	Nucleotide diversities between groups for the A-region of the field sample 312.....	115
23	Nucleotide diversities between groups for the A-region of the field sample 313.....	116
24	Nucleotide diversities between groups for the p23 gene region of the field sample 301.....	118
25	Nucleotide diversities between groups for the p23 gene region of the field sample 302.....	118
26	Nucleotide diversities between groups for the p23 gene region of the field sample 303.....	118
27	Nucleotide diversities between groups for the p23 gene region of the field sample 304.....	118
28	Nucleotide diversities between groups for the p23 gene region of the field sample 305.....	119
29	Nucleotide diversities between groups for the p23 gene region of the field sample 306.....	119
30	Nucleotide diversities between groups for the p23 gene region of the field sample 307.....	119
31	Nucleotide diversities between groups for the p23 gene region of the field sample 308.....	120
32	Nucleotide diversities between groups for the p23 gene region of the field sample 309.....	120

33	Nucleotide diversities between groups for the p23 gene region of the field sample 310.....	120
34	Nucleotide diversities between groups for the p23 gene region of the field sample 311.....	121
35	Nucleotide diversities between groups for the p23 gene region of the field sample 312.....	121
36	Nucleotide diversities between groups for the p23 gene region of the field sample 313.....	121
37	Nucleotide diversities within groups for the A-region of the greenhouse source samples.....	133
38	Nucleotide diversities within groups for the p23 gene region of the greenhouse source samples.....	133
39	Nucleotide diversities between groups for the A-region of the greenhouse sample MS12.ML.A.....	135
40	Nucleotide diversities between groups for the A-region of the greenhouse sample MS12.SR.A.....	135
41	Nucleotide diversities between groups for the A-region of the greenhouse sample MS35.ML.A.....	136
42	Nucleotide diversities between groups for the A-region of the greenhouse sample MS35.SR.A.....	136
43	Nucleotide diversities between groups for the p23 gene region of the greenhouse sample MS12.ML.p23.....	138
44	Nucleotide diversities between groups for the p23 gene region of the greenhouse sample MS12.SR.p23.....	138
45	Nucleotide diversities between groups for the p23 gene region of the greenhouse sample MS35.ML.p23.....	138
46	Nucleotide diversities between groups for the p23 gene region of the greenhouse sample MS35.SR.p23.....	139
47	Predicted amino acid sequences of the p23 protein from the field collected sample replicate 1 of GFMS 12 on Star Ruby (301).....	141
48	Predicted amino acid sequences of the p23 protein from the field collected sample replicate 2 of GFMS 12 on Star Ruby (302).....	141

49	Predicted amino acid sequences of the p23 protein from the field collected sample replicate 3 of GFMS 12 on Star Ruby (303).....	142
50	Predicted amino acid sequences of the p23 protein from the field collected sample replicate 4 of GFMS 12 on Star Ruby (313).....	142
51	Predicted amino acid sequences of the p23 protein from the field collected sample replicate 1 of GFMS 12 on Flame (304).....	143
52	Predicted amino acid sequences of the p23 protein from the field collected sample replicate 2 of GFMS 12 on Flame (305).....	143
53	Predicted amino acid sequences of the p23 protein from the field collected sample replicate 3 of GFMS 12 on Flame (306).....	144
54	Predicted amino acid sequences of the p23 protein from the field collected sample replicate 1 of GFMS 35 on Star Ruby (307).....	144
55	Predicted amino acid sequences of the p23 protein from the field collected sample replicate 2 of GFMS 35 on Star Ruby (308).....	145
56	Predicted amino acid sequences of the p23 protein from the field collected sample replicate 3 of GFMS 35 on Star Ruby (309).....	145
57	Predicted amino acid sequences of the p23 protein from the field collected sample replicate 1 of GFMS 35 on Flame (310).....	146
58	Predicted amino acid sequences of the p23 protein from the field collected sample replicate 2 of GFMS 35 on Flame (311).....	146
59	Predicted amino acid sequences of the p23 protein from the field collected sample replicate 3 of GFMS 35 on Flame (312).....	147

60	Predicted amino acid sequences of the p23 protein of the cloned sequences from the greenhouse collected reference sample GFMS 12 on Mexican Lime.....	148
61	Predicted amino acid sequences of the p23 protein of the cloned sequences from the greenhouse collected reference sample GFMS 12 on Star Ruby.....	149
62	Predicted amino acid sequences of the p23 protein of the cloned sequences from the greenhouse collected reference sample GFMS 35 on Mexican Lime.....	150
63	Predicted amino acid sequences of the p23 protein of the cloned sequences from the greenhouse collected reference sample GFMS 35 on Star Ruby.....	151

ABSTRACT

Citrus tristeza virus (CTV) is responsible for the most devastating viral disease of citrus crops and affects the profitability of citriculture on a worldwide scale. In South Africa, where CTV is endemic, the devastating effects of CTV severe-strain infections are reduced through the intentional inoculation of virus-free budwood with a mild-strain cross-protecting source. Cross-protection breakdown however occurs in grapefruit trees in South Africa and results in a reduction of yield and quality in grapefruit producing areas. Grapefruit cultivars are particularly sensitive to the stem-pitting effects of some severe CTV strains and despite the rigorous cross-protection scheme, severe stem-pitting and decline symptoms are still observed in the field. The GFMS 12 cross-protecting isolate was used as the cross-protecting source for grapefruit plantings in South Africa until its use was terminated in 2007. As early as 1993 however, severe stem-pitting was observed in the field. GFMS 35 has been employed as an alternate cross-protecting source, due to the apparent poor cross-protection ability of GFMS 12. Using nucleotide sequence analysis of a 5' variable region (A-region) and a 3' conserved region (p23 gene region), CTV population studies were performed on GFMS 12 and GFMS 35 pre-immunised Star Ruby and Flame trees in the field, in the hot production area of Malelane. The viral population of GFMS 12 and GFMS 35 sources, maintained on Mexican Lime and Star Ruby, plants were characterized as references. Severe VT-like strains dominated (up to 100% of clones) in all of the field samples. Of the two grapefruit cultivars sampled, no clear differences in their strain assemblage was obtained, suggesting that relative to each other, these cultivars played no role in shaping the CTV population strain composition that were observed. VT-like strain dominance was also observed in most of the sources maintained on Star Ruby. A shift in population strain composition was observed in the sources maintained on Mexican lime suggesting the important role that hosts may play in strain selection. A number of anomalous results were obtained for the sources that appeared not to follow the "general trend" of population strain composition observed in the other samples. These anomalies may have been caused by when the samples were collected, the age of the tissue material

collected, PCR bias during amplification or some other factor resulting in a population shift in the sources. From these results it is near impossible to pinpoint the exact mechanism responsible for CTV cross-protection breakdown. However, the scale of the complexity of CTV population dynamics in South Africa has become clear. From this it will be possible to begin changing the way that cross-protection candidates are evaluated in South Africa, from an entirely empirical approach to one that incorporates much needed molecular methods

Chapter 1

INTRODUCTION

Citrus tristeza virus (CTV) is a member of the viral genus *Closterovirus*, which produces filamentous virions, with dimensions of 2000nm x 11nm. CTV genomes are positive sense, single-stranded RNA (Karasev et al, 1995), approximately 20kb in length making them among the largest RNA genomes discovered to date (Dolja et al, 2006). CTV particles are limited to the phloem cells and their associated tissues where they occur as aggregated inclusion bodies (Brlansky and Lee, 1990). The CTV genome consists of 12 open reading frames together with 5' and 3' untranslated regions at the termini of the genomic RNA (gRNA) (Ruiz-Ruiz et al, 2006). The open reading frames overlap and are known to encode 19 protein products (Karasev et al, 1995).

CTV causes one of the most devastating and economically important viral diseases (Bar-Joseph et al, 1989), which reduces the profitability of citriculture worldwide (Herron et al. 2006). Movement of CTV to new regions is mainly due to the dispersal of infected budwood. Once established, it is able to spread via a number of aphid vectors (Moreno et al. 2008). It is estimated that CTV has been responsible for the death of tens of millions citrus trees propagated on sour orange rootstocks worldwide (Bar-Joseph et al, 1989). *Tristeza* is the Spanish word for sadness and is a description of the decline of infected citrus plants when sour orange is used as rootstock (Bertolini et al. 2008).

The economic losses incurred by CTV infections can be minimised by eradication of trees showing signs of CTV infection or through the use of cross-protecting CTV mild-strain isolates (Rubio et al, 1996), which involves the intentional infection of CTV free material with a CTV isolate known to exhibit only mild symptoms (van Vuuren and Manicom, 2005). Of all the commercial citrus species grown in Southern Africa and South America, grapefruit (*Citrus x paradisi*) is the most sensitive to CTV (Iglesias et al, 2008, van Vuuren and Manicom, 2005). GFMS (Grapefruit mild-strain) 12, sourced

from a 50 year-old Marsh type grapefruit tree in South Africa's Western Cape province was selected as the CTV cross-protecting source for grapefruit plantings in South Africa. However, in 1993 Star Ruby plantings pre-immunised with GFMS 12 began to show symptoms of severe stem-pitting (van Vuuren and Manicom, 2005). Due to the apparent poor protecting capacity of GFMS 12, GFMS 35 has subsequently been approved as the interim cross-protection source for grapefruit planting in South Africa (Luttig et al, 2002).

The objectives of this study were to establish the reasons for the observed cross-protection breakdown in grapefruit trees pre-immunised with the GFMS 12 cross-protecting source and to determine if the interim cross-protecting source, GFMS 35, is a suitable replacement for GFMS 12. The role that cultivars play in CTV population dynamics was also investigated. 13 samples were collected from a trial block in the hot Malelane production region. The samples consisted of both Star Ruby and Flame cultivars, pre-immunised with either GFMS 12 or GFMS 35. Populations of both the GFMS 12 and GFMS 35 populations on Star Ruby and Mexican Lime trees were studied and used as references, against which the field samples could be compared. Nucleotide sequence analysis of two genomic regions, the A-region (Rubio et al, 2001) and the p23 gene region (Sambade et al, 2003) were studied for each of the field isolates collected and the phylogenetic relationships between them were established. The nomenclature used to describe CTV variants can be confusing and can vary between publications. For the purposes of this study, genotype was used to define groups showing high similarity with the CTV genotype theory described by Hilf et al, (2005). Strain was used to describe specific sequence types falling within the same genotypic groups. Source was used to describe the CTV population present within individual living plants.

LITERATURE CITED

- Bar-Joseph M., Marcus R. and Lee R.F. (1989). The continuous challenge of Citrus Tristeza Virus Control. Annual Reviews in Phytopathology vol. 27 pp291-316
- Bertolini E., Moreno A., Capote N., Olmos A., de Luis A., Vidal E., Pérez-Panadés and Cambra M. (2008) 'Quantitative detection of *Citrus tristeza virus* in plant tissues and single aphids by real-time RT-PCR' European Journal of Plant Pathology vol. 120 pp177-188
- Brlansky R.H. and Lee R.F. (1990). Numbers of Inclusion Bodies Produced by Mild and Severe Strains of Citrus Tristeza Virus in Seven Citrus Hosts. Plant Disease vol. 74 pp297-299
- Dolja V.V., Kreuze J.F. and Valkonen J.P.T. (2006) 'Comparative and functional genomics of closteroviruses'. Virus Research vol. 117 pp38-51
- Herron C.M., Mirkov T.E., da Graca J.V. and Lee R.F. (2006). *Citrus tristeza virus* transmission by the *Toxoptera citricida* vector: In vitro acquisition and transmission and infectivity immunoneutralization experiments. Journal of Virological Methods vol. 134 pp205–211
- Hilf M.E., Mavrodieva V.A. and Garnsey S.M. (2005). Genetic Marker Analysis of a Global Collection of Isolates of *Citrus tristeza virus*: Characterization and Distribution of CTV Genotypes and Association with Symptoms. Phytopathology vol.95 pp909-917
- Iglesias N.G., Gago-Zachert S.P., Robledo G., Costa N., Plata M.I., Vera O., Grau O. and Semorile L.C. (2008). Population structure of *Citrus tristeza virus* from field Argentinean isolates. Virus Genes vol. 36 pp199-207
- Karasev A.V., Boyko V.P., Gowda S., Nikolaeva O.V., Hilf M.E., Koonin E.V., et al, (1995). 'Complete Sequence of the Citrus Tristeza Virus RNA Genome'. Virology vol. 208 pp511-520

Luttig M., van Vuuren S.P. and van der Vyver J.B. (2002). Differentiation of single aphid cultured sub-isolates of two South African *Citrus tristeza virus* isolates from grapefruit by single-strand conformation polymorphism. In: *Proc 15th Conf. IOCV*, Riverside, California.

Moreno P., Ambros S., Albiach-Marti M.R., Guerri J. and Pena L. (2008). 'Citrus tristeza virus: A pathogen that changed the course of the citrus industry'. *Molecular Plant Pathology* vol. 9 pp251-268

Rubio L., Ayllón M.A., Guerri J., Pappu H., Niblett C and Moreno P. (1996). Differentiation of citrus tristeza virus (CTV) isolates by single-strand conformation polymorphism analysis of the coat protein gene. *Annals of Applied Biology* vol. 129 pp479-489

Rubio L., Ayllón M.A., Kong P., Fernandez A., Polek M., Guerri J., Moreno P and Falk B. (2001). Genetic variation of *Citrus tristeza virus* isolates from California and Spain: Evidence for mixed infections and recombination. *Journal of Virology* vol. 75 pp8054-8062

Ruiz-Ruiz S., Moreno P., Guerri J. and Ambrós S. The complete nucleotide sequence of a severe stem pitting isolate of *Citrus tristeza virus* from Spain: comparison with isolates from different regions. *Archives of Virology* vol.151 pp387-398

Sambade A., Lopez C., Rubio L., Flores R., Guerri J. and Moreno P. (2003). Polymorphism of a specific region in gene *p23* of *Citrus tristeza virus* allows for the discrimination between mild and severe isolates. *Archives of Virology* vol. 148 pp2325-2340

van Vuuren S.P. and Manicom B.Q. (2005). The Response of Star Ruby Grapefruit to Different *Citrus tristeza virus* isolates. In: *Proc 16th Conf. IOCV*, 112-116 IOCV, Riverside, California.

Chapter 2

LITERATURE REVIEW

(2.1) CTV as an agent of Citrus disease

CTV isolates can generally be described as being either mild or severe, according to the types of symptoms that they elicit. The different symptoms produced by strains are probably a result of genomic differences, which is the result of error-prone replication (Cerni et al, 2008) by RNA dependant RNA polymerase (RdRp) (Albiach-Marti et al 2000). Mild isolates usually elicit superficial symptoms or no symptoms at all. Severe isolates however, are able to produce one or more of the following symptoms, depending on the strain of the infecting virus and the species of citrus being infected (Ruiz-Ruiz et al, 2007). General decline, seedling yellows, stem pitting and stunting, as well as low fruit yields and small fruit size (Bar-Joseph et al, 1989). Of all the symptoms, quick decline is the most dramatic, with a healthy looking tree undergoing complete collapse and death within as little as a few weeks (Moreno et al, 2008).

The severity of CTV symptoms depends largely on rootstock and scion combinations (Moreno et al, 2008). After an epidemic of foot-rot, a fungal disease caused by species of *Phytophthora*, growers were forced to use sour orange rootstocks (Moreno et al, 2008), which was used almost exclusively in Mediterranean regions as well as in the Americas. As a rootstock, sour orange was very attractive, since it is resistant to *Phytophthora*, is adaptable to poor soils and produces unrivalled fruit set and quality (Moreno et al, 2008). Growers were unaware however, that they had created a new host, severely sensitive to a then unknown pathogen, CTV (Moreno et al, 2008). Presently, most of the citrus plantings in Spain are still growing on sour orange rootstocks (Cambra et al, 2000), which is of major concern, since the Spanish citrus industry is at risk of being decimated by CTV. The introduction of the most efficient aphid vector (Herron et al, 2006) *Toxoptera citricida* in Spain may result in the complete demise of Spanish citriculture.

CTV has become established in almost all major citrus growing regions (Niblett et al, 2000) and in the past century has been responsible for the complete destruction of citrus industries in a number of countries (Albiach-Marti et al, 2000). Estimates reveal that CTV has killed approximately 38 million trees in the Americas (Niblett et al, 2000), more than 55 million in the Mediterranean countries of which 40 million of these were in the Valencian region of Spain (Cambra et al, 2000) and a further 5 million killed elsewhere around the world (Niblett et al, 2000). The dissemination of CTV within and between countries and continents has mainly been the result of illegally imported budwood or seedlings that are infected with CTV (Niblett et al, 2000, Herron et al, 2006), with the subsequent local spread of the virus through the resident aphid populations.

Aphids spread the virus in a semi-persistent manner (Bertolini et al, 2008, Ruiz-Ruiz et al, 2007) and a number of aphid species, namely *Toxoptera citricida* (brown citrus aphid), *Aphis gossypii*, and *Aphis spiraecola* are the most important vectors, with the brown citrus aphid (BrCA) being the most efficient transmitter of the virus (Cerni et al, 2008). Despite the major economic impact of this virus-aphid association a worryingly small amount of information is available on the specific workings of the CTV-BrCA relationship and the subsequent spread of the disease (Herron et al, 2006). This paucity information makes studying CTV epidemiology, as well as controlling the disease, a monumental task.

(2.2) CTV structure, genomics and epidemiology

CTV forms long flexuous filamentous particles, measuring 2000 x 11 nm in size (Gonsalves et al, 1978) and is among the largest known plant viruses (Karasev et al, 1995). The viral coat is made up of two capsid proteins, one of 25kDa (p25, CP) covering approximately 95% of the virion length and another of 27kDa (p27, CPm) covering the remaining 5% (Ayllon et al, 2001).

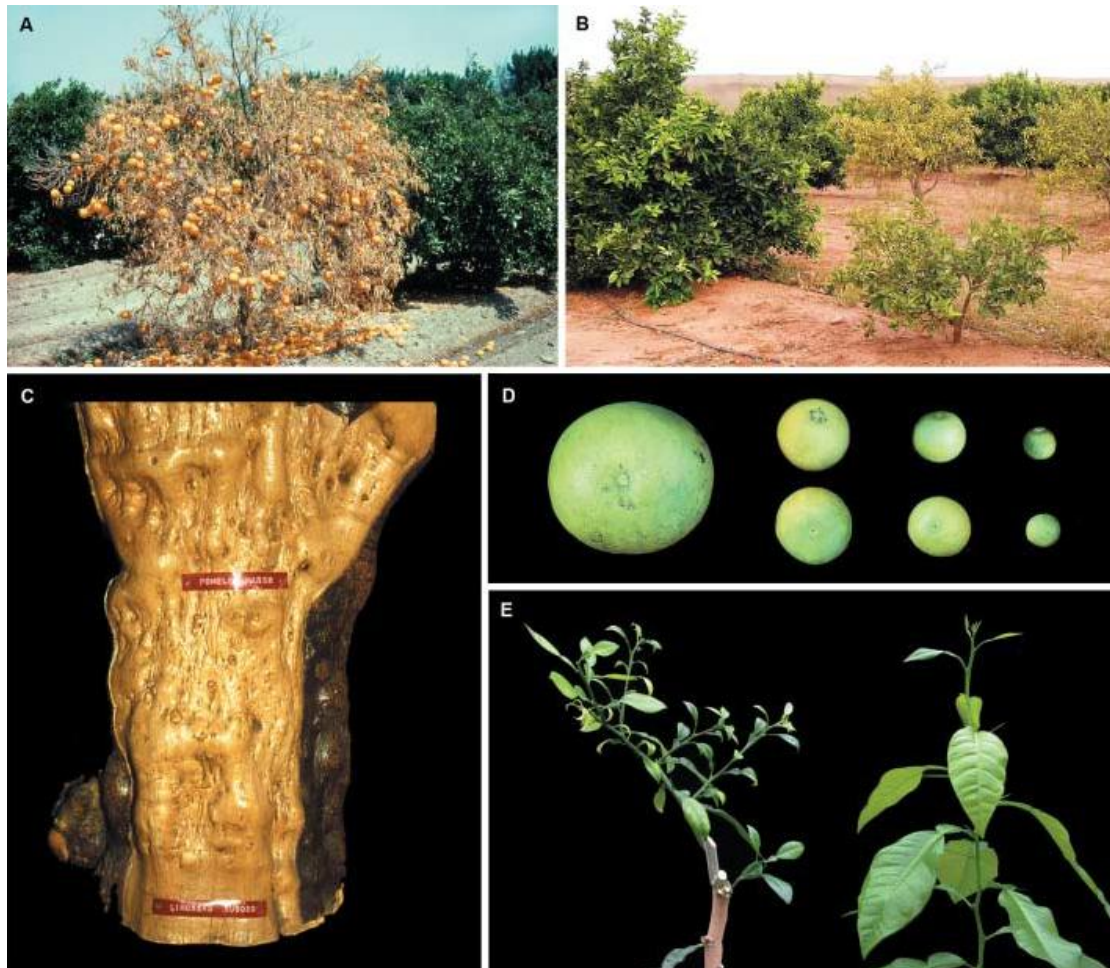


Figure 1: Illustrations showing the symptoms elicited by different severe CTV strains. A: Dramatic quick decline symptoms, with fruit still attached to the tree. B: General decline symptoms. C: Stem-pitting. D: Reduction in fruit size. E: Seedling yellows syndrome (Moreno et al, 2008).

The viral genome is made up a monopartite, single stranded, positive sense RNA molecule, varying in length between 19226 and 19296nt (Ayllon et al, 2001), depending on the isolate. The genome codes for 12 open reading frames (ORF's) along with a non-translated region (NTR) at the 5' and 3' termini (Karasev et al, 1995). ORF 1a and 1b form what is known as "the replication gene block", since their products are required for viral replication (Tatineni et al, 2008). ORF 1a encodes for a 349-kDa polyprotein, which possesses protease, methyltransferase-like and helicase-like domains (Karasev et al, 1995). The product of ORF 1b, an RNA-dependant RNA polymerase, is expressed via a +1 frameshift. ORF's 1a and 1b are expressed directly from the genomic RNA (Karasev et al, 1995). The 10 ORF's toward the 3'-end are expressed via subgenomic RNAs (sgRNAs) and encode for remainder of the CTV proteins known as p33, p6, p65, p61, p27, p25, p18,

p13, p20 and p23 (Hilf et al, 1995, Martin et al, 2009). The production of sub-genomic RNAs (sgRNA) is a strategy used by a number of positive-strand RNA viruses and allows for the regulation of production of different viral proteins. CTV produces large numbers of negative sgRNAs when compared with other members of the alpha-virus subgroup, however the reasons for this is still unknown (Satyanarayana et al, 2002)

Functional roles for p33, p13 and p18 have not been elucidated (Martin et al, 2009). p6 appears to be a hydrophobic membrane anchor protein but appears to play no apparent role in efficient viroin assembly (Satyanarayana et al, 2000). p65 (HSP70h) is a protein homolog of the cellular molecular chaperone HSP70 and members of the *Closteroviridae* family are the only viruses known to encode for such proteins (Satyanarayana et al, 2000). According to Satyanarayana et al, (2000), HSP70h, p61, p25 (CP) and p27 (CPm) are all essential for efficient viroin assembly. p61 shows a limited similarity with another cellular protein, HSP90 and it has been suggested that it may work synergistically with HSP70h. A characteristic symptom of *Closterovirus* infection is the formation of inclusion bodies in the phloem cells of the infected plants (Gowda et al, 2000). In a study by Gowda et al, (2000) it was shown that p20 has a high affinity for itself and is a major component of phloem cell inclusion bodies. In studies done on *Cauliflower mosaic virus* (CaMV), it was shown that similar inclusion bodies are sites for particle assembly (Himmelbach et al, 1996), however this appears to be untrue for CTV since very little CP has been detected within the inclusion bodies (Gowda et al, 2000). The function role of the CTV p20 is obscure, however it has been suggested that it may function as part of the CTV suppression of RNA silencing machinery (Lu et al, 2004). The p23 gene is situated at the extreme 3' end of the CTV genome and encodes for a 23-kDa RNA-binding protein, which accumulates earliest in viral replication (Satyanarayana et al, 2002). A study by López et al, (2000) proved that p23 has an RNA-binding capacity and it was proposed that it functions in either regulation or pathogenicity. Ghorbel et al, (2001) confirmed the pathogenic capacity of p23 by expressing it in Mexican Lime plants, which subsequently showed severe CTV symptoms, possibly through the interference of developmental processes of the host (Fagoaga et al, 2005). Deletion mutagenesis within the

p23 gene has demonstrated that it is required for the asymmetric accumulation of positive and negative sense sgRNA species (Satyanarayana et al, 2002), while Lu et al, (2004) suggest that p23 forms part of the machinery directed against the host RNA silencing defence mechanism.

Sequence analysis of the gRNA of CTV isolates has revealed that the 5'NTR is highly variable with as little as 44% sequence identity between strains, while the 3'NTR is highly conserved with sequence identity never being lower than 97% (Ayllón et al, 2001). Comparison of the VT and T36 genomes revealed an asymmetrical sequence identity distribution (Hilf et al, 1999) with a conserved level sequence divergence in the last 8 kb in the 3' proximal end with an abrupt reduction in sequence conservation in the first 11 kb of the 5' proximal end. Hilf et al, (1999) suggest that the VT and T36 genotypes represent two major "lineages" within the CTV complex. Their studies also showed that the T3 and T30 genotypes displayed a high degree of sequence similarity with VT and placed the three into a single group. Hilf et al, (1999) have proposed a "rudimentary CTV classification scheme" where T3, T30, VT and T36 each represent specific "genotypes". In a follow up study by Hilf et al, (2005), a phylogenetic approach, using four genetic markers supported the assignment of the four different genotypes. Their main goal was to develop a simple technique to classify CTV isolates and to provide more structure for current CTV classification. In continued research using multiple molecular marker primers, Roy and Bransky (2010), discovered that the B165 isolate is a novel genotype that occupies a distinct branch in a phylogenetic tree that was generated using nucleotide sequences from the ORF 2 gene region. CTV isolates can therefore be grouped as one of five different genotypes, namely T30, T36, VT, T3 (Hilf et al, 2005) and B165 (Roy and Bransky. 2010). T3 is recognised as a separate genotype (Hilf et al, 1999) but since it has not been fully sequenced, it is usually excluded from phylogenetic studies on CTV (Roy and Bransky. 2010).

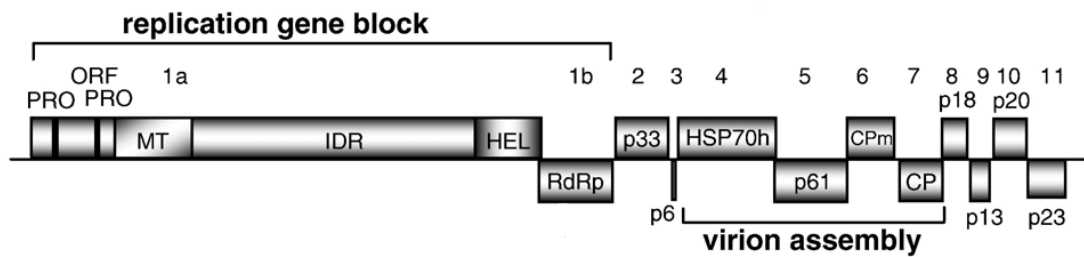


Figure 2: A schematic diagram depicting CTV genome organisation, with the ORF's represented as boxes with the protein products labelled (Tatineni et al, 2008).

Viruses present in plants are not clones of one another but rather a genetically heterogeneous population of different variants (Garcia-Arenal et al, 2001). These heterogeneous populations arise due to the accumulation of various mutations over time. RNA viruses show the highest levels of replication error rates (Sanz-Ramos et al, 2008), which is a function of the low copying fidelity of the viral RNA polymerase. These replication errors result in replicating populations that are highly diverse and are referred to as viral quasispecies (Sanz-Ramos et al, 2008). Even before the advent of molecular *in vitro* amplification techniques, the quasispecies nature of a number of RNA viruses was documented (Domingo, 2002). Variation in plant viruses was documented as early as 1926, through the isolation of different symptom variants from the same source (Garcia-Arenal et al, 2001). Molecular cloning and sequencing has provided researchers with the tools for the in depth study of viral quasispecies populations (Domingo, 2002).

The genomic determinants responsible for symptom expression remain largely unknown (Vives et al, 1999). One method of differentiating between mild and severe CTV isolates, involves the analysis of the predicted amino acid sequence of the p23 gene (Sambade et al, 2003). Amino acid motifs at positions 78-80 allow for the assignment of an isolate to one of three groups, mild, severe or atypical. The grouping of isolates into these groups is useful (Sambade et al, 2003) but whether this particular amino acid motif plays any role in symptom development remains unknown.

(2.3) Control of CTV through cross-protection and the problem of superinfection

Cross-protection is described as the phenomenon where a plant shows complete or partial resistance to a severe strain of a virus, after being infected by a mild or attenuated variant of the same virus (Gal-On and Shibolet, 2005 and Yoon et al, 2006). This approach is applied to crop or ornamental plant varieties in order to curb the damage caused by any subsequent severe-strain infection in the field (Fulton, 1986). A number of terms has been used to describe the technique, such as acquired immunity, prophylactic inoculation and antagonism but the term cross-protection was deemed most accurate in describing the reasons for its use, which is the protection against more severe strains (Fulton, 1986). A number of concerns against the use of cross-protection have arisen over the years. Firstly, the effectiveness of cross-protection to completely protect against certain severe plant virus strains has been called into question. However, Fulton (1986) argues the concept of “relative rather than complete” protection where a certain level of protection is superior to none at all, especially in cases where there is no other feasible alternative. Fulton (1986), lists a number of other concerns against cross-protection, among these are that propagating a virus may result in its rapid spread to other sensitive host plants. Secondly, synergism may result between the cross-protecting virus and another unrelated virus, resulting in severe and unforeseen disease symptoms. Potential synergism between CTV and another pathogenic agent may be the cause of a new disease called Citrus sudden death (CSD) (Gomes et al, 2008), however, further evidence is needed to substantiate this. A third concern adding to the uncertainty of applying cross-protection is the cost and labour required for the inoculation of an entire crop with a mild-strain virus. This concern is of particular importance to annual crops grown from seed and of least concern to perennial crop plants multiplied through vegetative propagation, such as citrus.

The best methods of CTV control is through effective quarantine in regions that are free of CTV or complete eradication of infected trees in regions with low incidence (Moreno et al, 2008). Eradication is not a feasible option in regions with widespread and high incidence of CTV infections that also have

effective vector populations to facilitate its spread. In this case, use of CTV resistant rootstocks is an option to curb negative economic effects (Moreno et al, 2008). Stem-pitting isolates however, are able to elicit symptoms regardless of the rootstock scion combinations used (Moreno et al, 2008). Severe stem-pitting isolates are present in the South African CTV population (Van Vuuren et al, 1993) and the only means of protecting against them is through cross-protection (Moreno et al, 2008). The use of resistant plant varieties is more favourable than implementing cross-protection schemes due to the reduced costs and labour required (Fulton, 1986), however the only way of producing resistant citrus is through transgenic modification, which is not feasible due to the large number of citrus cultivars and consumer concerns.

Cross-protecting sources are selected from relatively old but healthy looking plants (van Vuuren and Manicom, 2005), commonly in a field setting, that are surrounded other plants showing severe symptoms (Fulton, 1986). However, potential cross-protecting sources are usually evaluated empirically through biological trials (Folimonova, 2010), with no knowledge of the actual strain assemblage. This evaluation is time consuming, often taking many years, with most sources failing to protect (Folimonova, 2010) and those that do prove effective are usually suitable for only a few varieties. Due to factors such as the relationship between to host and isolate severity (Fulton, 1986) and the ability to replicate within certain hosts, different CTV cross-protecting sources are used for the various types of citrus being produced. All grapefruit trees planted after the implementation of the Southern African Citrus Improvement Scheme (CIP) were pre-immunised with the GFMS (Grapefruit mild-strain) 12 cross-protecting source (van Vuuren and Manicom, 2005). However, as early as 1993, evidence of severe cross-protection breakdown was observed in trees pre-immunised with GFMS 12 (van Vuuren and Manicom, 2005). The commercial use of GFMS 12 ceased in 2007 and since that time GFMS 35 has been used as the interim cross-protecting source.

In plant virology, the term superinfection usually refers to the failure of a cross-protecting viral isolate to completely protect against a severe isolate of the same virus (Fulton, 1978). Superinfection occurs when viruses, introduced

through secondary inoculations in the field, are allowed to become established within a plant that has been pre-immunised with a cross-protecting source. If a superinfecting strain is severe, this may result in the expression of disease symptoms and ultimately cross-protection breakdown. According to Fulton (1978), superinfection may occur due to the incomplete distribution or a low titre of the protecting strains within the plant or because the replicative processes of the protecting and challenge viruses may be sufficiently different that no interference occurs between them.

Superinfection exclusion or homologous interference is the principle on which cross-protection is based and refers to the prevention of a secondary infection due to the primary inoculation of a closely related virus (Folimonova et al, 2010). The exact mechanism of homologous interference is still unclear but among the most plausible possibilities are coat protein-mediated resistance and RNA interference (RNAi). The model of coat protein-mediated resistance suggests that the presence of existing coat proteins (CP) (from a primary inoculation) prevents the disassembly of an incoming virus of the same species (secondary inoculation), probably by binding to host factors involved in disassembly (Beachy, 1997) and in this way excludes superinfection. In the case of CTV however, previous studies have shown that apparent cross-protection extends from the infected to the uninfected cells (Folimonova et al, 2010) making coat protein-mediated resistance an unlikely model for superinfection exclusion in CTV. Due to this apparent protection of uninfected cells, the most plausible model for the mechanism of superinfection exclusion of CTV is RNAi (Folimonova et al, 2010). The phenomenon of RNAi was first discovered in plants expressing viral transgenes (Prins et al, 2008). The model describes the sequence-specific breakdown of dsRNA, formed through the binding of homologous transgenic RNA and infecting viral RNA (Lindbo et al, 1993). This mechanism should undoubtedly work in the same way as a cross-protecting isolate protects against superinfection. This model too however, does not fit the mechanism of superinfection exclusion of CTV since hybrid viruses containing 2.7 kb of exact homologous sequence failed to prevent the superinfection of one another (Folimonova et al, 2010). These findings indicate that superinfection

exclusion is probably achieved through a yet undefined molecular mechanism.

A study by Folimonova et al, (2010) has revealed how complicated the problem of CTV superinfection can be and issues that need to be addressed when selecting a cross-protecting source that will provide adequate protection against severe strain superinfection. Folimonova et al, (2010) determined that superinfection exclusion only occurred between variants of the same “genotype” (T30, VT, T68, T3 and T36). A primary infection of, for example VT, prevented the superinfection of a sequential inoculation of the same genotype (VT), while the presence one genotype from a primary infection had no influence on the establishment of a second heterologous genotype, within the same host. These results suggest that a complete change in the selection process of cross-protecting sources is required. According to Folimonova et al, (2010), the methodology for selection should now be relatively simple with the first objective being to identify the severe isolates within a region, which need to be controlled. Secondly, mild isolates need to be discovered or created, through the substitution of mild sequence determinants. An assemblage of mild strains can be created to produce a cross-protecting source that should protect against the severe field isolates present in a growing area.

(2.4) Methods for CTV characterization

Until the onset of molecular techniques, researchers relied on biological indexing on indicator hosts to characterize the pathogenic nature of CTV strains (Moreno et al, 2008). Since then, a variety of molecular typing methods have been previously used to characterize CTV populations. The availability of the Enzyme-linked immunosorbent assay (ELISA) revolutionised CTV detection, characterization and epidemiological studies (Moreno et al, 2008). Biological indexing and ELISA provide no information at the viruses genetic level. Availability and development of gene based characterization techniques allow for the continued improvement of isolate characterization, which has important implications for cross-protection schemes and epidemiological studies. Among these techniques are single-strand

conformation polymorphism (Rubio et al, 1996), restriction fragment length polymorphism (RFLP) (Jiang et al, 2008), bi-directional polymerase chain reaction (BD-PCR) (Jiang et al, 2008) and nucleotide sequence analysis (Iglesias et al, 2008, Jiang et al, 2008). Each of these methods possesses their own sets of advantages and disadvantages.

SSCP is a popular method among plant virologists (Garcia-Arenal et al, 2001). The technique allows for the differentiation between two DNA fragments, of the same size, varying by as little as a single nucleotide (Palacio et al, 1999) and can be useful in the determination of variance within viral populations (Chandler et al, 1999). The methodology involves the PCR amplification of a chosen genomic region followed by the denaturation of the double-stranded DNA (dsDNA) amplicon. Each single strand then takes on a specific folded conformation through internal base pairing, which is an inherent function of its nucleotide sequence (Shi et al, 1996). Small differences in the nucleotide sequence of the DNA fragment (as little as a single nucleotide) may change its overall conformation and hence alter its electrophoretic mobility (Rubio et al, 1996). Both DNA strands are then separated by non-denaturing polyacrylamide gel electrophoresis (PAGE) (Palacio et al, 1999).

SSCP analysis offers the advantages of being rapid and simple to carry out (Rubio et al, 1996), produces reliable and reproducible data (Kalvatchev et al, 2000), has high sensitivity being capable of detecting a single nucleotide difference between DNA fragments of 400bp in length (Chandler et al, 1999) and is applicable to the large-scale screening of viral populations (Xu et al, 2006). Among the disadvantages suffered by SSCP analysis is that nucleotide sequence analysis still needs to be performed to determine the exact number of nucleotide differences between two or more variants (Chandler et al, 1999) and to derive meaningful phylogenetic relationships within or between populations. Furthermore, single base pair differences may not always result in a conformational change and can therefore not be detected by SSCP (Chandler et al, 1999), which therefore can reduce the reliability of the data generated. Further disadvantages are that gel and electrophoresis conditions need to be optimised for each protocol (Xu et al, 2006), which can be time

consuming, occasionally the differences banding patterns can be too small to differentiate between two or more variants which are in fact different from one another (Rubio et al, 1996) and SSCP involves the use of toxic acrylamide.

Restriction fragment length polymorphism (RFLP) analysis is another molecular tool that can be used to evaluate the diversity of viral populations, as well as discriminating between variants. Variants possess unique restriction enzyme digest patterns and the differentiation of isolates is based on the relative positions of restriction enzyme digest sites (Hauser et al, 2000). Hauser et al, (2000) made use of RFLP analysis to clearly differentiate between three beet *Polerovirus* species, namely, *Beet mild yellowing virus* (BMV), *Brassica yellows virus* (BrYV) and *Beet chlorosis virus* (BChV). Although the study allowed for the accurate and reliable differentiation between the viral species, it is possible that RFLP analysis lacks sufficient sensitivity to differentiate between isolates of the same viral species.

Of the available techniques, nucleotide sequence analysis provides the highest level of resolution for CTV strains and population characterization. Usually selected regions of the genome are sequenced and subjected to phylogenetic analysis because full genome sequencing is costly and time consuming (Roy et al, 2005). In a review by Vigne et al, (2004), it is stated that a certain degree of variability in *Grapevine fanleaf virus* (GFLV) population was determined by RFLP and SSCP analyses, however an even higher degree of variability was suggested when comparative nucleotide sequencing analysis was utilised. These findings indicate that comparative sequencing analysis is superior to other methods for generating data for viral population and characterization studies. One of the potential disadvantages associated with comparative sequence analysis is primer-associated PCR selection (Vigne et al, 2004) of certain sequence types within a sample leading to the skewing of data, which does not truly represent the dominant sequence types within a viral population. Furthermore, mutations may be introduced into amplicons during the PCR reaction resulting in a distortion in the actual level of diversity within the population being characterized.

(2.5) Studies of population dynamics

The vast array of symptoms expressed by CTV strains is testament to the high variability within and between populations. Plant viruses are inherently heterogeneous (Garcia-Arenal et al, 2001) and CTV hosts contain a “quasispecies” population, which probably arise in part due to the error-prone properties of the RNA-dependant RNA-polymerase (Iglesias et al, 2008). Recombination events and the dispersal of infected tissue within and between regions also play a role in shaping CTV diversity (Moreno et al, 2008). Analysis of CTV populations are carried out to determine population structures of CTV within growing regions (Iglesias et al, 2008, Rubio et al, 2001) This information can be used to determine population complexity as well as which genotypes are present within regions and this knowledge can have an important influence on the CTV control measures that are put in place (Kong et al, 1999).

Phylogenetic analyses of either the 5' UTR sequences (Ayllón et al, 2001) or p23 gene (Sambade et al, 2003) are frequently used in phylogenetic analyses of CTV populations. Other 3' end coding regions have also been used (Iglesias et al, 2008). Five CTV genotypes are recognised when the 5' UTR sequence of a population is analysed and these are discussed under 2.2. Full genome sequences of these genotypes are placed on a phylogenetic tree when performing 5' UTR sequence analyses and perform the function of reference strains (*figure 3*). Full genome sequences of T318A (DQ151548), SY568 (AF001623), NUagA (AB046398) and VT (U56902) group within sequence type I (VT genotype, severe stem-pitting isolates). T30 (AF260651) and T385 (Y18420) group within sequence type II (T30 genotype, mild isolates). T36 (U16304), Qaha from Egypt (AY340974), Mexican CTV isolate (DQ272579) group as sequence type III (T36 genotype, intermediate characteristics) (Moreno et al, 2008). According to Roy and Brankisy. (2010), the B165 isolate groups as a separate group on a phylogenetic tree and is now classified a sequence type IV for 5' UTR sequence analyses. Phylogenetic analysis of the 3' p23 gene region shows the same isolates grouping together.

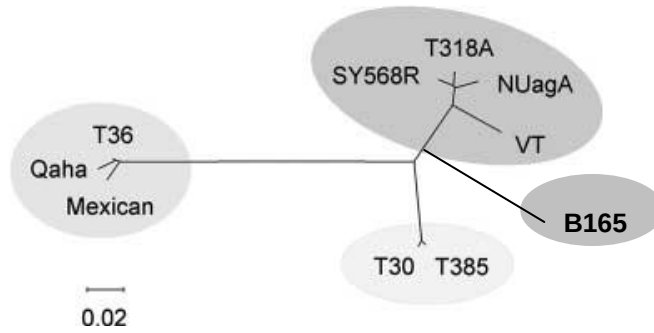


Figure 3: Grouping of the three sequence types obtained from 5' UTR sequence analysis. The newly discovered sequence type IV of B165 groups at a point between the T30 and T36 groups (Moreno et al, 2008). The B165 isolate forms a unique grouping whose approximate position is shown between the T36 and T30 groups.

Literature Cited

- Ayllón M.A., Lopez C., Navas-Castillo J., Garnsey S.M., Guerri J., Flores R. and Moreno P. (2001). 'Polymorphism of the 5' terminal region of *Citrus tristeza virus* (CTV) RNA: Incidence of three sequence types in isolates of different origin and pathogenicity'. Archives of Virology vol. 146 pp27-40
- Beachy R.N. (1997). Mechanisms and applications of pathogen-derived resistance in transgenic plants. Current Opinion in Biotechnology vol. 8 pp215-220
- Bertolini E., Moreno A., Capote N., Olmos A., de Luis A., Vidal E., Pérez-Panadés and Cambra M. (2008) 'Quantitative detection of *Citrus tristeza virus* in plant tissues and single aphids by real-time RT-PCR' European Journal of Plant Pathology vol. 120 pp177-188
- Cambra M., Gorris M.T., Marroquin C., Roman M.P., Olmos A., Martínez M.C., de Mendoza A.H., López A. and Navarro L. (2000). 'Incidence and epidemiology of *Citrus tristeza virus* in the Valencian Community of Spain'. Virus Research vol. 71 pp85-95
- Cerni S., Ruscic J., Nolasco G., Gatin Z., Krajacic M. and Skoric D. (2008) 'Stem pitting and seedling yellows symptoms of *Citrus tristeza virus* infection may be determined by minor sequence variants'. Virus Genes vol. 36 pp241-249
- Chandler L.J. and Nordoff N.G. (1999). Identification of genetic variation among St. Louis encephalitis virus isolates, using single-strand conformation polymorphism analysis. Journal of Virological Methods vol. 80 pp169-178
- Dolja V.V., Kreuze J.F. and Valkonen J.P.T. (2006) 'Comparative and functional genomics of closteroviruses'. Virus Research vol. 117 pp38-51
- Domingo E. (2002). Quasispecies Theory in Virology. Journal of Virology vol. 76 pp463-465

Fagoaga C., López C., Moreno P., Navarro L., Flores R and Peña L. (2005). Viral-Like Symptoms Induced by the Ectopic Expression of the *p23* Gene of *Citrus tristeza virus* Are Citrus Specific and Do Not Correlate with the Pathogenicity of the Virus Strain. *Molecular Plant-Microbe Interactions* vol. 18 pp435-445

Folimonova S.Y., Robertson C.J., Shilts T., Folimonov A.S., Hilf M.E., Garnsey S.M. and Dawson W.O. (2010). Infection with Strains of *Citrus Tristeza Virus* Does Not Exclude Superinfection by Other Strains of the Virus. *Journal of Virology* vol. 84 pp1314-1325

Fulton F.W. (1978). Superinfection by Strains of Tobacco Streak Virus. *Virology* vol. 85 pp1-8

Fulton F.W. (1986). Practices and precautions in the use of cross protection for plant virus disease control. *Annual Reviews in Phytopathology* vol 24 pp67-81

Gal-On A. and Shibolet Y.M. (2005). Cross-protection, p. 261-288. *In* G. Loebenstein and J.P. Carr (ed.), *Natural resistance mechanisms of plants to viruses*. Springer, Dordrecht, The Netherlands.

Garcia-Arenal F., Fraile A. and Malpica J.M. (2001). Variability and Genetic Structure of Plant Virus Populations. *Annual Reviews in Phytopathology* vol. 39 pp157-186

Ghorbel R., López C., Fagoaga C., Moreno P., Navarro L., Flores R and Peña L. (2001). Transgenic citrus plants expressing the citrus tristeza virus *p23* protein exhibit viral-like symptoms. *Molecular Plant Pathology* vol. 2 pp27-36

Gomes C.P.C., Nagata T., de Jesus W.C., Nito C.R.B., Pappas G.J. and Martin D.P. (2008). Genetic variation and recombination of RdRp and HSP 70h genes of *Citrus tristeza virus* isolates from orange trees showing symptoms of citrus sudden death disease. *Virology Journal* vol 5 pp1-7

Gonsalves D., Purcifull D.E. and Garnsey S.M. (1978). Purification and Serology of Citrus Tristeza Virus. *Phytopathology* 68 pp553-559

Gowda S., Satyanarayana T., Davis C.L., Navas-Castillo J., Albiach-Marti M.R., Mawassi M., Valkov N., Bar-Joseph M., Moreno P and Dawson W.O. (2000). The p20 Gene Product of *Citrus Tristeza Virus* Accumulates in the Amorphous Inclusion Bodies. *Virology* 274 pp246-254

Hauser S., Weber C., Vetter G., Stevens M., Beuve M. and Lemaire O. (2000). Improved detection and differentiation of poleroviruses infecting beet or rape by multiplex RT-PCR. *Journal of Virological Methods* vol. 89 pp11-21

Herron C.M., Mirkov T.E., da Graca J.V. and R.F. Lee (2006). *Citrus tristeza virus* transmission by the *Toxoptera citricida* vector: In vitro acquisition and transmission and infectivity immunoneutralization experiments. *Journal of Virological Methods* vol. 134 pp205–211

Hilf M.E., Karasev A.V., Pappu H.R., Gumpf D.J., Niblett C.L. and Garnsey S.M (1995). Characterization of Citrus Tristeza Virus Subgenomic RNAs in Infected Tissue. *Virology* vo. 208 pp576-582

Hilf M.E., Karasev A.V., Albiach-Marti M.R., Dawson W.O. and Garnsey S.M. (1999). Two Paths of Sequence Divergence in the Citrus Tristeza Virus Complex. *Phytopathology* vol. 89 pp336-342

Hilf M.E., Mavrodieva V.A. and Garnsey S.M. (2005). Genetic Marker Analysis of a Global Collection of Isolates of *Citrus tristeza virus*: Characterization and Distribution of CTV Genotypes and Association with Symptoms. *Phytopathology* vol.95 pp909-917

Himmelbach A., Chapdelaine Y. and Hohn T. (1996). Interaction between Cauliflower Mosaic Virus Inclusion Body Protein and Capsid Protein: Implications for Viral Assembly. *Virology* vol. 217 pp147-157

Iglesias N.G., Gago-Zachert S.P., Robledo G., Costa N., Plata M.I., Vera O., Grau O. and Semorile L.C. (2008). Population structure of *Citrus tristeza virus* from field Argentinean isolates. *Virus Genes* vol. 36 pp199-207

Jiang B., Hong N., Wang G., Hu J., Zhang J., Wang C., Liu Y and Fan X. (2008). Characterization of *Citrus tristeza virus* strains from southern China based on analysis of restriction patterns and sequences of their coat protein genes. *Virus Genes* vol. 37 pp185-192

Kalvatchev Z., Walder R., Garzaro D. and Barrios M. (2000). Detection of Genetic Diversity Among Bovine Immunodeficiency Virus Populations by Single-Strand Conformation Polymorphism Analysis. *Viral Immunology* vol. 13 pp373-381

Karasev A.V., Boyko V.P., Gowda S., Nikolaeva O.V., Hilf M.E., Koonin E.V., Niblett C.L., Cline K., Gumpf D.J., Lee R.F., Garnsey S.M., Lewandowski D.J and Dawson W.O. (1995). 'Complete Sequence of the Citrus Tristeza Virus RNA Genome'. *Virology* vol. 208 pp511-520

Kong P., Rubio L., Polek M. and Falk B.W. (1999). Population Structure and Genetic Diversity within California *Citrus tristeza virus* (CTV) Isolates. *Virus Genes*. vol. 21 pp139-145

Lindbo J.A., Silva-Rosales L., Proebsting W.M. and Dougherty W.G. (1993). Induction of a Highly Specific Antiviral State in Transgenic Plants: Implications for Regulation of Gene Expression and Virus Resistance. *The Plant Cell* vol. 5 pp1749-1759

López C., Navas-Castillo J., Gowda S., Moreno P. and Flores R. (2000). The 23-kDa Protein Coded by the 3'-Terminal Gene of Citrus Tristeza Virus Is an RNA-Binding Protein. *Virology* vol. 269 pp462-470

Lu R., Folimonov A., Shintaku M., Wan-Xiang L., Falk B.W., Dawson W.O and Ding S. (2004). Three distinct suppressors of RNA silencing encoded by a 20-kb viral RNA genome. *Proceedings of the National Academy of Sciences* vol. 101 pp15742-15747

Marroquin C., Olmos A., Gorris M.T., Bertolini E., Martinez M.C., Carbonell E.A., de Mendoza A.H. and Cambra M. (2004). Estimation of the number of aphids carrying Citrus tristeza virus that visit adult trees. *Virus Research* vol 100 pp 101-108

Martin S., Sambade A., Rubio L., Vives M.C., Moya P., Guerri J., Elena S.F. and Moreno P. (2009). Contribution of recombination and selection to molecular evolution of *Citrus tristeza virus*. *Journal of General Virology* vol 90 pp1527-1538

Moreno P., Ambros S., Albiach-Marti M.R., Guerri J. and Pena L. (2008). '*Citrus tristeza virus*: A pathogen that changed the course of the citrus industry'. *Molecular Plant Pathology* vol. 9 pp251-268

Niblett C.L., Genc H., Cevik B., Halbert S., Brown L., Nolasco G., Bonacalza B., Manjunath K.L., Febres V.J., Pappu H.R., and Lee R.F. (2000). Progress on strain differentiation of *Citrus tristeza virus* and its application to the epidemiology of citrus tristeza disease. *Virus Research* vol. 71 pp97-106

Palacio A. and Duran-Vila N. (1999). Single-strand conformation polymorphism (SSCP) analysis as a tool for viroid characterization. *Journal of Virological Methods* vol. 77 pp27-36

Prins M., Laimer M., Noris E., Schubert J., Wassenegger M and Tepfer M. (2008). Strategies for antiviral resistance in transgenic plants. *Molecular Plant Pathology* vol. 9 pp73-83

Roy A., Manjunath K.L. and Bransky R.H. (2005). Assessment of sequence diversity in the 5'-terminal region of *Citrus tristeza virus* from India. *Virus Research* vol.113 pp132-142

Roy A. and Brlansky R.H. (2010). Genome Analysis of an Orange Stem Pitting *Citrus Tristeza Virus* Isolate Reveals a Novel Recombinant Genotype. *Virus Research* vol 151 pp118-130

Rubio L., Ayllón M.A., Guerri J., Pappu H., Niblett C and Moreno P. (1996). Differentiation of citrus tristeza virus (CTV) isolates by single-strand conformation polymorphism analysis of the coat protein gene. *Annals of Applied Biology* vol. 129 pp479-489

Rubio L., Ayllón M.A., Kong P., Fernandez A., Polek M., Guerri J., Moreno P. and Falk B.W. (2001). Genetic Variation of *Citrus Tristeza Virus* Isolates from California and Spain: Evidence for Mixed Infections and Recombination. *Journal of Virology*. vol. 75 pp8054-8062

Ruiz-Ruiz S., Moreno P., Guerri J. and Ambrós S. (2006). The complete nucleotide sequence of a severe stem pitting isolate of *Citrus tristeza virus* from Spain: comparison with isolates from different origins. *Archives of Virology* vol. 151 pp387-398

Ruiz-Ruiz S., Moreno P., Guerri J. and Ambros S. (2007) 'A real-time RT-PCR assay for detection and absolute quantitation of *Citrus tristeza virus* in different plant tissues'. *Journal of Virological Methods* vol. 145 pp96-105

Sambade A., Lopez C., Rubio L., Flores R., Guerri J. and Moreno P. (2003). Polymorphism of a specific region in gene *p23* of *Citrus tristeza virus* allows for the discrimination between mild and severe isolates. *Archives of Virology* vol. 148 pp2325-2340

Sanz-Ramos M., Segundo F.D., Escarmis C., Domingo E. and Sevilla N. (2008). Hidden Virulence Determinants in a Viral Quasispecies In Vivo. *Journal of Virology* vol. 82 pp10465-10476

Satyanarayana T., Gowda S., Mawassi M., Albiach-Marti M.R., Ayllón M.A., Robertson C., Garnsey S.M. and Dawson W.O. (2000). Closterovirus Encoded HSP70 Homolog and p61 in Addition to Both Coat Proteins Function in Efficient Virion Assembly. *Virology* vol. 278 pp253-265

Satyanarayana T., Bar-Joseph M., Mawassi M., Albiach-Marti M.R., Ayllon M.A., Gowda S., Hilf M.E., Moreno P., Garnsey S.M., and Dawson W.O. (2001). Amplification of *Citrus Tristeza Virus* from a cDNA Clone and Infection of *Citrus* Trees. *Virology* vol. 280 pp87-96

Satyanarayana T., Gowda S., Ayllón M.A., Albiach-Marti M.R., Rabindran S. and Dawson W.O. (2002). The p23 Protein of *Citrus tristeza virus* controls asymmetrical RNA accumulation. *Journal of Virology* vol. 76 pp473-483

Shi N., Chen J., Wilson T.M.A., Macfarlane S.A., Antoniw J.F. and Adams M.J. (1996). Single-strand conformation polymorphism analysis of RT-PCR products of UK isolates of barley yellow mosaic virus. *Virus Research* vol. 44 pp1-9

Tatineni S., Robertson C.J., Garnsey S.M., Bar-Joseph M., Gowda S. and Dawson W.O. (2008) Three genes of *Citrus tristeza virus* are dispensable for infection and movement throughout some varieties of citrus trees. *Virology* 376 pp297-307

van Vuuren S.P., Collins R.P. and da Graca J.V. (1993). 'Evaluation of Citrus Tristeza Virus Isolates for Cross Protection of Grapefruit in South Africa'. *Plant Disease* vol. 77 pp24-27

van Vuuren S.P. and Manicom B.Q. (2005). The Response of Star Ruby Grapefruit to Different *Citrus tristeza virus* isolates. In: *Proc 16th Conf. IOCV*, 112-116 IOCV, Riverside, California

Vigne E., Bergdoll M., Guyader S. and Fuchs M. (2004). Population structure and genetic variability within isolates of *Grapevine fanleaf virus* from a naturally infected vineyard in France: evidence for mixed infection and recombination. *Journal of General Virology* vol. 85 pp2435-2445

Vives M.C., Rubio L., López C., Navas-Castillo J., Albiach-Marti M.R., Dawson W.O., Guerri J., Flores R. and Moreno P. (1999). The complete genome sequence of the major components of a mild citrus tristeza virus isolate. *Journal of General Virology* vol 80 pp811-816

Xu W.X., Hong N., Zhang J.K. and Wang G.P. (2006). Improving the sensitivity of single-strand conformation polymorphism (SSCP) to study the variability of PLMVd. *Journal of Virological Methods* vol. 135 pp276-28

Yoon J.Y., Ahn H.I., Kim M., Tsuda S. and Ryu K.I. (2006). '*Pepper mild mottle virus* pathogenicity determinants and cross protection effect of attenuated mutants in pepper'. *Virus Research* vol. 118 pp23-30

Chapter 3

MATERIALS AND METHODS

(3.1) Experimental block and sampling

Thirteen samples were collected from the ARC experimental block Malelane H7 (GPS co-ordinates 25°27'55.55"S; 31°34'58.65"E) (van Vuuren and Manicom, 2005) from Star Ruby and Flame trees. Cultivar, pre-immunising source population and sample replicate numbers are listed in *Table 1*.

Table 1: List of replicate samples collected from the ARC experimental block Malelane H7

Cross-protecting source	Cultivar	Replicate sample numbers
GFMS 12	Star Ruby	301; 302; 303; 313
GFMS 12	Flame	304; 305; 306
GFMS 35	Star Ruby	307; 308; 309
GFMS 35	Flame	310; 311; 312

Four replicates of the GFMS 12 on Star Ruby combination were taken because of the particularly severe cross-protection breakdown symptoms associated with these plants. Samples were collected during the hot summer month of February 2009. Young shoots with green bark were collected with leaves still attached. 3 to 5 stems were taken per tree, at approximately equal intervals around the circumference of the tree.

Material used for the characterization of GFMS 12 and GFMS 35 pre-immunising sources were collected from Star Ruby and Mexican Lime plants maintained in an insect-free greenhouse. The temperatures within the greenhouse were not strictly regulated and varied according to the prevailing climatic conditions.

(3.2) RNA extractions

All collected samples were subjected to total RNA extraction. Green bark from young shoots and leaf midribs were macerated using liquid nitrogen and a mortar and pestle. A total RNA extraction was performed on the powdered

plant tissue using a Promega® SV Total RNA extraction kit (Promega, Madison, WI, USA), according to the manufacturers protocol.

(3.3) cDNA synthesis of the A-fragment and p23 gene region

12 µl of total RNA extract was used as template for production of cDNA of the A-fragment and the p23 gene region, in the presence of 50 pmol of their respective antisense primers, A-R and PM51 (Table 2), 10U *Avian myeloblastosis virus* reverse transcriptase (AMV-RT) (Roche, Mannheim, Germany), 5U RNase inhibitor (Roche, Mannheim, Germany), 1x RT buffer (Roche, Mannheim, Germany) and 0.1 mM dNTP mix (Promega, Madison, WI, USA). Reverse transcription was carried by incubation at 42°C for 1 hour (Rubio et al, 2001, modified by Scott, unpublished data). In cases where the A-region proved difficult to amplify, the cDNA step was carried out at 48°C to minimize the formation of secondary structures, for 1.5 hours (Scott, personal communication)

(3.4) PCR amplification of the A-fragment and p23 gene region

PCR amplification of the p23 gene and the A-fragment was carried out by using 10µl of the cDNA as template to a reaction mix containing 5µl of 1x PCR reaction buffer (Bioline, London, UK), 2.5mM MgCl₂ (Bioline, London, UK), 0.14mM dNTP mix (Promega, Madison, WI, USA), 2µM forward primer, 2µM reverse primer (Table 2), 2.5 U DNA polymerase (Bioline, London, UK), 2µl of 20µg/µl Bovine serum albumin (BSA) (Promega, Madison, WI, USA). PCR grade water (Sigma-Aldrich, St. Louis, MO, USA) was added to a final reaction volume of 50µl. The PCR reaction conditions used were: 1 cycle of 92°C for 2min. 40 cycles of 92°C for 30s, 55°C for 45s and 72°C for 1min. 1 cycle of 72°C for 10min (Rubio et al, 2001, modified by Scott, unpublished data).

Table 2: Primer sequences used for the amplification of the p23 gene and A-fragment. PM50 and PM51 were used to amplify the p23 gene and A-forward and A-reverse were used to amplify the A-region

Primer name	Sequence 5'-3'	Amplicon size (bp)	Reference
PM50	ACTAACTTTAATTCTGAACA	697	Sambade et al, (2003)
PM51	AACTTATTCCGTCCACTTC	697	Sambade et al, (2003)
A-forward	ACGTGTTCTGTGAAACGCGG	528	Rubio et al, (2001)
A-reverse	GTCGATAACTCGACAAACGAGC	528	Rubio et al, (2001)

(3.5) Cloning of the A-fragment and p23 gene region PCR products

RT-PCR products of the A-fragment and p23 gene region were run on a 1% agarose gel and purified using a Promega, Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI), according to the manufacturers instructions. Concentrations of the purified DNA products were determined by UV spectroscopy using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Purified PCR products of the p23 gene and A-fragment were cloned into the p-GEM T Easy® vector (Promega, Madison, WI, USA) according to the manufacturers specifications and used to transform competent *E. coli* JM109 cells (Promega, Madison, WI, USA) according to the manufacturers specifications. The transformants were grown on Luria-Bertani (LB) agar plates, containing 50µg/ml ampicillin (amp) (United States Biochemical, Cleveland, OH, USA) and augmented with 30µl of 20mM bromo-chloro-indolyl-galactopyranoside (X-gal) solution (Fermentas, Vilnius, Lithuania) and 100µl of a 100mM isopropyl β -D-1 thiogalactopyranoside (IPTG) solution (Sigma-Aldrich, St. Louis, MO, USA). Potentially recombinant

colonies were selected using the blue/white screening method. The selected colonies were grown in LB broth containing 50µg/ml amp, overnight, at 37°C in a shaking incubator (200rpm). 100 recombinant colonies were chosen per field sample analysed for the A-region, 50 per field sample analysed for the p23 gene region and between 60 and 150 recombinant colonies were chosen per gene sequence analysed per greenhouse samples collected.

(3.6) Recombinant plasmid extraction, verification and sequencing

Each of the selected recombinant colonies was subjected to the alkaline lysis plasmid miniprep (Sambrook 2001), in order to isolate potentially recombinant plasmids. After extraction, each recombinant plasmid was tested to ensure that they contained an insert of the expected size. A PCR protocol, making use of the vector specific primers T7 (5'- TAA TAC GAC TCA CTA TAG GG-3') and SP6 (5'- ATT TAG GTG ACA CTA TAG AA -3') (Promega, pGEM®-T and pGEM®-T Easy Vector Systems, technical manual), were used to amplify the insert between these two vector specific promoter regions. PCR reaction conditions were the same as those described under 3.4. The PCR products were run on a 1% agarose gel and plasmids whose PCR products were of the correct size were selected for the sequencing of its insert. 60 clones from each A-region clone sets of samples collected from the field and 30 clones from each field p23 gene region clone sets of field collected samples were sequenced and between 60 and 150 clones were sequenced for each of the greenhouse samples collected. Unidirectional Sanger sequencing was performed on each of the selected clones. Sequencing reactions were set-up in the reverse direction, using the vector specific SP6 primer. Each sequencing reaction contained the following reaction cocktail: 1µl BigDye® Terminator mix v3.1 (Applied Biosystems, Foster City, CA, USA), 2.25µl 5x BigDye® v3.1 sequencing buffer (Applied Biosystems, Foster City, CA, USA), 0.75µl SP6 primer (2µM) and molecular grade water (Sigma-Aldrich, St. Louis, MO, USA) to a final reaction volume of 10µl. Sequencing cycling conditions used were as follows: 94°C for 1 minute, 25 cycles of 94°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes. The sequences were submitted to the African Centre for Gene Technologies, Automated Sequencing Facility, Department of Genetics, University of Pretoria, South Africa and sequenced

using an ABI Prism® 3130XL Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

(3.7) Phylogenetic analysis

Screening of sequence identities was done using the National Centre for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (<http://www.ncbi.nlm.nih.gov>) (Altschul et al, 1990). The CLC Main Workbench 5 software package (Aarhus, Denmark) was used to view the sequence chromatograms, evaluate their quality and completeness, correct any errors therein and to produce the reverse complement of each sequence. Complete reverse complement sequences were aligned using the CLUSTAL W alignment software (EBI, Cambridgeshire, England).

Cognate regions from ten fully sequenced CTV strains were used as reference sequences in each alignment. The reference sequences were retrieved from the NCBI database and their Genbank accession numbers (in brackets) are listed as follows: B165 (EU076703) (Roy and Bransky, 2010), Mexican CTV isolate (DQ272579) (Quiroz et al, unpublished data), NUagA (AB046398) (Suastika et al, unpublished data), NZRB-TH28 (FJ525433) (Harper et al, 2010), Qaha from Egypt (AY340974) (Abdelmaksoud and Gamal El-din, unpublished data), SY568 (AF001623) (Yang et al, 1999), T30 (AF260651) (Alibiach-Marti et al, 2000), T36 (U16304) (Pappu et al, 1994), T318A (DQ151548) (Ruiz-Ruiz et al, 2006), T385 (Y18420) (Vives et al, 1999) and VT (U56902) (Mawassi M et al, 1993). In the case of the A-fragment alignments the VT defective RNA (AY206452) (Che et al, 2002) was also used as a reference sequence. In the case of the p23 gene region analyses, the HA16-5 (GQ454870) (Melzer et al, 2010) full genome was included as a reference. With the exception of AY206452 only fully sequenced CTV genomes were used as references. The number of references used was considered to be sufficient for the purposes of this study, where the objectives were to get a broad overview of the genotypes present in the field and within the cross-protecting sources. Previous studies have used a similar number of references to the current study (Barzegar et al, 2005; Iglesias et al, 2008; Melzer et al, 2010; Roy et al, 2005). Vector sequence still present in each

alignment was trimmed using the BioEdit sequence alignment editor (Isis Pharmaceuticals, Carlsbad, CA, USA). Phylogenetic dendrograms were produced, using the MEGA 4.1 software package (Tamura et al, 2007), using the p-distance model (Nei and Kumar, 2000). Dendrograms were produced as unrooted, neighbour-joining trees, with a bootstrap test of phylogeny of 1000 replicates.

Clones on the A-region trees were grouped into six groups, namely 1a (VT-like), 1b (T30-like), 1c (Unknown, for sequences that did not fit into any of the conventional groups), 2 (B165-like) 3a (Resistance breaking-like) and 3b (T36-like). Clones on the p23 gene region trees were grouped into four groups, namely, 1a (VT-like), 1b (HA15-6-like), 2 (T36-like) and 3 (T30-like). Estimations of recombination events were determined using the Recombination detection program v1.08 (Martin et al, 2005), running the GENECONV (Padidam et al, 1999) algorithm

Literature Cited

- Alibiach-Marti M.R., Mawassi M., Gowda S., Satyanarayana T., Hilf M.E., Shanker S. Almira E.C., Vives M.C., López C., Guerri J., Flores R., Moreno P., Garnsey S.M. and Dawson W.O. (2000). 'Sequences of *Citrus tristeza virus* Separated in Time and Space are Essentially Identical'. *Journal of Virology* vol. 74 pp6856-6865
- Altschul S.F., Gish W., Miller W., Myers E.W. and Lipman D.J. (1990). Basic Local Alignment Search Tool. *Journal of Molecular Biology* vol. 215 pp403-410
- Barzegar A., Sohi H.H., Rahimian H. (2005). Comparative Sequence Analysis of Coat Protein Gene of Iranian *Citrus tristeza virus* Isolates. *Journal of Phytopathology* vol. 153 pp457-463
- Che X., Mawassi M. and Bar-Joseph M. (2002). A novel class of large and infectious defective RNAs of *Citrus tristeza virus*. *Virology* vol. 298 pp133-145
- Harper S.J., Dawson T.E. and Pearson M.N. (2010). Isolates of *Citrus tristeza virus* that overcome *Poncirus trifoliata* resistance comprise a novel strain. *Archives of Virology* vol. 155 pp471-480
- Iglesias N.G., Gago-Zachert S.P., Robledo G., Costa N., Plata M.I., Vera O., Grau O. and Semorile L.C. (2008). Population structure of *Citrus tristeza virus* from field Argentinean isolates. *Virus Genes* vol. 36 pp199-207
- Martin D.P., Williamson C. and Posada D. (2005). RDP2: recombination detection and analysis from sequence alignments. *Bioinformatics* vol. 21 pp260-262
- Mawassi,M., Gafny,R. and Bar-Joseph,M. (1993). Nucleotide sequence of the coat protein gene of citrus tristeza virus: comparison of biologically diverse isolates collected in Israel. *Virus Genes* vol. 7 pp265-275

Melzer M.J., Borth W.B., Sether D.M., Ferreira S., Gonsalves D. and Hu J.S. (2010). Genetic diversity and evidence for recent modular recombination in Hawaiian *Citrus tristeza virus*. *Virus Genes* vol. 40 pp111-118

Nei M. and Kumar S. (2000). *Molecular Evolution and Phylogenetics*. Oxford University Press, New York.

Padidam M., Sawyer S. and Fauquet C.M. (1999). Possible emergence of new geminiviruses by frequent recombination. *Virology* vol. 265 pp218-225

Pappu, H.R., Karasev, A.V., Anderson, E.J., Pappu, S.S., Hilf, M.E., Febres, V., Eckloff, R.M.G., McCaffery, M., Boyko, V., Gowda, S., Dolja V.V. and Koonin E.V. (1994). Nucleotide sequence organisation of eight 3' open reading frames of the citrus tristeza closterovirus genome. *Virology* vol. 199 pp35-46

Roy A., Manjunath K.L. and Bransky R.H. (2005). Assessment of sequence diversity in the 5'-terminal region of *Citrus tristeza virus* from India. *Virus Research* vol 113 132-142

Roy A. and Bransky R.H. (2010). Genome Analysis of an Orange Stem Pitting *Citrus Tristeza Virus* Isolate Reveals a Novel Recombinant Genotype. *Virus Research* vol. 151 pp118-130

Rubio L., Ayllón M.A., Kong P., Fernandez A., Polek M., Guerri J., Moreno P. and Falk B.W. (2001). Genetic Variation of *Citrus Tristeza Virus* Isolates from California and Spain: Evidence for Mixed Infections and Recombination. *Journal of Virology*. vol. 75 pp8054-8062

Ruiz-Ruiz S., Moreno P., Guerri J. and Ambrós S. (2006). The complete nucleotide sequence of a severe stem pitting isolate of *Citrus tristeza virus* from Spain: comparison with isolates from different origins. *Archives of Virology* vol. 151 pp387-398

Sambade A., Lopez C., Rubio L., Flores R., Guerri J. and Moreno P. (2003). Polymorphism of a specific region in gene *p23* of *Citrus tristeza virus* allows for the discrimination between mild and severe isolates. *Archives of Virology* vol. 148 pp2325-2340

Sambrook J. (2001). *Molecular Cloning: 'A laboratory manual'*. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York.

Tamura K, Dudley J, Nei M & Kumar S (2007) *MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0*. *Molecular Biology and Evolution* vol.24 pp1596-1599

van Vuuren S.P. and Manicom B.Q. (2005). The Response of Star Ruby Grapefruit to Different *Citrus tristeza virus* isolates. In: *Proc 16th Conf. IOCV*, 112-116 IOCV, Riverside, California

Vives M.C., Rubio L., López C., Navas-Castillo J., Albiach-Marti M.R., Dawson W.O., Guerri J., Flores R. and Moreno P. (1999). The complete genome sequence of the major components of a mild citrus tristeza virus isolate. *Journal of General Virology* vol 80 pp811-816

Yang Z.N., Mathews D.M., Dodds J.A. and Mirkov T.E. (1999). Molecular Characterization of an Isolate of Citrus Tristeza Virus that Causes Severe Symptoms in Sweet Orange. *Virus Genes* vol. 19 pp131-142

Chapter 4

RESULTS

(4.1) Visual symptom evaluation of sampled field plants

Four replicate samples were collected from Star Ruby trees pre-immunised with GFMS 12. Samples 302 and 303 showed symptoms of mild stem-pitting and decline but overall, appeared healthy with good leaf coverage. Samples 301 and 313 displayed severe stem-pitting and decline with 313 showing particularly severe stunting symptoms.

Samples 304, 305 and 306 from Flame trees pre-immunised with GFMS 12 all showed symptoms of mild stem-pitting. 304 and 306 displayed decline symptoms with stunting noted on 304.

Mild stem-pitting was observed on samples 307 and 309 from Star Ruby trees pre-immunised with GFMS 35 but overall the plants appeared healthy with dense leaf coverage. Sample 308 however showed symptoms of severe decline and stem-pitting.

Samples 311 and 312, from Flame trees pre-immunised with GFMS 35 both showed symptoms of severe stem-pitting, severe decline and mild stunting. Sample 310 however showed only mild stem-pitting.

(4.2) PCR amplification of the A-region and p23 gene region of the field and greenhouse collected samples.

The A-region and p23 gene were successfully amplified from all samples using the PCR protocol described under 3.4. The PCR amplicons were purified and run on a gel (*Figure 4*) to confirm the correct band size and the presence of the amplicon before performing the cloning experiments.

(4.3) Incidence of strains within field samples.

It was assumed that the number of clones of a given strain reflects its relative proportion in the actual viral populations. Therefore, strain dominance in each of the field samples was determined by counting the number of clones grouping with each of the reference groups (3.9). Summary trees for each replicate combination are shown in *Figures 6 – 13*. VT-like strains showed the greatest incidence in all of the field samples, ranging from 78.7% to 100% of clones in the A-region (*Table 3*) and 75% to 100% of clones in the p23 gene region (*Table 4*). The presence of T30-like strains ranged from 0% to 20.8% of clones in the A-region and 0% to 24.1% of clones in p23 gene region.

Minor sequence types that did not fit into any of the conventional groups were observed during A-region analysis of four of the thirteen field samples (*Table 3*). BLAST analysis of these minor variants showed that they grouped closely (93% identity) to the sequence of AY652911 (Baja California p349) (Herrera-Isidron et al, unpublished data). The published sequence does not cover the entire A-region and was therefore not used as a reference sequence in the construction of dendrograms. The cognate region of the full genome reference sequence with which these anomalous sequences show the highest identity (87-88%) is with the A-region of the VT full genome reference sequence (U56902). The samples showing the anomalous sequences (301, 306, 312 and 313) correspond to plants in the field showing symptoms of severe decline.

Analysis of the p23 gene region of the field samples revealed another minor sequence type, which did not cluster with any of the reference groups. BLAST analysis revealed that these clones showed the highest identity with clones from a sample called HA 16-5 from a study by Melzer et al (2010). These sequence types were only observed in three out of the thirteen samples and constituted 3.6% to 8.3% of the population in these samples (*Table 4*). Analysis of the phylogenetic trees of the p23 gene region shows that these sequence types group at an intermediate position between the VT and T36 groups. This suggests that these are possible recombinants, a hypothesis that is supported by the study by Melzer et al, (2010). Appendix 8, *Figure 8.1*,

shows the phylogenetic tree of the p23 gene region of field sample 310. The figure supports the overall trend off VT-like dominance in the field. The possible recombinant sequence types can also be seen on this tree.

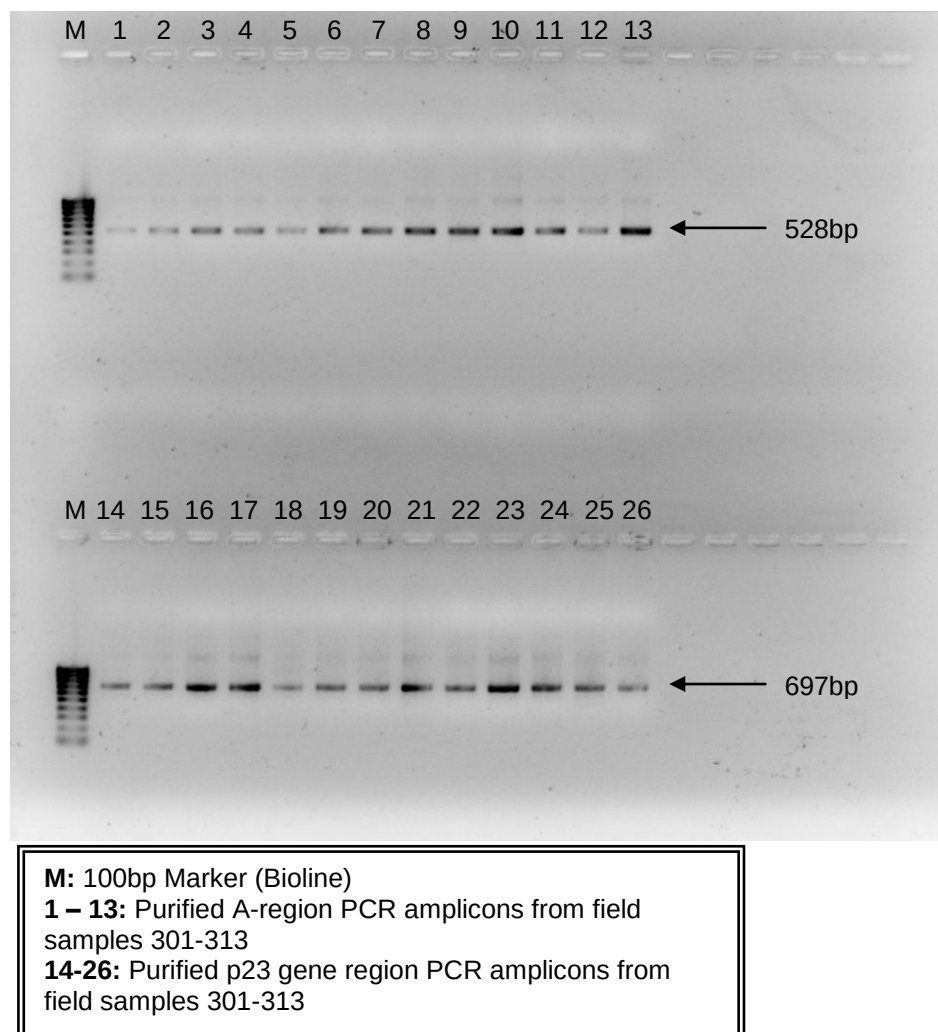


Figure 4: Agarose gel photograph of the purified PCR amplicons, amplified from the samples collected from the field

Table 3: Number of clones of the A-region analysis within each field sample, grouping with reference CTV strains. Values in brackets represent the percentage of the total number of clones sequenced.

Sample number	Gr 1a (VT)	Gr 1b (T30)	Gr 1c (Unk)	Gr 2 (B165)	Gr 3a (RB)	Gr 3b (T36)	Total seq's
Star Ruby pre-immunised with GFMS 12							
301	49 (90.7%)	0	5 (9.3%)	0	0	0	54
302	47 (100%)	0	0	0	0	0	47
303	49 (98%)	1 (2%)	0	0	0	0	50
313	47 (87%)	0	7 (13%)	0	0	0	54
Flame pre-immunised with GFMS 12							
304	47 (97.9%)	1 (2.1%)	0	0	0	0	48
305	51 (94.4%)	3 (5.6%)	0	0	0	0	54
306	42 (87.5%)	1 (2.1%)	5 (10.4%)	0	0	0	48
Star Ruby pre-immunised with GFMS 35							
307	37 (78.7%)	9 (19.1%)	1 (2.1%)	0	0	0	47
308	55 (100%)	0	0	0	0	0	55
309	56 (100%)	0	0	0	0	0	56
Flame pre-immunised with GFMS 35							
310	42 (79.2%)	11 (20.8%)	0	0	0	0	53
311	53 (98.1%)	1 (1.9%)	0	0	0	0	54
312	46 (88.5%)	0	6 (11.5%)	0	0	0	52

Table 4: Number of clones of the p23 gene region analysis within each field sample, grouping with reference CTV strains. Values in brackets represent the percentage of the total number of clones sequenced.

Sample number	Group 1a (VT)	Group 1b (HA15-6)	Group 2 (T36)	Group 3 (T30)	Total seq's
Star Ruby pre-immunised with GFMS 12					
301	19 (76%)	0	0	6 (24%)	25
302	25 (100%)	0	0	0	25
303	26 (96.3%)	0	0	1 (3.7%)	27
313	21 (91.3%)	0	0	2 (8.7%)	23
Flame pre-immunised with GFMS 12					
304	21 (75%)	1 (3.6%)	0	6 (21.4%)	28
305	9 (69.2%)	3 (23%)	0	1 (7.7%)	13
306	25 (89.3%)	2 (7.1%)	0	1 (3.6%)	28
Star Ruby pre-immunised with GFMS 35					
307	25 (100%)	0	0	0	25
308	28 (93.3%)	0	0	2 (6.7%)	30
309	22 (84.6%)	0	0	4 (15.4%)	26
Flame pre-immunised with GFMS 35					
310	18 (75%)	2 (8.3%)	0	4 (16.7%)	24
311	22 (84.6%)	0	0	4 (15.4%)	26
312	22 (75.9%)	0	0	7 (24.1%)	29

(4.4) Incidence of strains within the greenhouse collected reference samples

The incidence of CTV strains in the greenhouse collected reference samples yielded both expected and anomalous results when compared with the general trend of previously analysed results. The A-region analysis of the Star Ruby plant pre-immunised with Grapefruit mild-strain 35 (MS35.ML.A, *Table 5*), yielded the expected result of a predominance of VT-like strains (73.1%), which is in agreement with the field samples where VT-like strains

were present in the highest numbers. This was also supported by a 100% dominance of VT-like strains of the p23 gene region of Grapefruit mild-strain 12 on Star Ruby (MS12.SR.p23) (*Table 6*). The dynamics of the CTV populations shifted however when Mexican Lime was used as the host. In the case of the p23 gene region analysis of GFMS 12 on Mexican Lime (MS12.ML.p23) (*Table 6*) an almost even occurrence of VT-like (44.8%) and T30-like (55.2%) strains was observed. The A-region analysis of GFMS 12 on Mexican Lime (MS12.ML.A) (*Table 5*) yielded a 100% incidence of B165-like strains. This was “trend” was supported in the A-region analysis of Grapefruit mild-strain 35 on Mexican Lime (MS35.ML.A), which yielded a 94.5% incidence of B165-like strains. The discrepancy between the strain incidence observed between the A-region and p23 gene region analyses of the Mexican Lime samples is a result of the difference in reference contig grouping for these regions. The B165-like form a unique grouping in an A-region analysis and fall within the VT-like group upon p23 gene region analysis. Phylogenetic analysis of the remainder of the greenhouse collected samples revealed unexpected and anomalous results. The A-region analysis of Grapefruit mild-strain 12 on Star Ruby (*Table 5*) yielded results that did not adhere to the general “trend” of high VT-like dominance that was expected from Star Ruby as a host. The p23 gene region analysis of GFMS 35 on Mexican Lime (MS35.ML.p23) yielded an unexpected 48.4% incidence of strains grouping with the HA15-6 reference contig (GQ454870). The 51.6% incidence of T30-like strains compares well with the even distribution of T30-like and VT-like strains in MS12.ML.p23.

Table 5: Number of clones from the A-region analysis within each source population sample, collected from the greenhouse, grouping with reference CTV strains. The following abbreviations are used in the naming of the samples: MS12=Grapefruit mild-strain 12; MS35=Grapefruit mild-strain 35; ML=Mexican Lime; SR=Star Ruby and A=A-region.

Sample name	Gr 1a (VT)	Gr 1b (T30)	Gr 2 (B165)	Gr 3a (RB)	Gr 3b (T36)	Total seq's
MS12.ML.A	-	-	53 (100%)	-	-	53
MS12.SR.A	2 (5.3%)	20 (52.6%)	16 (42.1%)	-	-	38
MS35.ML.A	3 (5.5%)	-	52 (94.5%)	-	-	55
MS35.SR.A	87 (73.1%)	20 (16.8%)	4 (3.4%)	8 (6.7%)	-	119

Table 6: Number of clones from the p23 gene region analysis within each source population sample, collected from the greenhouse, grouping with reference CTV strains. The following abbreviations are used in the naming of the samples: MS12=Grapefruit mild-strain 12; MS35=Grapefruit mild-strain 35; ML=Mexican Lime; SR=Star Ruby and p23=p23 gene region.

Sample name	Group 1a (VT)	Group 1b (HA15-6)	Group 2 (T36)	Group 3 (T30)	Total seq's
MS12.ML.p23	26 (44.8%)	-	-	32 (55.2%)	58
MS12.SR.p23	60 (100%)	-	-	-	60
MS35.ML.p23	-	44 (48.4%)	-	47 (51.6%)	91
MS35.SR.p23	-	26 (37.7%)	-	43 (62.3%)	69

(4.5) Predicted amino acid sequence analysis of the p23 gene region

The predicted amino acid sequence at positions 78-80 of the CTV p23 protein can be used to differentiate between mild, severe and atypical isolates (*Figure 5*) (Sambade et al, 2003). Analysis of this amino acid motif of the clones produced from the field collected samples showed that specific amino acid sequences correspond to a very high degree (100% in most cases) with the genotypic groups present on the phylogenetic tree. The VT-like clones consistently displayed a Gly⁷⁸, Ser⁷⁹, Arg⁸⁰ (GSR) or Ala⁷⁸, Ser⁷⁹, Arg⁸⁰ (ASR)

motif. The presence of the Ser⁷⁹, Arg⁸⁰ motif is considered indicative severe strains and Sambade et al, (2003) discovered that the VT strain is severe, further backing up this data. The p23 78-80 amino acid motif of the T30-like clones consistently showed a sequence of Ala⁷⁸, Leu⁷⁹, Lys⁸⁰ (ALK), which is indicative of mild-strain isolates. This is further supported by the fact that Sambade et al, (2003) considered T30-like strains to be mild. Cloned sequence data grouping close to the HA 15-6 reference contig (Genbank accession: GQ454870) displayed a consistent amino acid sequence of Gly⁷⁸, Leu⁷⁹, Lys⁸⁰ (GLK) at the 78-80 position motif. According to Sambade et al, (2003) this sequence is indicative of atypical strains. Atypical strains are thought to produce symptoms of a variable nature. Some anomalous sequences were observed at positions 78-80 but their incidence was low and was probably the result of errors during PCR amplifications or sequencing reactions. The almost complete correlation of the predicted amino acid sequences to the groups present on the phylogenetic trees based on nucleotide sequence data of the field samples, further supports the reliability of these phylogenetic trees. Refer to Appendix 17 for a complete set of tables containing the sequences of the p23 78-80 amino acid motif of all of the clones relating to the field collected samples.

K	g..
T36	g..
Galego 50	g.r
C-268-2	g..
C-270-3	g..
C-269-6	.sr
Barão	.sr
VT	.sr
T388	.sr
T305	.sr
Cald-CB	.sr
Val-CB	.sr
Consensus	ALK
78-80	

Figure 5: Multiple protein alignment of the p23 gene of various strains of CTV (Sambade et al, (2003). The amino acid sequences at positions 78-80 potentially be used to assign CTV strains to either mild, severe or atypical groups.

The predicted amino acid sequences of the greenhouse collected reference samples yielded results which also support the grouping on the phylogenetic trees, however some differences, when compared with the field collected samples were observed. For example, the clones representing the VT-like

strains displayed only the Ala⁷⁸, Ser⁷⁹, Arg⁸⁰ (ASR) motif and there were no instances of the Gly⁷⁸, Ser⁷⁹, Arg⁸⁰ (GSR) motif in any of the greenhouse plant derived clones. The HA 15-6-like clones consistently displayed the Gly⁷⁸, Leu⁷⁹, Lys⁸⁰ (GLK), again suggesting that these clones are atypical according to the strain classification of Sambade et al, (2003). The T30-like strains consistently showed the Ala⁷⁸, Leu⁷⁹, Lys⁸⁰ (ALK) motif, which was also the case for the field-collected samples. A low number of unusual or anomalous motifs were observed. Some of these had unexpected stops within the reading frame and the resultant predicted amino acid sequences were probably incorrect due to errors during PCR amplification or during the sequencing reaction. Three clones from the reference population of GFMS 12 on Mexican Lime showed a unique 78-80 amino acid motif of Ala⁷⁸, Thr⁷⁹, Arg⁸⁰ (ATR), which may have been the result of a transversion of the first nucleotide of the 79th codon (TCT to ACT) during the PCR or sequencing reaction. Three other anomalous amino acid sequences were observed at this motif and these may all have resulted from erroneous transversions. The RSR sequence may have arisen due to GGT to CGT transversion of the 78th codon, the G*R (where * represents a stop codon) from a TCA to TAA transversion of the 79th codon and GSG from a transversion at the 80th codon from CGT to GGT. The correspondence of the predicted amino acid sequences to the grouping of the clones on the phylogenetic trees supports the reliability of these phylogenetic trees. Refer to Appendix 17 for a complete set of tables containing the sequences of the p23 78-80 amino acid motif of all of the clones relating to the greenhouse collected samples.

(4.6) Recombination analysis

The number of potential recombination events present within a particular cloned sequence was determined using the GENECONV algorithm (Padidam et al, 1999), since it is considered to be the most robust for estimating recombination events (Aaskov et al, 2007; Posada, 2002). Evidence of recombination was only found amongst A-region clones of samples 301, 302 and 313 and in the p23 gene region clones of samples 301, 303, 306, 308, 309, 310, 312, 313. Amongst the reference samples, evidence of recombination was only found in the A-region and p23 gene of GFMS 12 on

Mexican Lime and in the p23 gene of GFMS 35 on both Mexican Lime and Star Ruby. The BLAST tool was used to determine the recombinant strain compositions of individual clones displaying evidence of recombination using the RDP2 program (Martin et al, 2005). This data is listed in *Table 7* and 8.

Table 7: Field collected samples with clones showing evidence of recombination events using the GENECONV algorithm. The number of clones with identical recombination patterns are listed in brackets.

Sample number	Strain recombination pattern
A-region of 301	VT/T318A (39)
A-region of 312	VT/T385/T318A (10); T385/SY568 (18, 35, 58)
A-region of 313	SY568/T385 (14, 21, 37, 38, 57, 58)
p23 gene region of 301	T318A/VT/T318A (4, 14, 28); VT/HA 15-6/T318A (6, 24); T385/T318A (11)
p23 gene region of 303	T385/T318A (2); T385/NUagA (24);
p23 gene region of 306	T318A/NZRB TH-28 (30)
p23 gene region of 308	T318A/NZRB TH-28 (37)
p23 gene region of 309	T318A/VT/NUagA (14, 15, 16, 25); T318A/VT (36)
p23 gene region of 310	NZRB TH-28/T385 (3); T318A/T385 (4, 7, 9, 15); T318A/NZRB TH-28 (13); T318A/T30 (11)
p23 gene region of 312	T318A/NZRB TH-28/NUagA (1); T318A/T385 (7)
p23 gene region of 313	T318A/NZRB TH-28 (16); NZRB TH-28/T318A (18)

Table 8: Reference samples with clones showing evidence of recombination events using the GENECONV algorithm. Clones with identical recombination patterns are listed in brackets.

Sample number	Strain recombination pattern
A-region of GFMS 12 on Mexican Lime	B165/NZRB TH-28 (56)
p23 gene region of GFMS 12 on Mexican Lime	VT/NZRB TH-28/VT (32); VT/NZRB TH-28/VT (70)
p23 gene region of GFMS 35 on Mexican Lime	HA 15-6/NZRB TH-28 (2); NZRB TH-28/HA 15-6/NZRB TH-28 (25);
p23 gene region of GFMS 35 on Star Ruby	NZRB TH-28/HA 15-6 (2, 3, 7, 9, 10, 11, 13, 14, 16, 17, 19, 25, 30, 31, 35, 37, 38, 40, 47, 48, 50, 51, 53, 56, 58, 59, 62, 69, 70, 74);

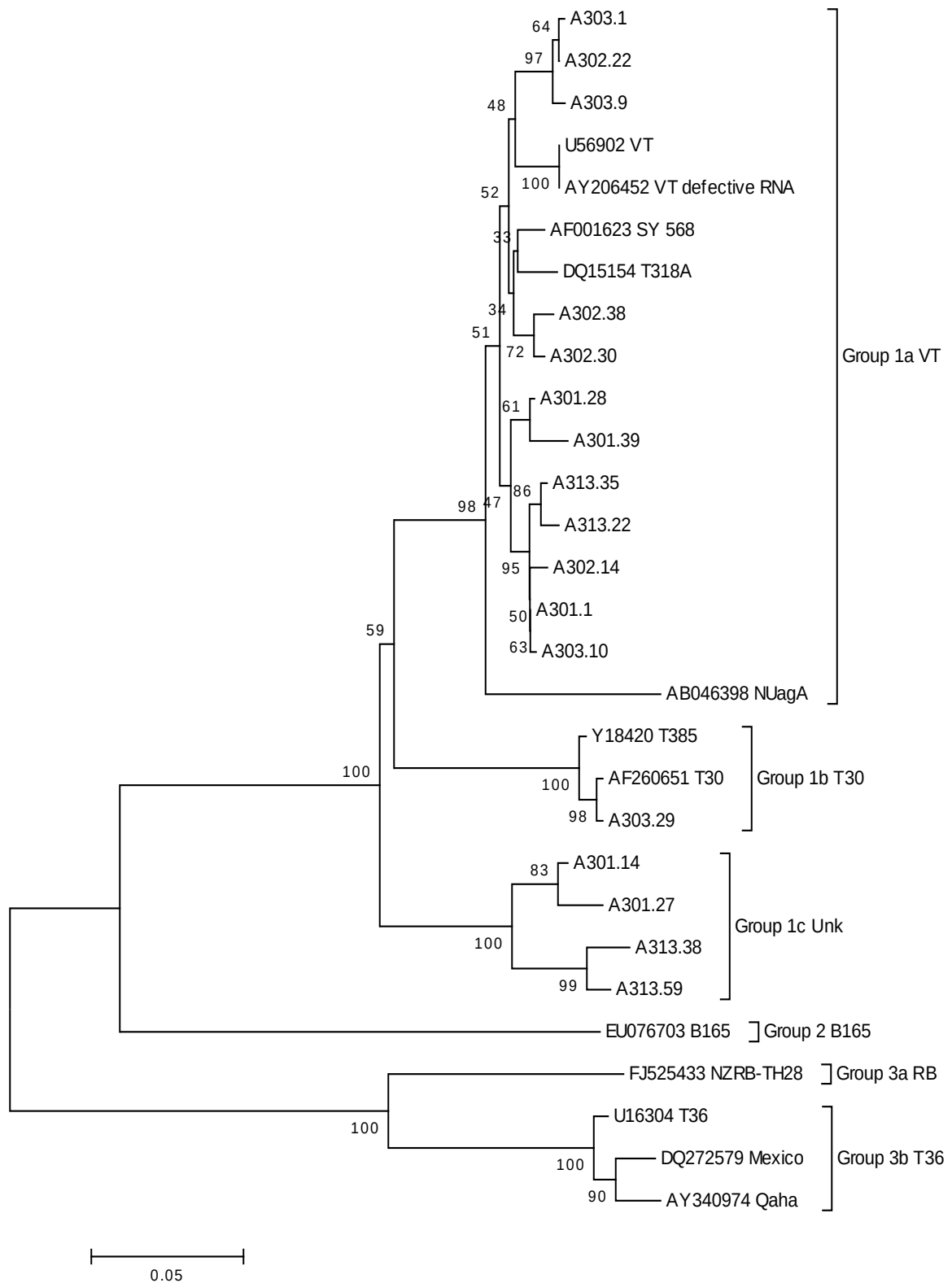


Figure 6: Summary tree for the A-region of field sample replicates of GFMS 12 on Star Ruby produced from the fundamental phylogenetic trees shown in Appendix 1. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence). Clone numbers are representative of samples and specific clone numbers. For example 301 is the sample number and .14 denotes a specific clone. The clones within this tree represent a group of clones showing at least 99% similarity to those present in groups on the fundamental phylogenetic trees.

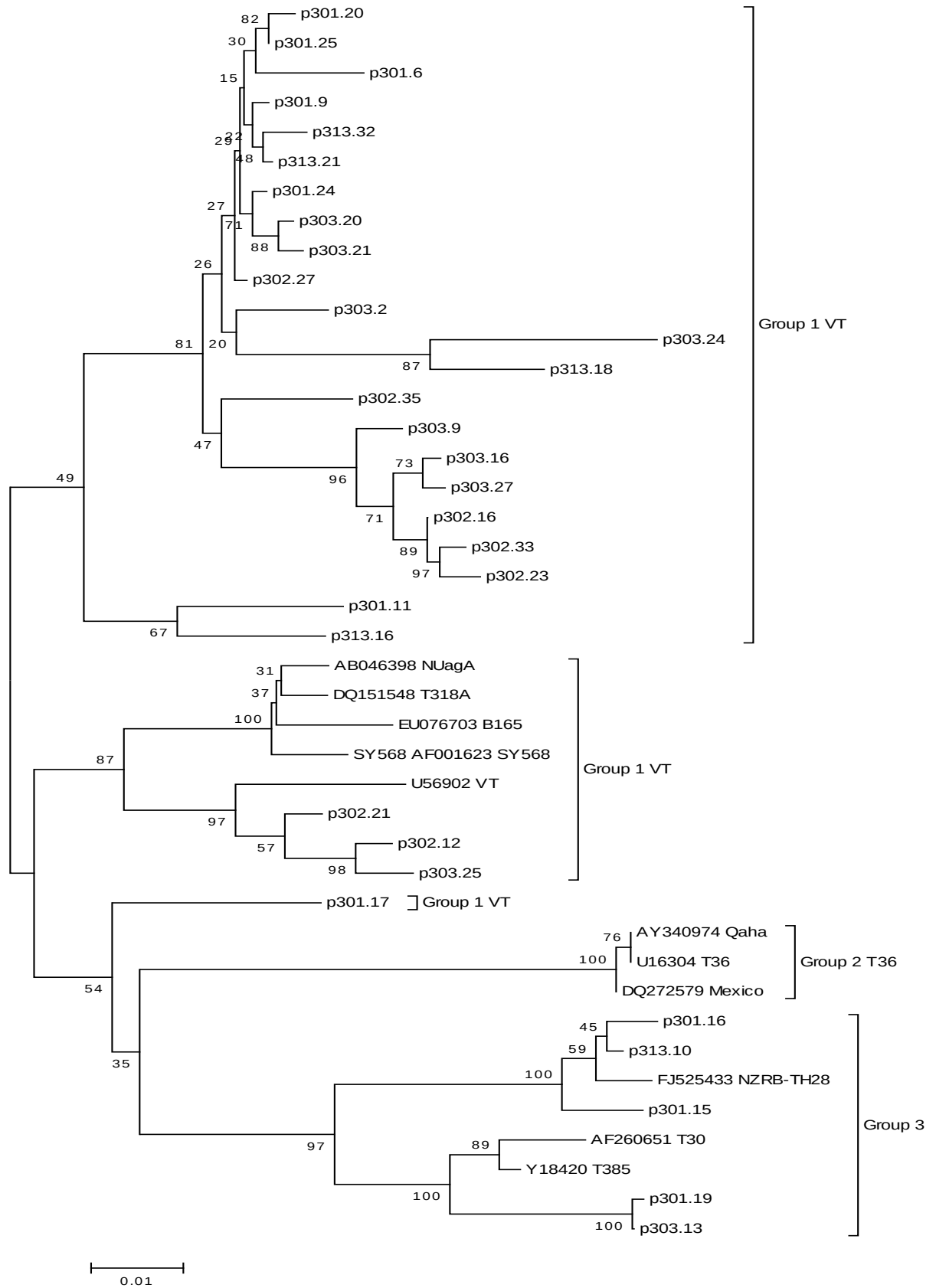


Figure 7: Summary tree for the p23 gene region of field sample replicates of GFMS 12 on Star Ruby produced from the fundamental phylogenetic trees shown in Appendix 5. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence). Clone numbers are representative of samples and specific clone numbers. For example 302 is the sample number and .12 denotes a specific clone. The clones within this tree represent a group of clones showing at least 99% similarity to those present in groups on the fundamental phylogenetic trees.

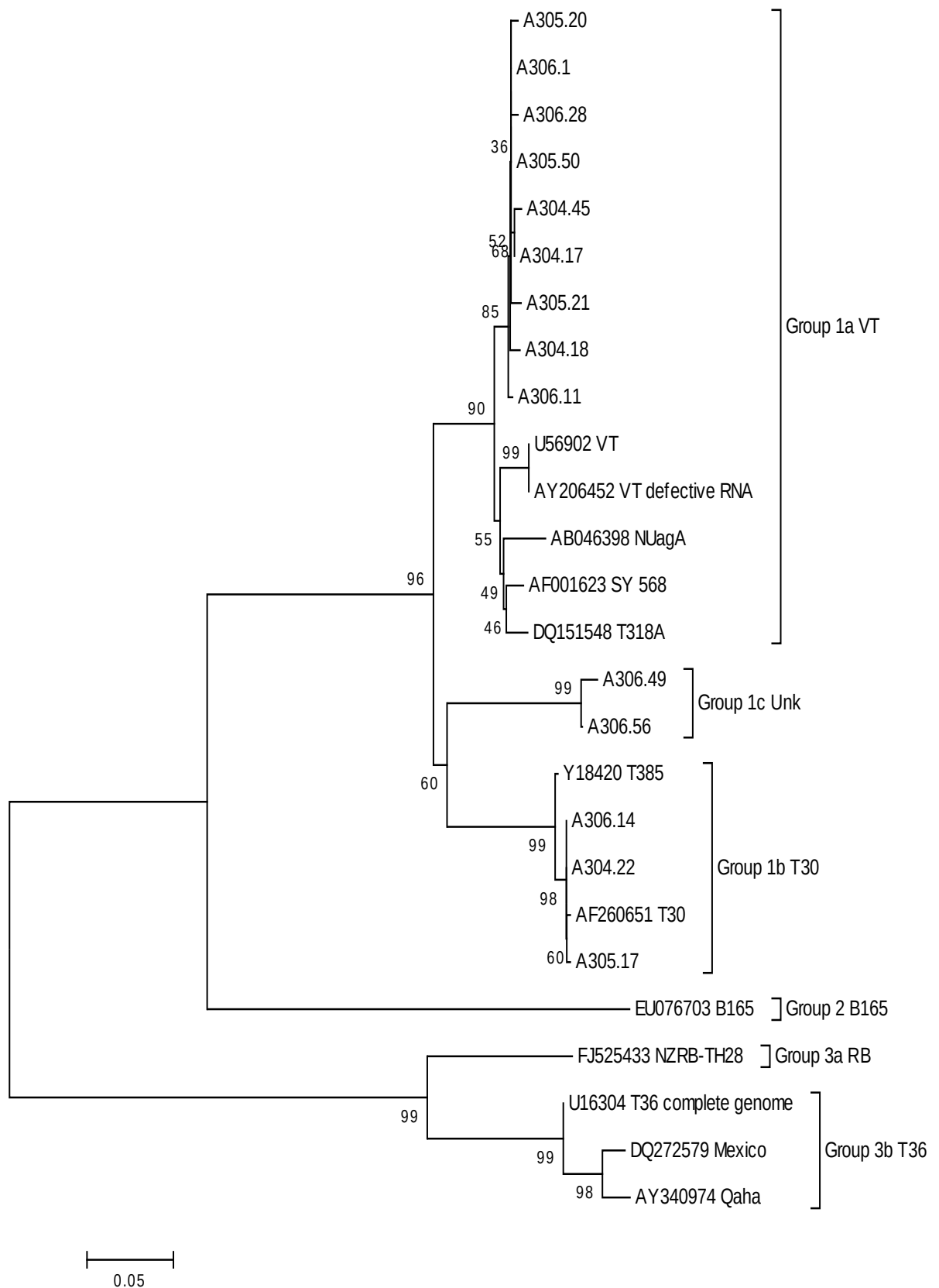


Figure 8: Summary tree for the A-region of field sample replicates of GFMS 12 on Flame produced from the fundamental phylogenetic trees shown in Appendix 2. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence). Clone numbers are representative of samples and specific clone numbers. For example 304 is the sample number and .22 denotes a specific clone. The clones within this tree represent a group of clones showing at least 99% similarity to those present in groups on the fundamental phylogenetic trees.



Figure 9: Summary tree for the p23 gene region of field sample replicates of GFMS 12 on Flame produced from the fundamental phylogenetic trees shown in Appendix 6. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence). Clone numbers are representative of samples and specific clone numbers. For example 304 is the sample number and .84 denotes a specific clone. The clones within this tree represent a group of clones showing at least 99% similarity to those present in groups on the fundamental phylogenetic trees.

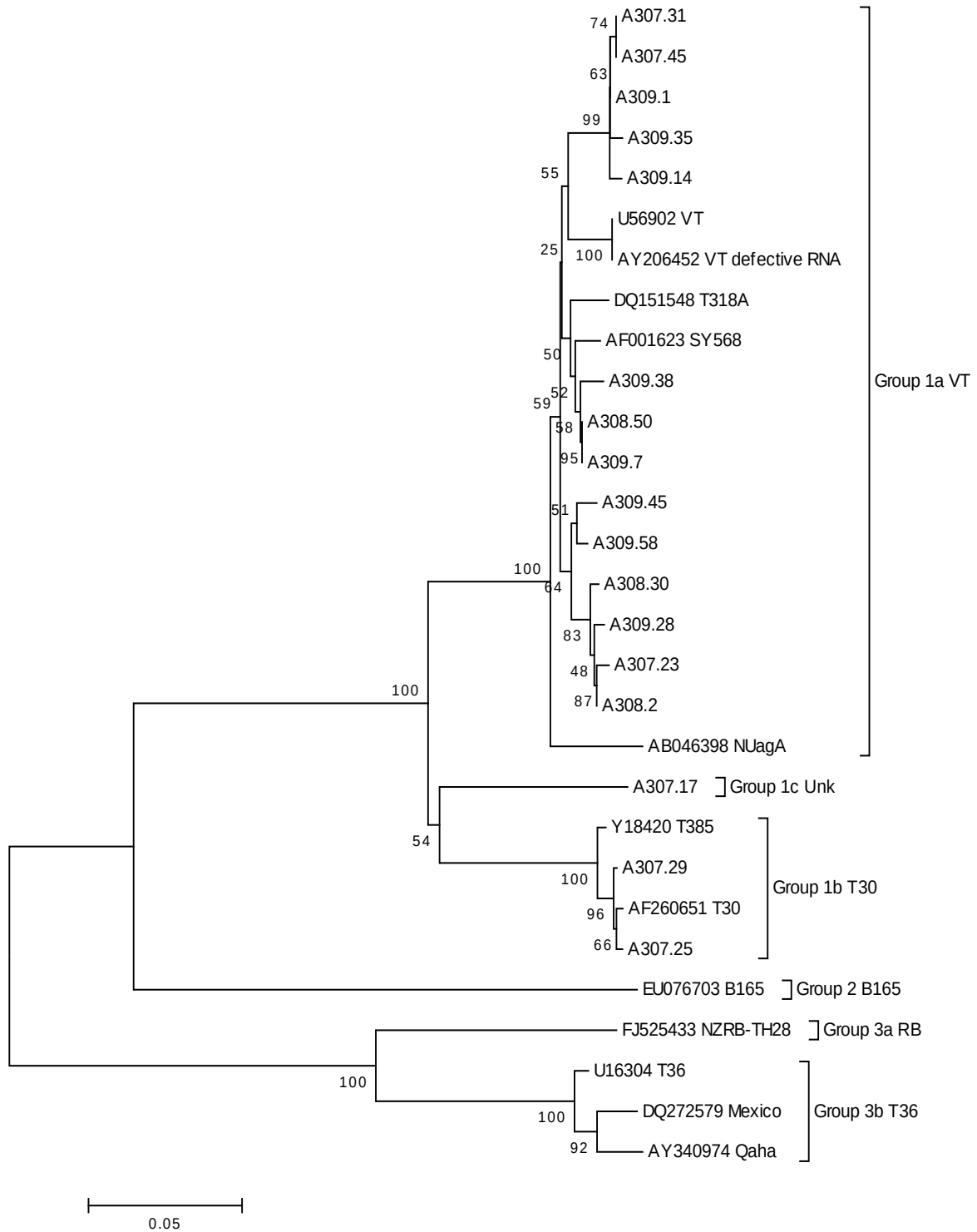


Figure 10: Summary tree for the A-region of field sample replicates of GFMS 35 on Star Ruby produced from the fundamental phylogenetic trees shown in Appendix 3. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence). Clone numbers are representative of samples and specific clone numbers. For example 307 is the sample number and .25 denotes a specific clone. The clones within this tree represent a group of clones showing at least 99% similarity to those present in groups on the fundamental phylogenetic trees.

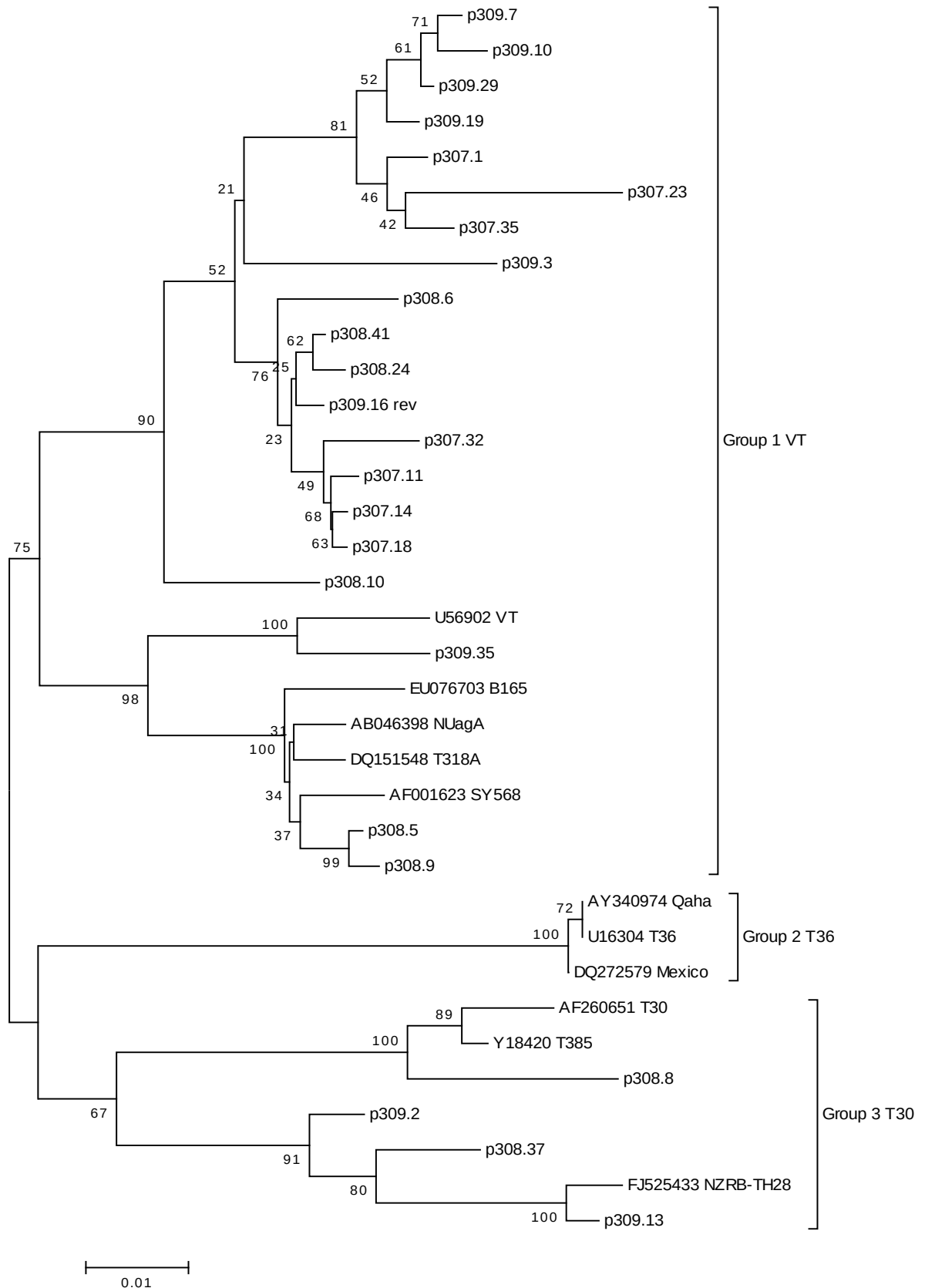


Figure 11: Summary tree for the p23 gene region of field sample replicates of GFMS 35 on Star Ruby produced from the fundamental phylogenetic trees shown in Appendix 7. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence). Clone numbers are representative of samples and specific clone numbers. For example 308 is the sample number and .8 denotes a specific clone. The clones within this tree represent a group of clones showing at least 99% similarity to those present in groups on the fundamental phylogenetic trees.

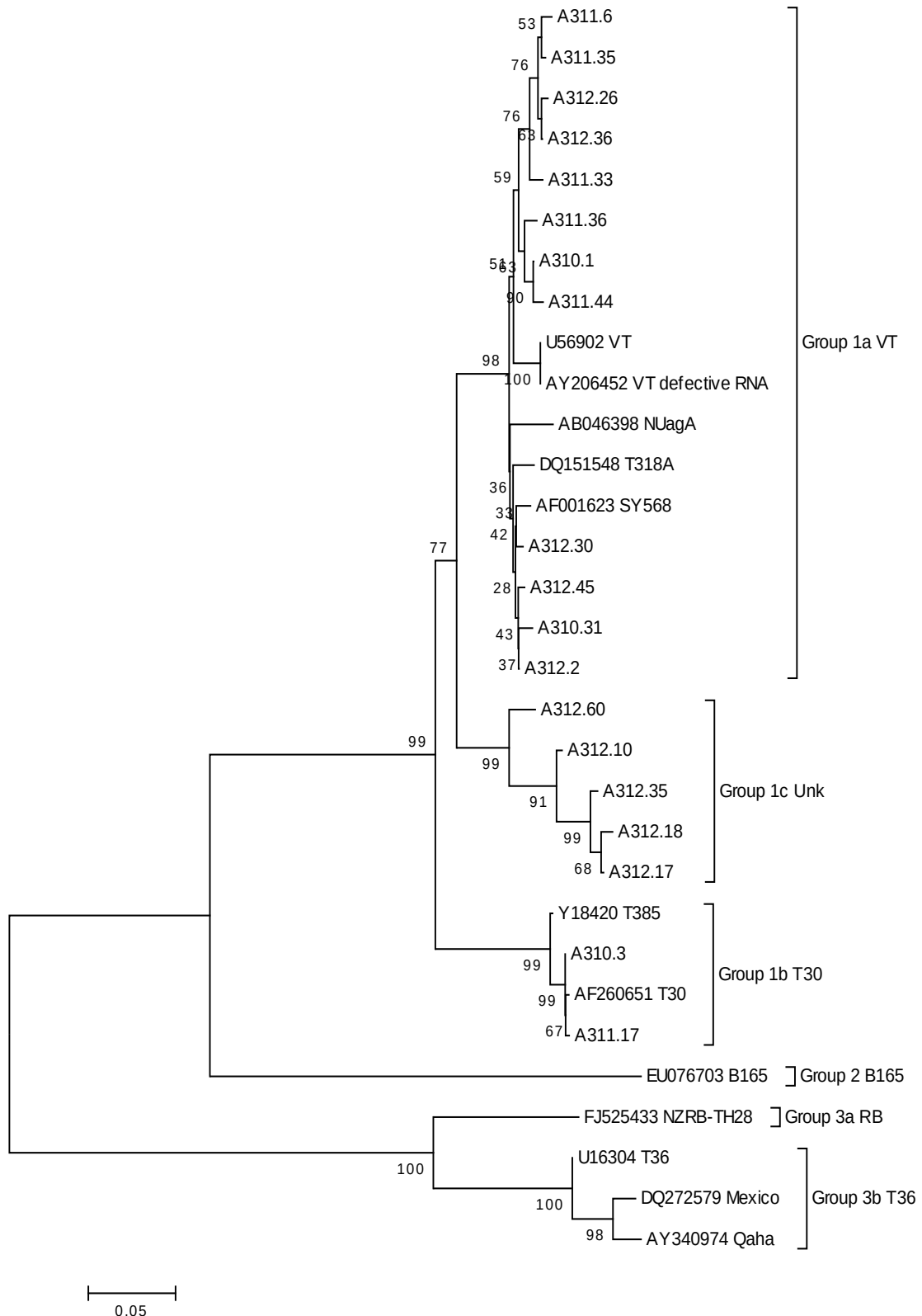


Figure 12: Summary tree for the A-region of field sample replicates of GFMS 35 on Flame produced from the fundamental phylogenetic trees shown in Appendix 4. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence). Clone numbers are representative of samples and specific clone numbers. For example 310 is the sample number and .17 denotes a specific clone. The clones within this tree represent a group of clones showing at least 99% similarity to those present in groups on the fundamental phylogenetic trees.

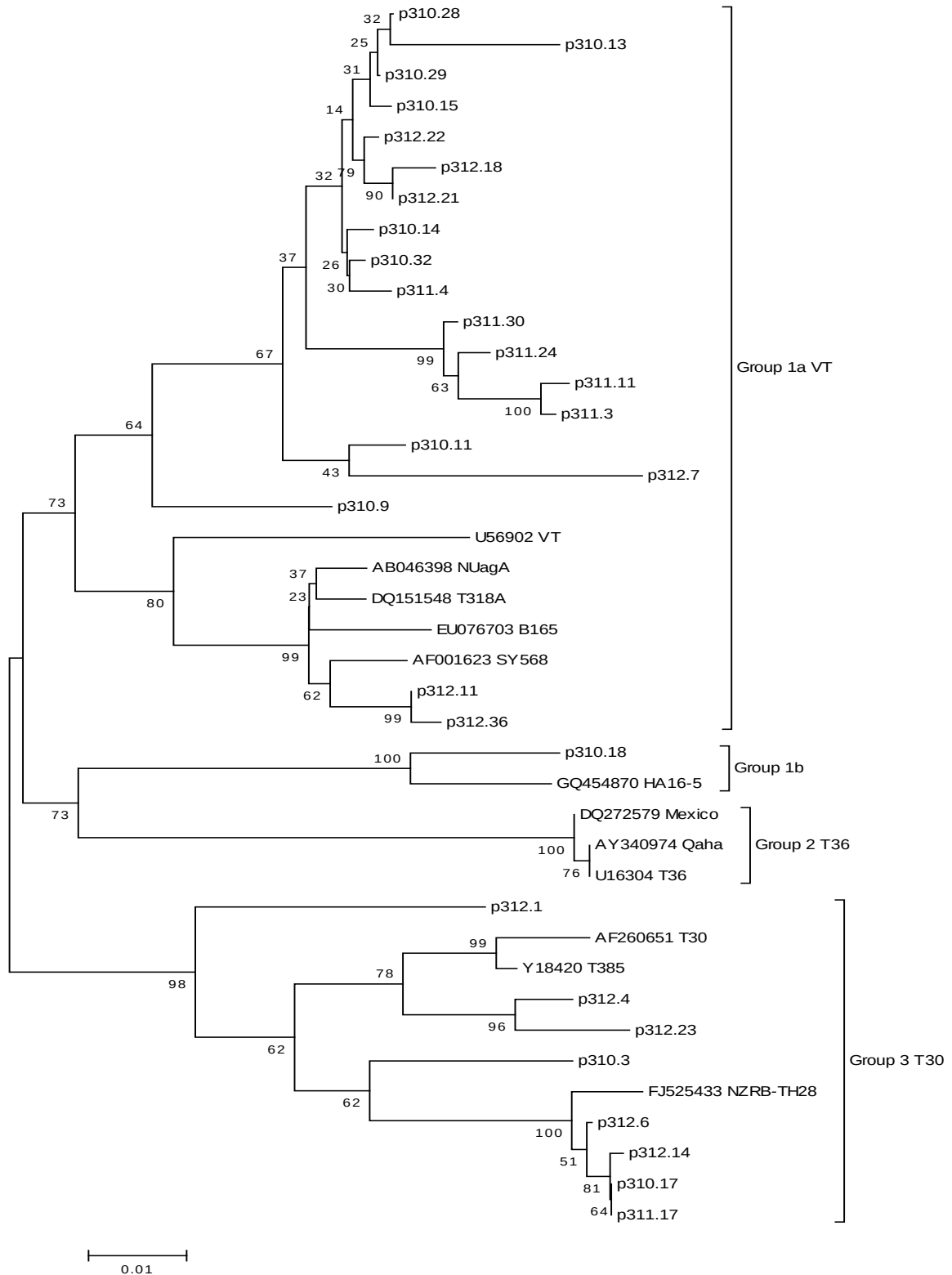


Figure 13: Summary tree for the p23 gene region of field sample replicates of GFMS 35 on Flame produced from the fundamental phylogenetic trees shown in Appendix 8. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence). Clone numbers are representative of samples and specific clone numbers. For example 312 is the sample number and .6 denotes a specific clone. The clones within this tree represent a group of clones showing at least 99% similarity to those present in groups on the fundamental phylogenetic trees.

Literature Cited

Aaskov J., Buzakott K., Field E., Lowry K., Berlioz-Arthaud A. and Holmes H.C. (2007). Multiple recombinant dengue type I viruses in an isolate from a dengue patient. *Journal of General Virology* vol. 88 pp3334-3340

Martin D.P., Williamson C. and Posada D. (2005). RDP2: recombination detection and analysis from sequence alignments. *Bioinformatics* vol. 21 pp260-262

Melzer M.J., Borth W.B., Sether D.M., Ferreira S., Gonsalves D. and Hu J.S. (2010). Genetic diversity and evidence for recent modular recombination in Hawaiian *Citrus tristeza virus*. *Virus Genes* vol 40 pp111-118

Padidam M., Sawyer S. and Fauquet C.M. (1999). Possible emergence of new geminiviruses by frequent recombination. *Virology* vol. 265 pp218-225

Posada D. (2002). Evaluation of Methods for Detecting Recombination from DNA Sequences: Empirical Data. *Molecular Biology and Evolution* vol 19 pp708-717

Sambade A., Lopez C., Rubio L., Flores R., Guerri J. and Moreno P. (2003). Polymorphism of a specific region in gene *p23* of *Citrus tristeza virus* allows for the discrimination between mild and severe isolates. *Archives of Virology* vol. 148 pp2325-2340

Chapter 5

DISCUSSION

The objective of this study was to determine whether severe-strain superinfection or a severe component of the preimmunising populations is responsible for cross-protection breakdown observed in grapefruit in the field. This was done by comparing the viral populations of the original pre-immunising sources with those found in pre-immunised plants in the field, displaying symptoms of cross-protection breakdown. Data was generated through the mass cloning of amplified PCR products of the A-region and p23 gene of Star Ruby and Flame grapefruit cultivars, pre-immunized with either GFMS 12 or GFMS 35, from the field, in the hot citrus production area of Malelane, South Africa. GFMS 12 and GFMS 35 source populations maintained on either Star Ruby or Mexican Lime plants, kept in an insect proof greenhouse, were used as reference populations. These references were considered to be representative the two pre-immunising sources, without the influence of additional CTV strains that may have been introduced by the aphid vector. Between 13 and 56 clones were analysed for each gene region for the field-collected samples. While those for the reference samples ranged from 38 to 119. It was assumed that this number of clones would provide a relatively accurate representation of the strain composition of each CTV population being characterised. The numbers of clones sequenced in this study are at least comparable or in most cases higher than those of other studies that have characterised CTV isolates, with similar methods (Davino et al, 2005; Iglesias et al, 2005a; Iglesias et al, 2005b, Iglesias et al, 2008; Ramos et al, 2005).

Cross-protection has long been considered a generally poor mechanism of protection against plant viral pathogens due to an incomplete or ineffective protection. However, the use of cross-protection against CTV is often considered a unique success story (Fulton, 1986). The issue of cross-protection breakdown has continued to tarnish the success of CTV cross-protection strategies and without adequate molecular-based research the factors responsible for cross-protection breakdown have been very difficult to

determine. The traditional empirical approach, used to evaluate the cross-protecting capacity of CTV isolates, yields no information regarding the assemblage of strains within the population and requires long-term field performance trials (van Vuuren et al, 2000c) that are labour intensive. Severe sequence types, present at very low levels within the population, may become dominant and express symptoms when placed under different climatic conditions (Broadbent et al, 1996), transmitted by a vector (Roy and Brlansky, 2009) or within a different host (Ayllón et al, 1999), probably due to varying replicative capacities of specific strains in different hosts (Targon et al, 2000). Superinfection can often occur if a pre-immunizing source has poor systemic movement capabilities, leaving young flush tissue virus-free, exposing them to possible severe-strain inoculations by aphids (Zhou et al, 2002a).

It has been suggested that either superinfection or a severe component of the original pre-immunizing source may be responsible for symptom expression, depending on the cultivar and cross-protecting source used. Pelosi et al, (2000) found that severe-strain superinfection occurred in almost all sweet oranges from a trial while an adjacent grapefruit block showed almost no superinfection of severe strains. van der Vyver et al, (2002) showed that CTV strain incidence in grapefruit trees, pre-immunised with either GFMS 12 or GFMS 35, in the field changed during a 10 year period. In that study, additional CTV variants were found within the CTV populations, leading van der Vyver et al, (2002) to suggest that superinfection was responsible for the change in strain incidence. This also showed that GFMS 12 and GFMS 35 both lack one of the essential characteristics of a cross-protecting source, which is stability within the host (Souza et al, 2002).

Without full-genome data, there appears to be no definitive protocol for grouping CTV cloned sequence data according to sequence similarity. Strain delineation based on partial sequence data is especially challenging with CTV since sequence similarity between strains varies along the genome and increases drastically from the 3' to 5' ends. Cloned sequence data, as shown on a phylogenetic tree, was grouped according to references at first on a visual basis and the reliability of these groups was confirmed according to the following sequence similarity thresholds. In a study by Hilf et al, (2005) the

K17 (marker position: ~4850-5250nt, 5' half) and POL (marker position: ~10780-11500, 3' half) markers of various CTV isolates were sequenced. The K17 marker showed a 95-99% intragroup similarity for the T36, T30 and VT groups, while the POL marker showed an 89-99% intragroup similarity for the same groups. Since the K17 marker is close to the 5' end of the CTV genome, a 95-99% similarity threshold was used as a guideline for assigning cloned A-region sequence data to the relevant reference groups. Despite the POL showing a lower intragroup similarity than the K17 marker, the intergroup similarity for the POL marker was higher and was probably a function of the overall higher sequence conservation of the 3' end of the CTV genome. Using this data as a guideline, a 90-99% similarity threshold was used for assigning cloned p23 gene region sequence data to the relevant reference groups.

In the current study, VT-like strains appear to dominate in samples collected from grapefruit trees grown in the field, regardless of the cultivar or cross-protecting source used. Analysis of the A-region clones (between 47-56 clones per tree replicate) of replicate trees of *Citrus x paradisi* cv. Star Ruby showed an incidence of 79-100% of VT-like strains within each replicates viral population, while replicate trees of *Citrus x paradisi* cv. Flame (48-54 clones per tree replicate) showed an incidence of 79-98% incidence of VT-like strains. Analysis of the p23 gene clones (between 23-30 clones per tree replicate) of *Citrus x paradisi* cv. Star Ruby showed an incidence of 85-100% VT-like strains, while replicates of *Citrus x paradisi* cv. Flame (13-29 clones per tree replicate) showed an incidence of 69-90% incidence of VT-like strains. The predicted amino acid sequences at positions 78-80 of the p23 gene is reported to differentiate between severe, mild and atypical CTV strains (Sambade et al, 2003). Although it is unclear whether this amino acid motif plays a direct role in CTV pathogenicity, clones that were considered to be mild or severe according to their predicted amino acid sequences showed an almost perfect correlation to groupings that are considered mild or severe on the phylogenetic tree.

The fundamental cause of the VT-like dominance is difficult to determine but one of the accepted hypotheses is the occurrence of superinfection of VT-like strains within grapefruit trees in the field. Superinfection usually refers to

the failure of a cross-protecting viral isolate to completely protect against a severe isolate of the same virus (Fulton, 1978). Iglesias et al, (2008) evaluated the CTV population of 11 samples isolated from pigmented grapefruits grown in commercial Argentinean orchards. Three genomic regions were analysed among which was the p23 gene. 10 clones were produced for each of the field isolates, which is probably too few to present an accurate estimation of each populations diversity but none the less showed that VT-like sequence types were present in the greatest numbers in the field collected samples reinforcing the results obtained in the current study. The cultivation of pigmented grapefruit cultivars is severely affected by stem-pitting (Iglesias et al, 2005b). The correlation between severe stem-pitting and the presence of VT as the predominant sequence type suggests that VT-like strains are probably responsible for severe stem-pitting symptoms on grapefruits and possibly on other citrus species/cultivars as well. The results from the current study, coupled with those obtained by Iglesias et al, (2008), suggest that the pigmented grapefruit cultivars possibly select for VT-like strains within the CTV population infecting them. Although no significant difference in strain incidence in trees pre-immunised with either GFMS 12 and GFMS 35 was observed, Luttig et al, (2002) showed that the original pre-immunising sources did differ from each other. This was done through SSCP analysis.

Harper et al, (2010) found that VT-like strains are the most abundant CTV sequence variants present in the New Zealand CTV population and are often present as mixtures with Resistance Breaking-like (RB-like) strains, with a relative paucity of T36-like strains. Roy et al, (2005) characterised 21 CTV isolates from India using multiple molecular markers (MMM) in the 5' proximal end and found that a similar picture may exist in India. 19 isolates were amplified by the VT-specific primers, 5 isolates by the T30-specific primers and 1 isolate by the T36-specific primers. Although there was some cross-amplification between the primers this still demonstrates that VT-like strains are the most prevalent with a relative scarcity of T36-like strains. The data from the current study has produced a similar picture, where in most cases VT-like strains appeared almost exclusively on the Star Ruby grapefruit trees sampled in the field and was one of the most common genotypes on the

greenhouse maintained Star Ruby plants. Hilf et al, (2005) characterised 14 CTV isolates from South Africa maintained on sweet orange and classified 9 of these as VT-like, using marker analysis. However, it is likely that diverse CTV strains present at varying levels of strain incidence will be observed when sampling different host species and cultivars. CTV populations on grapefruit trees have been the most extensively characterised on a molecular level, in South Africa, (Scott, unpublished data and Read, unpublished data). The strain incidence on other citrus species and cultivars within South African orchards is not well known and would be a worthwhile area of further research.

Minor sequence types may be important in eliciting disease symptoms despite being present in relatively low titres (Cerni et al, 2008). Minor sequence types with the highest incidence, expressed as a percentage value of all the clones analysed for a particular sample, in the A-region sequence analysis were T30-like strains (2-19% for Star Ruby replicates and 2-21% for Flame replicates). Another minor sequence types obtained for the A-region analysis of the field samples was of an unknown variant. Upon BLAST analysis this variant showed a 93% sequence similarity to a partially complete contig of the Baja California p349 (Genbank: AY652911) sequence (Herrera-Isidron, unpublished data). The incidence of the unknown sequence type was 2-13% amongst strains for Star Ruby replicates and 10-12% for Flame replicates. The sequence of these clones only showed an 88% sequence similarity to the VT genotype. Interestingly, trees with CTV populations containing this unknown sequence type showed very severe CTV symptoms. These variants may be recombinants within the region that was analysed (A-region) or could potentially be entirely new genotypes. In either case, further investigations into the origin of this sequence type needs to be carried out. Pure sources of this strain need to be isolated followed by whole genome sequencing, which will give more insight into the origins of this minor sequence type and information on its recombination history.

In p23 gene region analysis, minor sequence types included T30-like strains (4-24% incidence in Star Ruby and 4-24% incidence in Flame) and HA 15-6-like strains (0% incidence in Star Ruby and 4-23% incidence in Flame). The HA 15-6 strain is a known recombinant variant from Hawaiian citrus

orchards (Melzer et al, 2010). Differentiation of these sequence types were only observed in the p23 gene analyses (for both field and reference populations) and not in the A-region, which further reinforces the evidence that the HA 15-6 strains observed in this study may also be recombinants that may have similar properties to the Hawaiian strain. Iglesias et al, (2005a) characterized the p23 gene region of an Argentinean CTV pre-immunizing source, from a Henninger Ruby Red grapefruit and showed that the population consisted of an equal incidence of VT-like and T30-like strains. This demonstrates that the T30 genotype is capable of replicating to relatively high titres in pigmented grapefruit cultivars but the reason this was not observed in the current study was probably due to differences in environmental conditions. RB-like strains were represented as another minor sequence type in the CTV populations from the plants in the field. According to Harper et al, (2010) the origin of the New Zealand RB-like strains cannot be accurately determined however citrus planting material has been imported into New Zealand from a multitude of countries, among which is South Africa. Since these unique sequence types are present in both South Africa and New Zealand, it is possible that South Africa is their country of origin. The results of Harper et al, (2010) and from the current study both depict a preponderance of VT-like strains present in mixtures with minor sequence types. This further emphasises the idea that large-scale CTV diversity studies need to be conducted in South Africa's climatically diverse citrus production areas.

Evaluation of the phylogenetic data produced for the greenhouse maintained reference populations collected from the greenhouse supports the hypothesis that host species and/or cultivar plays a role in shaping CTV population structures. In the case of the A-region data analysis, Star Ruby pre-immunised with GFMS 35 showed a 73% incidence of VT-like strains (n clones = 119) suggesting that red grapefruit cultivars select for these sequence types. This conclusion was again reinforced in the case of the p23 gene region analysis where a Star Ruby tree pre-immunised with GFMS 12 showed a 100% incidence of VT-like strains (n clones = 60). A different incidence of strains was observed when evaluating the Mexican Lime host in the greenhouse. In the case of the p23 gene analysis of a Mexican Lime plant pre-immunised with GFMS 12, the population comprised of 44.8% VT-like

strains and 55.2% T30-like strains (n clones = 58). Similarly, a Mexican Lime plant pre-immunised with GFMS 35 (n clones = 91) produced a phylogenetic tree with a similar shape to that of GFMS 12, however the T30-like strains (52% incidence) displayed a shared dominance with an anomalous sequence type which grouped most closely to a full genome sequence called HA15-6 (48% incidence) from Hawaii (Melzer et al, 2010). The A-region analysis of Mexican Lime plants harbouring the GFMS 12 and GFMS 35 pre-immunising sources showed a 100% and 94.5% incidence of B165 respectively. This is in contrast to the data obtained for GFMS 12 and GFMS 35 sources on Star Ruby. Meyer et al, (2005) performed SSCP analysis on the CTV p25 gene region of GFMS 12 and GFMS 35 on various grapefruit cultivars including Star Ruby and Flame. GFMS 35 on Star Ruby and Flame showed similar SSCP profiles, which is in agreement with the field data from the current study where no differences were observed for the GFMS 35 populations on these two cultivars. Interestingly, Meyer et al, (2005) found altered SSCP profiles for GFMS 12 on Star Ruby and Flame, which is in contrast to the data from the current study where no differences were observed in the GFMS 12 populations from Star Ruby or Flame. However, some differences in GFMS 12 and GFMS 35 maintained on Star Ruby in the greenhouse were observed in some data, in the absence of the insect vector but whether this is a true reflection of the population structure of these two sources is unknown and other data from the current study suggest no large differences between GFMS 12 and GFMS 35 sources.

Recombination events are probably responsible for the incongruent phylogenetic data from the two different genomic regions, as described previously (Rubio et al, 2001) and B165 sequence variants are known to have arisen through a number of recombination events with multiple genotypes (Roy et al, 2010). Another possibility for the existence of the incongruent phylogenetic data is the reduced strain/genotype resolving capacity toward the 3' end of the genome due to high nucleotide conservation. In some cases, anomalous sequence types appear to be dominant where VT-like strains were expected to dominate. These sequence types may represent recombinants that have an increased capacity to replicate, which has allowed them to become dominant sequence types. Sequencing analysis of small

gene regions provides limited phylogenetic resolution and future research should include the whole genome sequencing of these anomalous sequence types. This will provide information about their genotypic origins and recombination history. The reason why such a relatively high incidence of the HA15-6-like strains is present in some greenhouse maintained samples remains unknown. Should further populations of this strain be found, research should be directed toward isolating a pure source of the strain, possibly through single aphid transmissions, performing pathogenicity trials on indicators and characterizing the strain at the whole genome level.

The presence of recombinants in a viral population presents problems during phylogenetic analyses because they can lead to incongruent phylogenetic trees (Martin et al, 2009). Furthermore, a single phylogenetic tree is not able to illustrate the complete evolutionary history of a recombinant sequence type and even a single recombinant in an alignment can compromise the accuracy of conclusions made when analysing phylogenetic data (Martin et al, 2005). Further potential reasons for the unusual CTV population structures observed for many of the greenhouse maintained plants could be that samples were collected, as needed, at different times of the year and it is possible that seasonal conditions may have played a role in shifting the population structure. The effect that fluctuations in environmental conditions have on CTV dynamics has been well documented (da Graça et al, 1984; van Vuuren, 2002). The collection of young flush sample material may have skewed the actual population structures in these plants due to the differential movement and distribution of CTV strains within different host species (Folimonova et al, 2008) and pigmented grapefruit cultivars have been shown to have particularly uneven CTV distribution within a single plant (Meyer et al, 2005; Zhou et al, 2002a).

The clones of “unknown” genotypic origin that are present in the phylogeny of the A-region of sample 312 can be attributed to recombination events, since four out of the six “unknown” clones have been shown to be recombinants of strains within the VT and T30 genotypic groups. The same pattern was observed in the A-region phylogeny of sample 313 where six of the seven “unknown” sequences showed evidence of recombination.

The p23 gene region phylogeny of sample 301 had six recombinant clones, which share high similarity with a number of different strains and showed three unique recombination patterns. Some of these clones (p301.6, p301.11) grouped away from the major branches on the phylogenetic tree, grouping alone to form unique branches (see *figure 5.1*). Clone p301.24 occupies a unique phylogenetic branch (*figure 5.3*) and shows evidence of recombination between strains within the VT and T30 genotypes. The same type of grouping was observed for clone p306.30, a VT/T30 recombinant. Clones p309.14, 15, 16, 25 all occupy a unique branch (*figure 7.3*) and show a recombination between different strains of the same genotype. This demonstrates that even inter-strain recombination can have an effect on the phylogeny of a viral population. Analysis of the p23 gene region of sample 310 showed that seven clones appeared to be recombinants with four unique strain recombination patterns. Clones p310.3, 9, 11, 13 grouped alone (*figure 8.1*). Recombination data from sample 312 again demonstrates how recombination events can skew conclusions made from phylogenetic trees. Clone p312.1 showed a recombinant pattern of VT/T30/VT genotypes and grouping away from the main branches within the T30-like group of a phylogenetic tree (*figure 8.3*), while clone p301.7 showing a VT/T30 pattern branches alone within the VT-like group of the same phylogenetic tree. Therefore, despite the two clones having similar recombination patterns they occupy very different phylogenetic positions. This again demonstrates the complexity added by recombination events to CTV and other virus' phylogenetics. Clones p313.16, 18 also occupy unique phylogenetic branches but are within the same genotype group (*figure 5.4*).

Analysis of the greenhouse reference samples also yielded some evidence of recombination in some samples. A single clone was shown to represent a recombinant in the phylogeny of the A-region of GFMS 12 on Mexican Lime, which occupies a unique position on the phylogenetic tree (*figure 12.1*). The p23 gene region of GFMS 12 on Mexican Lime showed evidence of recombination for two clones, 12.ML.23.32, 70, both of which, display two different patterns of VT/T30/VT, however, 12.ML.23.32 clusters within the T30-like branch and 12.ML.23.70 clusters within the VT-like branch (*figure*

13.1). This data once again provides evidence for the skewing effect that recombination events have on phylogenetics. The phylogenetic tree of the p23 gene region of GFMS 35 on Star Ruby (*figure 13.4*) showed very unusual groupings when compared with the p23 gene region phylogenies of the other reference samples (*figures 13.1, 13.2, 13.3*). A group of clones appearing to cluster within the T30-like group but almost all of these clones were shown to be recombinants, with a strain pattern of NZRB TH-28/HA 15-6. The large numbers of these recombinant sequence types and their high sequence similarity suggests that it is well adapted to this host-environment combination. The recombination data obtained from the current study has demonstrated how recombinant sequence types can influence the overall conclusions drawn from a phylogenetic tree (Martin et al, 2005). Most of the recombinant sequence types were represented as a single or a few clones and are probably of little significance to the overall pathogenicity of a CTV population. However, some recombinant sequence types replicated to high numbers, which could potentially alter the symptom severity of a CTV population.

The significance that hosts play in the variation of CTV titres has developed into a dogma in CTV pathology (Folimonova et al, 2008). Brlansky and Lee (1990) produced one of the first pieces of evidence to support the theory of selection by host species, which states that CTV strains produce differing numbers of inclusion bodies within the phloem cells and this appears to have a direct correlation on the severity of symptoms. Therefore, depending on its relative ability to replicate within a specific host, an infection by a suspected mild-strain may result in the manifestation of severe symptoms in a different host species where its replicating capabilities are increased. This emphasizes that cross-protecting sources should be selected to be host-specific and that the strain composition of the sources be thoroughly researched prior to commercial dissemination.

Folimonova et al, (2010) revealed how complicated the problem of CTV superinfection can be and issues that need to be addressed when selecting a cross-protecting source that will provide adequate protection against severe-strain superinfection. Cross-protection is based on superinfection exclusion,

also known as homologous interference and refers to the prevention of a secondary infection due to the primary inoculation of a closely related virus. Folimonova et al, (2010) determined that superinfection exclusion only occurred between variants of the same genotype (T30, VT, T3 or T36). A primary infection of, for example VT, prevented the superinfection of a subsequent inoculation of the same genotype (VT), while the presence one genotype from a primary infection had no influence on the establishment of a second heterologous genotype, within the same host. This data explains the results of Zhou et al, (2002b), where it was found that a cross-protecting source afforded better protection against a severe isolate that showed high sequence similarity to the protecting source.

According to Folimonova et al, (2010) the methodology for selecting cross-protecting sources should now be relatively simple, with the first objective being to identify the severe isolates within a region, requiring control. Secondly, mild isolates need to be discovered or created, through the substitution of mild sequence determinants. An assemblage of mild strains can be created to produce a characterised cross-protecting population that should protect against the severe field isolates present in a growing area. The high incidence of VT-like strains in South African grapefruit CTV populations is probably responsible for the decline of grapefruit trees in South Africa. Pathogenicity trials conducted by Ayllón et al, (2001) confirmed that VT-like strains are responsible for severe stem-pitting symptoms on sweet orange and grapefruit cultivars. Although cross-protection breakdown continues to be a problem, especially on grapefruits, the cultivation of grapefruit in South Africa would probably not be economically viable without the implementation of the, albeit imperfect, cross-protection scheme. van Vuuren et al, (2000a) showed that crop values of cv. Marsh grapefruit trees pre-immunised with either GFMS 12 and GFMS 35 pre-immunising populations were higher than those of the virus free control plants. However, both GFMS 12 and GFMS 35 were considered “poor performers” in empirical field performance trials with GFMS 35 producing symptom severity similar do GFSS (Grapefruit Severe Strain) 1 (van Vuuren et al, 2000b). This data demonstrates that cross-protection in South Africa is, at least in some cases, working but the mechanisms that have allowed these “mild” protecting sources to partially

protect against the onset of symptoms, despite containing severe components themselves, remains uncertain.

Providing unequivocal answers to the research questions of this study is not possible without further research. The results have merely hinted at the complexity of CTV population dynamics in South Africa and the possible reasons for cross-protection breakdown. However, it has also been very important in representing the scale of research still required in the area of CTV population studies, as well as the way in which this research should be directed. It may also direct a paradigm shift in the way CTV cross-protecting sources are evaluated for use within the Southern African Citrus Improvement Scheme from an empirical to a more directed approach, where molecular methods are included in evaluations. A combination of severe-strain superinfection and shifts of dominance within the original protecting source population could be responsible for the observed cross-protection breakdown in South Africa. The significance of the role that either of these factors may play in CTV population dynamics is probably affected by environmental and host variations. Complete baseline knowledge of CTV diversity in South Africa's different citrus production areas will allow for the more efficient control of the disease and the implementation of improved cross-protection schemes. The data required for this baseline of knowledge will come from evaluating CTV strain incidence present in all of the commercially grown citrus species and/or cultivars in South Africa. Studying the diversity of populations within different geographic regions will provide insight into how climatic conditions shape CTV population structures and to what degree this will influence future pathogen and vector control strategies. The South African citrus industry is making use of a very diverse selection of cultivars grown under diverse climatic conditions (Luttig et al, 2002). These variable climatic conditions in South Africa have been shown to have a significant effect on CTV symptom expression even in regions that are relatively close to one another (van Vuuren, 2002) and therefore probably also has an impact on CTV population dynamics. Baseline knowledge can be established through mass sampling and whole genome resequencing of the viral populations on the multitude of commercial citrus cultivars in all South African production areas. These populations may either be diverse among geographic locations or genetically

uniform due to the impact of cross-protection (Bar-Joseph et al, 2002), which makes use of a selection of the “same” protecting sources. The dominance of South African VT-like strains in pigmented grapefruit from the current study and in sweet orange cultivars (Zablocki, unpublished data) could be due cross-protection strain uniformity.

Full-length cDNA clones can be produced for each of the genotypes present within the larger South African CTV population. The pathogenicity of CTV can be studied through complete or partial gene knockouts in the full-length clones. Clones with inactivated pathogenicity can be used to create cross-protecting sources to protect against symptom inducing strains present in the field. Once the required data has been generated, cross-protecting sources, suited for each cultivar and climate, can be produced. This is necessary because it is known that one protecting source is not sufficient to protect all cultivars (van Vuuren et al, 2000b) due to differing replicative capacities among different strains or genotypes (Targon et al, 2000). Souza et al, (2000) showed that the CTV diversity within a naturally infected plant can drop as time progresses and may be due to competitive strain interactions or selection by the host. This reinforces the idea that protecting sources need to be produced for each cultivar and possibly each production area. Reaching the goal of having a much-improved CTV cross-protection scheme still lies some distance in the future. However, current CTV research in South Africa is making reaching this goal much more realistic and the direction of future research much more clear.

Literature Cited

- Ayllón M.A., Rubio L., Moya A., Guerri J. and Moreno P. (1999). The Haplotype Distribution of Two Genes of Citrus Tristeza Virus Is Altered after Host Change or Aphid Transmission. *Virology* vol 255 pp32-39
- Ayllón M.A., Lopez C., Navas-Castillo J., Garnsey S.M., Guerri J., Flores R. and Moreno P. (2001). Polymorphism of the 5' terminal region of *Citrus tristeza virus* (CTV) RNA: Incidence of three sequence types in isolates of different origin and pathogenicity. *Archives of Virology* vol. 146 pp27-40
- Bar-Joseph M., Che X., Mawassi M., Gowda S., Satyanarayana T., Ayllón M.A., Albiach-Marti M.R., Garnsey S.M. and Dawson W.O. (2002). The Continuous Challenge of *Citrus tristeza virus* Molecular Research. In: *Proc. 15th Conf. IOCV*, 1-7. IOCV, Riverside, CA.
- Brlansky R.H. and Lee R.F. (1990). Numbers of Inclusion Bodies Produced by Mild and Severe Strains of Citrus Tristeza Virus in Seven Citrus Hosts. *Plant Disease* vol. 74 pp297-299
- Broadbent P., Brlansky R.H. and Indsto J. (1996). Biological Characterization of Australian Isolates of Citrus Tristeza Virus and Separation of Subisolates by Single Aphid transmissions. *Plant Disease* vol. 80 pp329-333
- Cerni S., Ruscic J., Nolasco G., Gatin Z., Krajacic M. and Skoric D. (2008) 'Stem pitting and seedling yellows symptoms of *Citrus tristeza virus* infection may be determined by minor sequence variants'. *Virus Genes* vol. 36 pp241-249
- da Graça J.V., Marias L.J. and von Broemsen L.A. (1984). Severe tristeza stem pitting decline of young grapefruit in South Africa. In: *Proc. 9th Conf. IOCV*, 62-65. IOCV, Riverside, CA.

Davino S., Guardo M., Sorrentino G., Caruso A. and Davino M. (2005). Variability Among Italian *Citrus tristeza virus* Isolates Revealed by SSCP Analysis, Cloning and Sequencing. In: *Proc. 16th Conf. IOCV*, 137-142. IOCV, Riverside, CA.

Folimonova S.Y., Folimonov A.S., Tatineni S. and Dawson W.O. (2008). *Citrus Tristeza Virus*: Survival at the Edge of the Movement Continuum. *Journal of Virology* vol. 82 pp6546-6556

Folimonova S.Y., Robertson C.J., Shilts T., Folimonov A.S., Hilf M.E., Garnsey S.M. and Dawson W.O. (2010). Infection with Strains of *Citrus Tristeza Virus* Does Not Exclude Superinfection by Other Strains of the Virus. *Journal of Virology* vol. 84 pp1314-1325

Fulton F.W. (1978). Superinfection by Strains of Tobacco Streak Virus. *Virology* vol. 85 pp1-8

Fulton F.W. (1986). Practices and precautions in the use of cross protection for plant virus disease control. *Annual Reviews of Phytopathology* vol 24 pp67-81

Harper S.J., Dawson T.E. and Pearson M.N. (2010). Isolates of *Citrus tristeza virus* that overcome *Poncirus trifoliata* resistance comprise a novel strain. *Archives of Virology* vol. 155 pp471-480

Hilf M.E., Mavrodieva V.A. and Garnsey S.M. (2005). Genetic Marker Analysis of a Global Collection of Isolates of *Citrus tristeza virus*: Characterization and Distribution of CTV Genotypes and Association with Symptoms. *Phytopathology* vol.95 pp909-917

Iglesias N.G., Marengo J., Riquelme K., Costa N., Plata M.I. and Semorile L. (2005a). Characterization of the Population Structure of a Grapefruit Isolate of *Citrus tristeza virus* (CTV) Selected for Pre-immunization Assays in Argentina. In: *Proc. 16th Conf. IOCV*, 150-158. IOCV, Riverside, CA.

Iglesias N.G., Riquelme K., Marengo J., Costa N., Plata M.I. and Semorile L. (2005b). Genetic Structure of *Citrus tristeza virus* (CTV) Populations from Field Argentinean Grapefruit Isolates. In: *Proc. 16th Conf. IOCV*, 143-149. IOCV, Riverside, CA.

Iglesias N.G., Gago-Zachert S.P., Robledo G., Costa N., Plata M.I., Vera O., Grau O. and Semorile L.C. (2008). Population structure of *Citrus tristeza virus* from field Argentinean isolates. *Virus Genes* vol. 36 pp199-207

Luttig M., van Vuuren S.P. and van der Vyver J.B. (2002). Differentiation of Single Aphid Cultured Sub-Isolates of Two South African *Citrus tristeza virus* Isolates from Grapefruit by Single-Strand Conformation Polymorphism. In: *Proc. 15th Conf. IOCV*, 187-196. IOCV, Riverside, CA.

Martin D.P., Williamson C. and Posada D. (2005). RDP2: recombination detection and analysis from sequence alignments. *Bioinformatics* vol 21 pp260-262

Martin S., Sambade A., Rubio L., Vives M.C., Moya P., Guerri J., Elena S.F. and Moreno P. (2009). Contribution of recombination and selection to molecular evolution of *Citrus tristeza virus*. *Journal of General Virology* vol 90 pp1527-1538

Melzer M.J., Borth W.B., Sether D.M., Ferreira S., Gonsalves D. and Hu J.S. (2010). Genetic diversity and evidence for recent modular recombination in Hawaiian *Citrus tristeza virus*. *Virus Genes* vol 40 pp111-118

Meyer J.B., van Vuuren S.P., Luttig M., Manicom B.Q. and da Graça J.V. (2005). Strain Prevalence of *Citrus tristeza virus* Cross-Protecting Isolates Altered by Red Grapefruit Hosts. In: *Proc. 16th Conf. IOCV*, 205-212. IOCV, Riverside, CA.

Pelosi R.R., Rundell P.A., Cohen M. and Powell C.A. (2000). Evaluation of a Sixteen-Year Citrus Tristeza Virus Cross-Protection Trial in Florida. In: *Proc. 14th Conf. IOCV*, 111-114. IOCV, Riverside, CA.

Ramos C., Castillo J.C., Fernández O., Rangel B., Manjunath K.L. and Lee R.F. (2005). Molecular Characterization of *Citrus tristeza virus* Isolates from Panama. In: *Proc. 16th Conf. IOCV*, 159-164. IOCV, Riverside, CA.

Roy A., Manjunath K.L. and Bransky R.H. (2005). Assessment of sequence diversity in the 5'-terminal region of *Citrus tristeza virus* from India. *Virus Research* vol 113 132-142

Roy A. and Bransky R.H. (2009). Population Dynamics of a Florida *Citrus tristeza virus* Isolate and Aphid-Transmitted Subisolates: Identification of Three Genotypic Groups and Recombinants After Aphid Transmission. *Phytopathology* vol 99 pp1297-1306

Roy A. and Bransky R.H. (2010). Genome Analysis of an Orange Stem Pitting *Citrus Tristeza Virus* Isolate Reveals a Novel Recombinant Genotype. *Virus Research* vol 151 pp118-130

Rubio L., Ayllón M.A., Kong P., Fernandez A., Polek M., Guerri J., Moreno P. and Falk B.W. (2001). Genetic Variation of *Citrus Tristeza Virus* Isolates from California and Spain: Evidence for Mixed Infections and Recombination. *Journal of Virology*. vol. 75 pp8054-8062

Sambade A., Lopez C., Rubio L., Flores R., Guerri J. and Moreno P. (2003). Polymorphism of a specific region in gene *p23* of *Citrus tristeza virus* allows for the discrimination between mild and severe isolates. *Archives of Virology* vol. 148 pp2325-2340

Souza A.A., Carvalho S.A., Targon M.L.P.N., Müller G.W. and Machado M.A. (2000). Characterization of Citrus Tristeza Virus Isolates by SSCP of the Coat Protein Gene in Initially Healthy Sweet Orange Varieties After Three Years of Field Exposure. In: *Proc. 14th Conf. IOCV*, 50-56. IOCV, Riverside, CA

Souza A.A., Müller G.W., Targon M.L.P.N., Takita M.A. and Machado M.A. (2002). Stability of the Mild Protective 'PIAC' Isolate of *Citrus tristeza virus*. In: *Proc. 15th Conf. IOCV*, 131-135. IOCV, Riverside, CA

Targon M.L.P.N., Machado M.A., Carvalho S.A., Souza A.A. and Müller G.W. (2000). Differential Replication of a Mild and a Severe Citrus Tristeza Virus Isolate in Species and Varieties of Citrus. In: *Proc. 14th Conf. IOCV*, 127-130. IOCV, Riverside, CA

van der Vyver J.B., van Vuuren S.P., Luttig M. and da Graça J.V. (2002). Changes in the *Citrus tristeza virus* Status of Pre-immunised Grapefruit Field Trees. In: *Proc. 15th Conf. IOCV*, 175-185. IOCV, Riverside, CA

van Vuuren S.P. and da Graça J.V. (2000a). Reduction in 'Marsh' grapefruit tree size infected with citrus tristeza virus populations. *Journal of Horticultural Science & Biotechnology* vol. 75 pp542-545

van Vuuren S.P. and van der Vyver J.B. (2000b). Comparison of South African Pre-Immunizing Citrus Tristeza Virus Isolates with Foreign Isolates in Three Grapefruit Selections. In: *Proc. 14th Conf. IOCV*, 50-56. IOCV, Riverside, CA

van Vuuren S.P., van der Vyver J.B. and Luttig M. (2000c). Diversity Among Sub-Isolates of Cross-Protecting Citrus Tristeza Virus Isolates in South Africa. In: *Proc. 14th Conf. IOCV*, 103-110. IOCV, Riverside, CA

van Vuuren S.P. (2002). Effects of *Citrus tristeza virus* Isolates on Two Tolerant Commercial Scions on Different Rootstocks in South Africa. In: *Proc. 15th Conf. IOCV*, 31-38. IOCV, Riverside, CA

Zhou C.Y., Broadbent P., Hailstones D.L., Bowyer J. and Connor R. (2002a). Movement and Titer of *Citrus tristeza virus* (Pre-immunizing Isolate PB61) Within Seedlings and Field Trees. In: *Proc. 15th Conf. IOCV*, 39-47. IOCV, Riverside, CA

Zhou C.Y., Hailstones D.L., Broadbent P., Connor R. and Bowyer J. (2002b). Studies on Mild Strain Cross Protection Against Stem-Pitting *Citrus tristeza virus*. In: *Proc. 15th Conf. IOCV*, 151-157. IOCV, Riverside, CA

Appendix 1

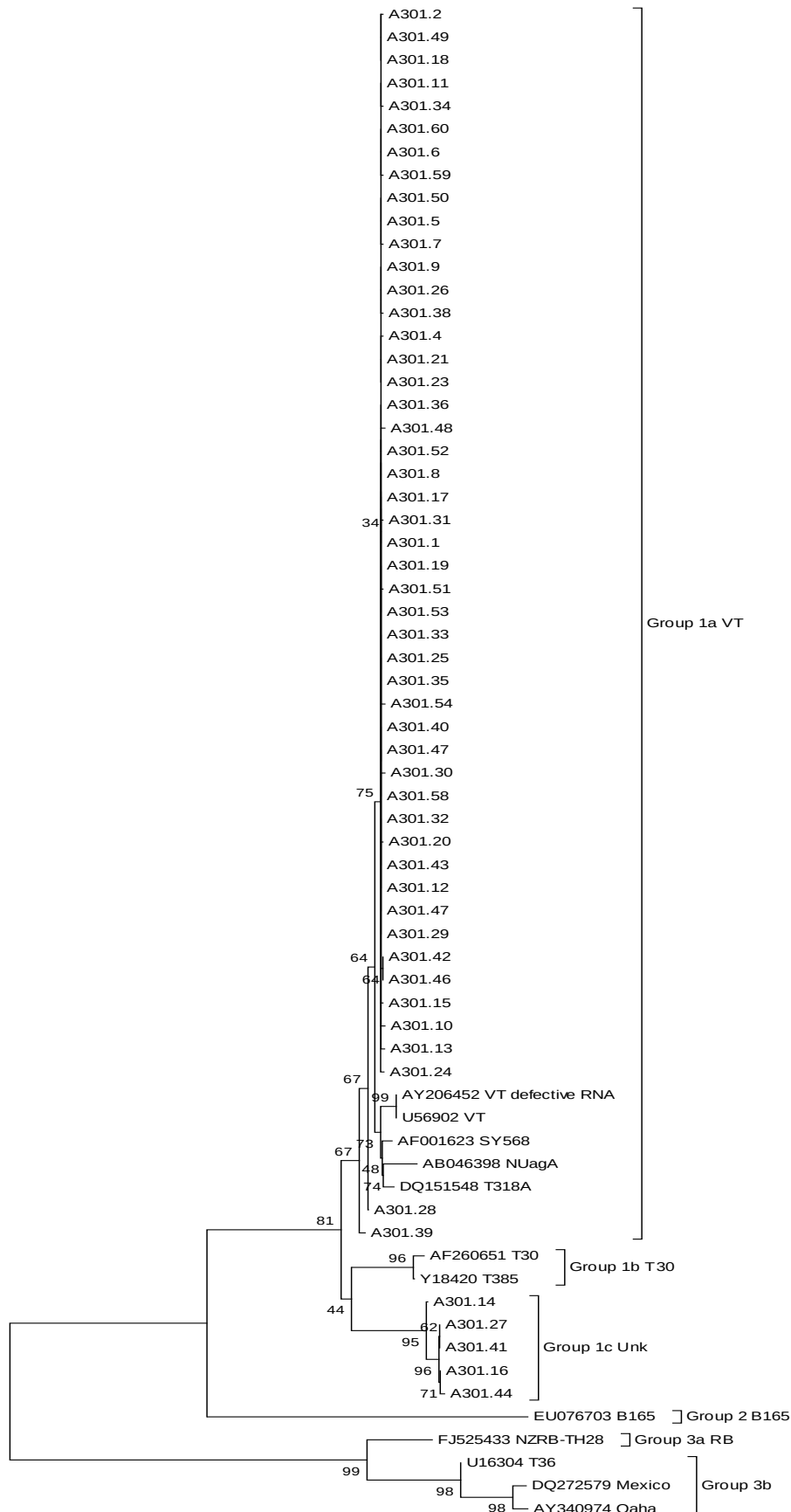


Figure 1.1: Phylogenetic tree of the A-fragment of sample replicate 1 of GFMS 12 on Star Ruby. All groups share a 95-99% intragroup sequence similarity. Group 1a contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the T30 reference contigs. Group 1c contains clones grouping none of the available reference contigs. Group 2 contains clones grouping with the B165 reference contig. Group 3a contains clones grouping with the New Zealand Resistance Breaking reference contig. Group 3b contains clones grouping with the T36 reference contigs. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

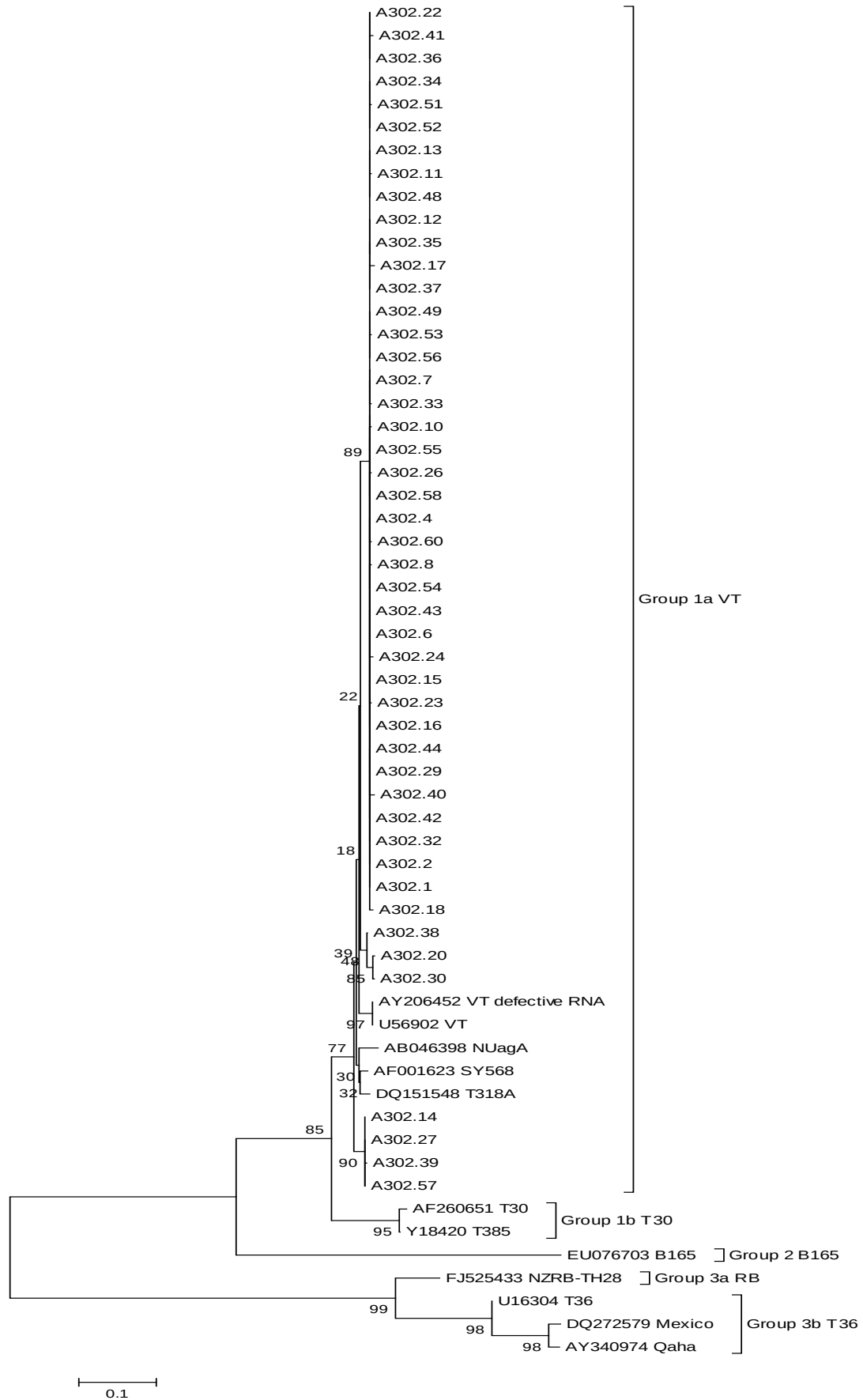


Figure 1.2: Phylogenetic tree of the A-fragment of sample replicate 2 of GFMS 12 on Star Ruby. All groups share a 95-99% intragroup sequence similarity. Group 1a contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the T30 reference contigs. Group 2 contains clones grouping with the B165 reference contig. Group 3a contains clones grouping with the New Zealand Resistance Breaking reference contig. Group 3b contains clones grouping with the T36 reference contigs. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

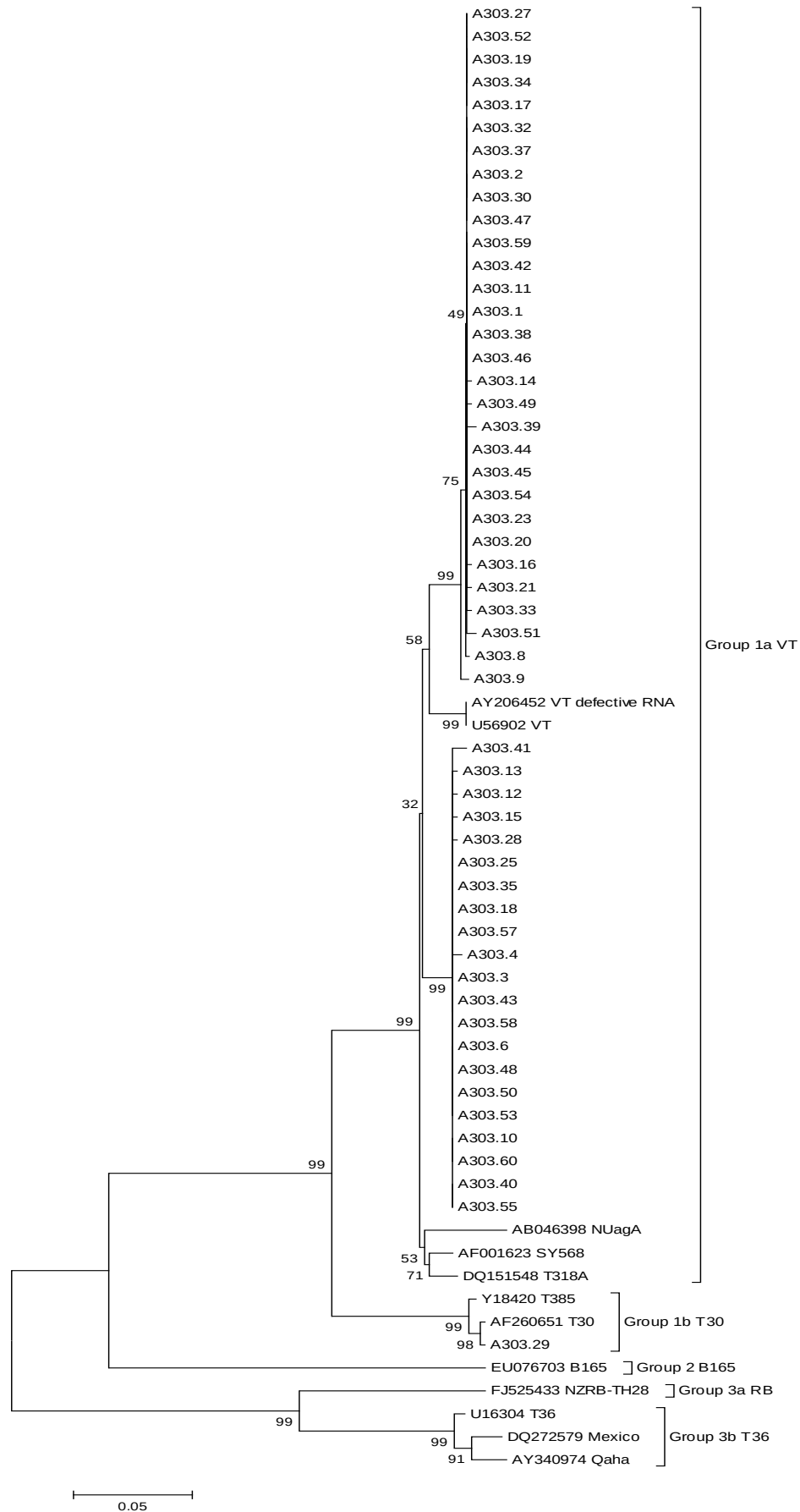
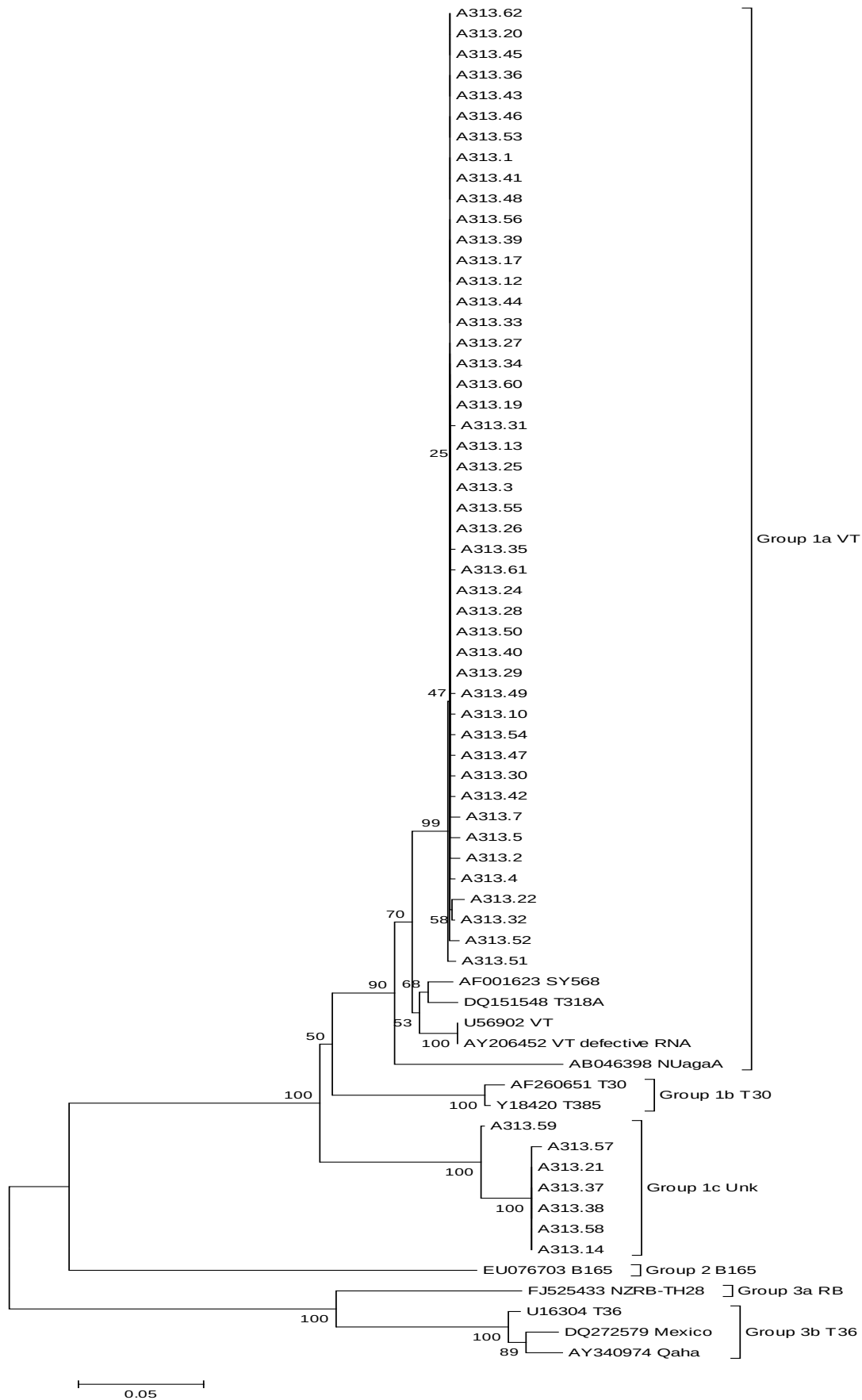


Figure 1.3: Phylogenetic tree of the A-fragment of sample replicate 3 of GFMS 12 on Star Ruby. All groups share a 95-99% intragroup sequence similarity. Group 1a contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the T30 reference contigs. Group 2 contains clones grouping with the B165 reference contig. Group 3a contains clones grouping with the New Zealand Resistance Breaking reference contig. Group 3b contains clones grouping with the T36 reference contigs. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).



Appendix 2

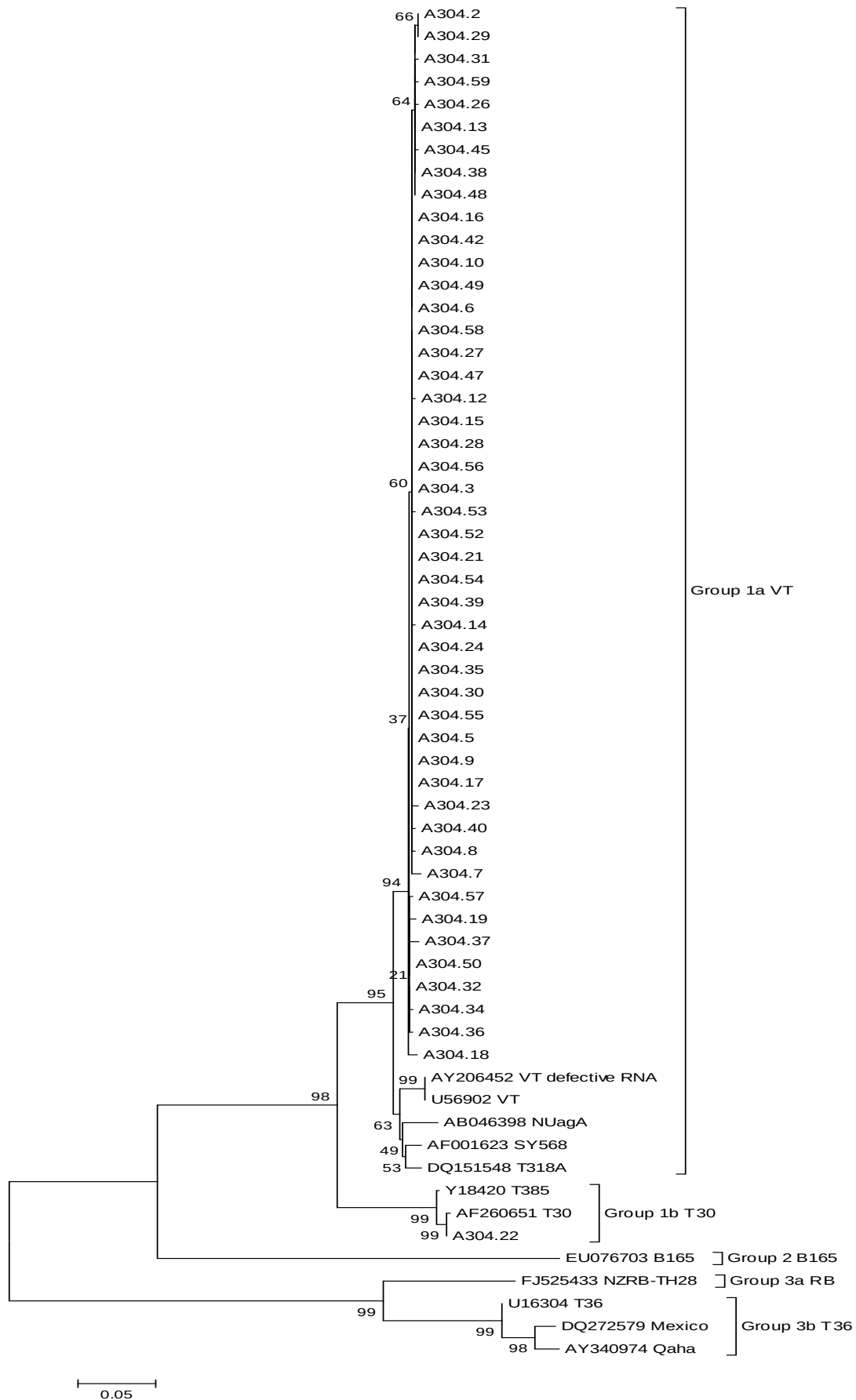


Figure 2.1: Phylogenetic tree of the A-fragment of sample replicate 1 of GFMS 12 on Flame. All groups share a 95-99% intragroup sequence similarity. Group 1a contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the T30 reference contigs. Group 2 contains clones grouping with the B165 reference contig. Group 3a contains clones grouping with the New Zealand Resistance Breaking reference contig. Group 3b contains clones grouping with the T36 reference contigs. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

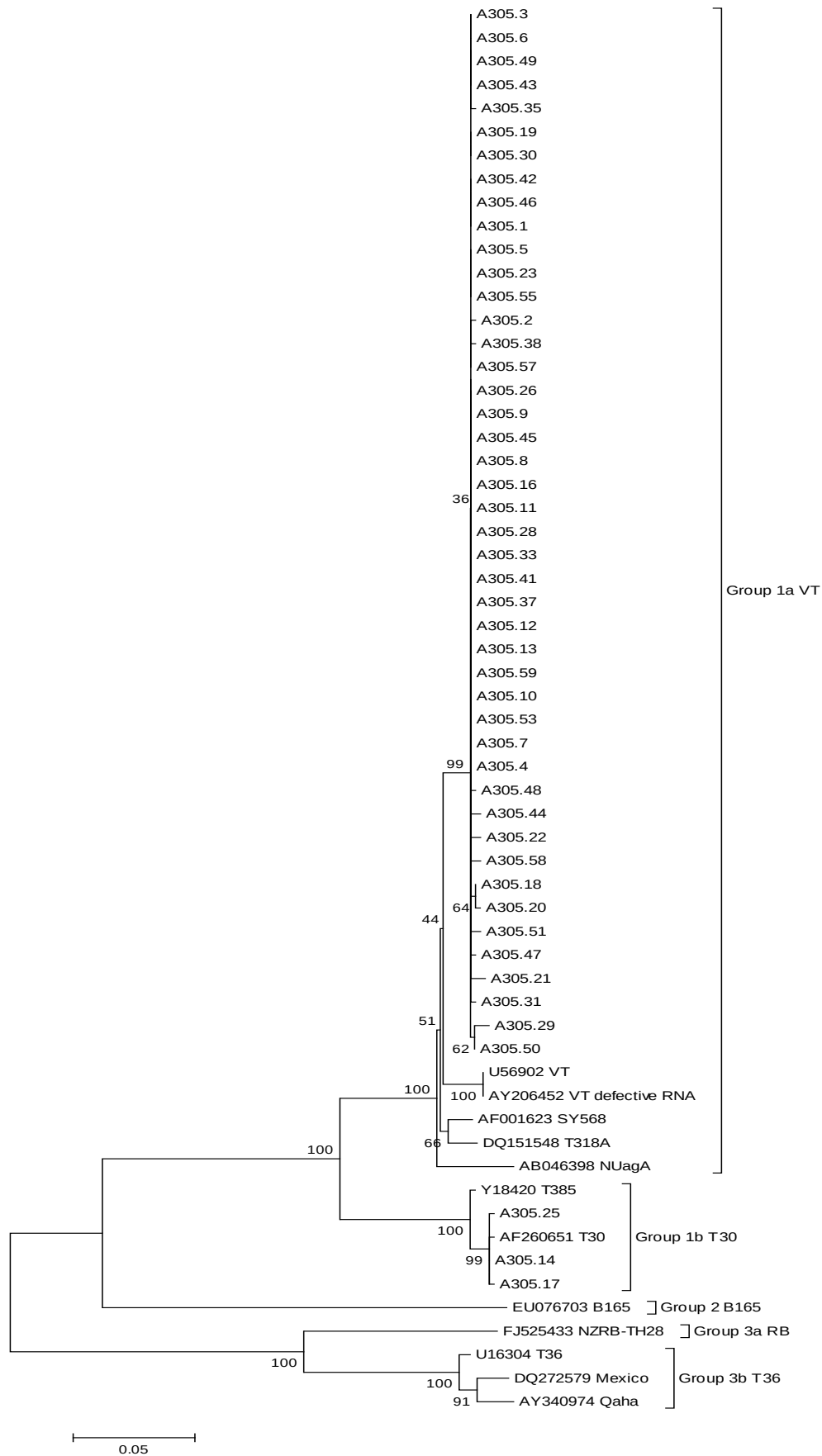


Figure 2.2: Phylogenetic tree of the A-fragment of sample replicate 2 of GFMS 12 on Flame. All groups share a 95-99% intragroup sequence similarity. Group 1a contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the T30 reference contigs. Group 2 contains clones grouping with the B165 reference contig. Group 3a contains clones grouping with the New Zealand Resistance Breaking reference contig. Group 3b contains clones grouping with the T36 reference contigs. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

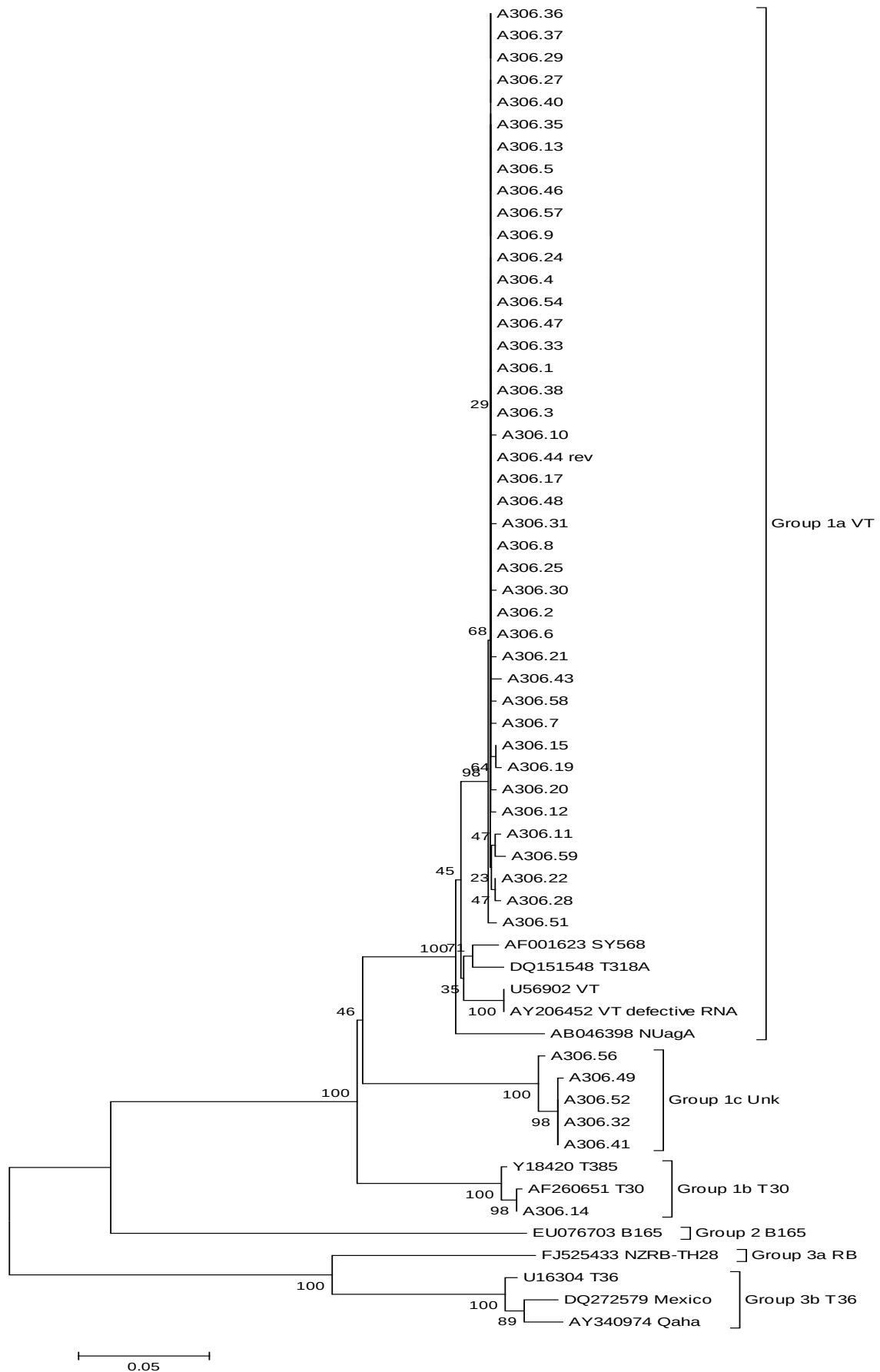


Figure 2.3: Phylogenetic tree of the A-fragment of sample replicate 3 of GFMS 12 on Flame. . All groups share a 95-99% intragroup sequence similarity. Group 1a contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the T30 reference contigs. Group 1c contains clones grouping none of the available reference contigs. Group 2 contains clones grouping with the B165 reference contig. Group 3a contains clones grouping with the New Zealand Resistance Breaking reference contig. Group 3b contains clones grouping with the T36 reference contigs. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

Appendix 3

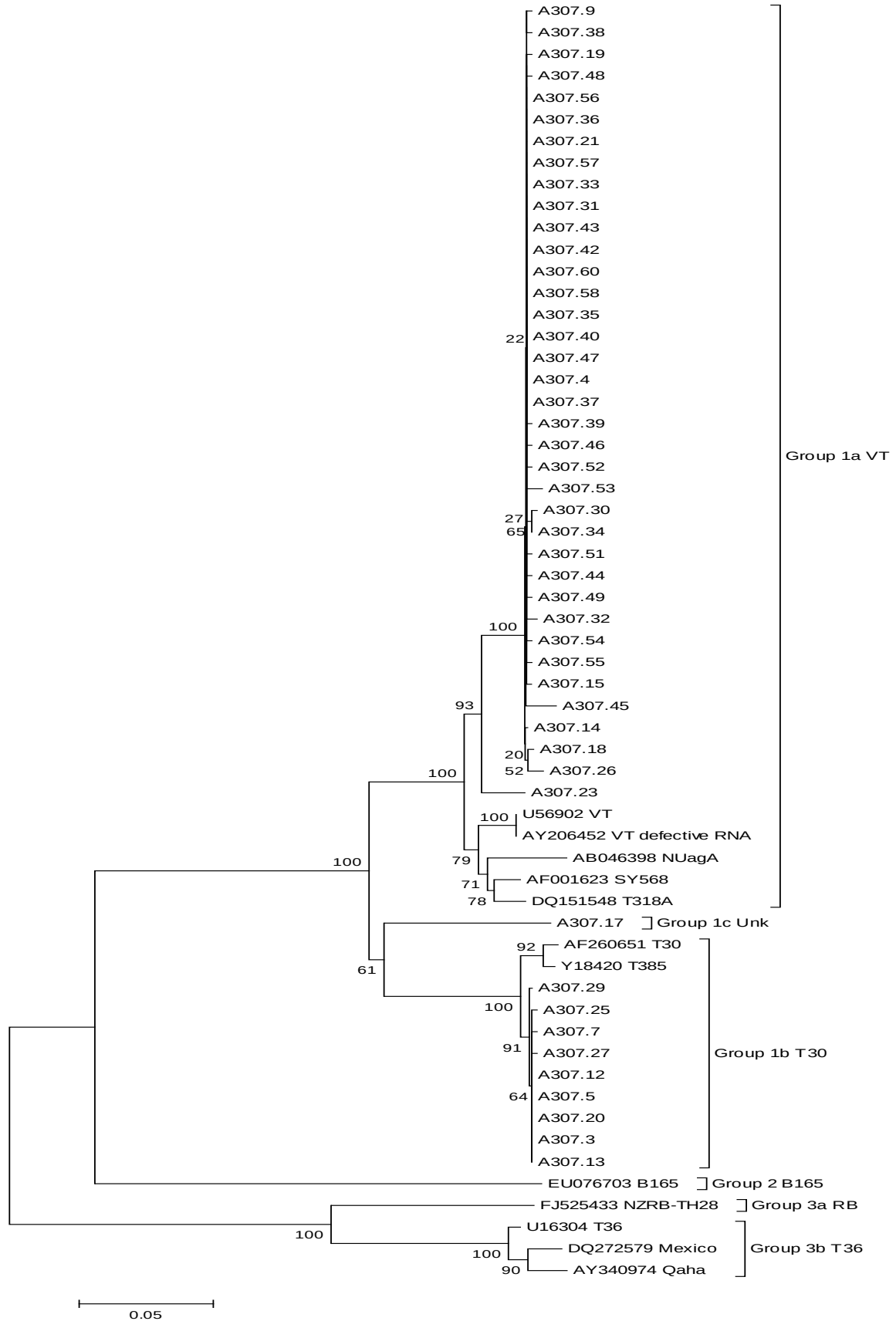


Figure 3.1: Phylogenetic tree of the A-fragment of sample replicate 1 of GFMS 35 on Star Ruby. All groups share a 95-99% intragroup sequence similarity. Group 1a contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the T30 reference contigs. Group 1c contains clones grouping none of the available reference contigs. Group 2 contains clones grouping with the B165 reference contig. Group 3a contains clones grouping with the New Zealand Resistance Breaking reference contig. Group 3b contains clones grouping with the T36 reference contigs. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

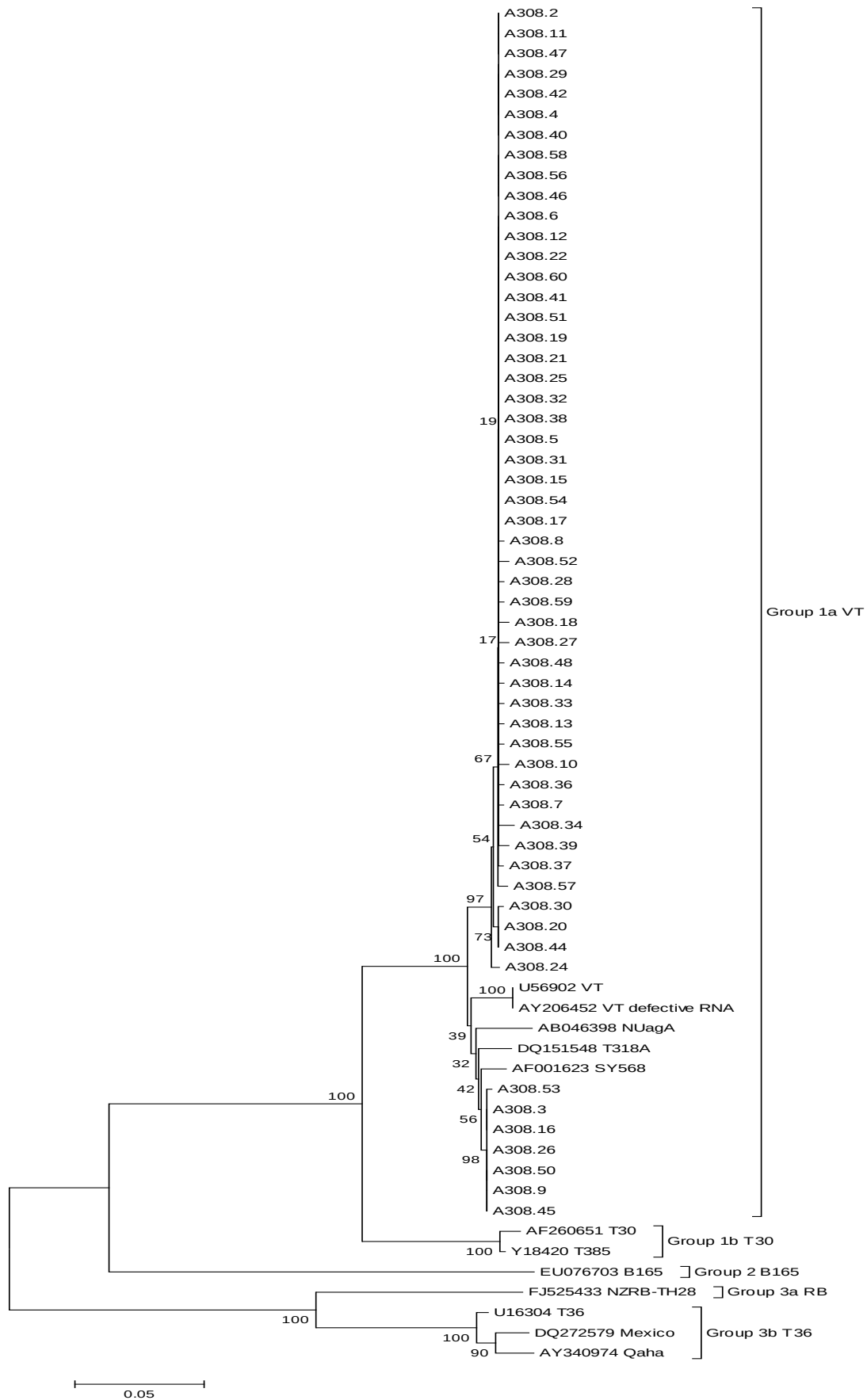


Figure 3.2: Phylogenetic tree of the A-fragment of sample replicate 2 of GFMS 35 on Star Ruby. All groups share a 95-99% intragroup sequence similarity. Group 1a contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the T30 reference contigs. Group 2 contains clones grouping with the B165 reference contig. Group 3a contains clones grouping with the New Zealand Resistance Breaking reference contig. Group 3b contains clones grouping with the T36 reference contigs. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

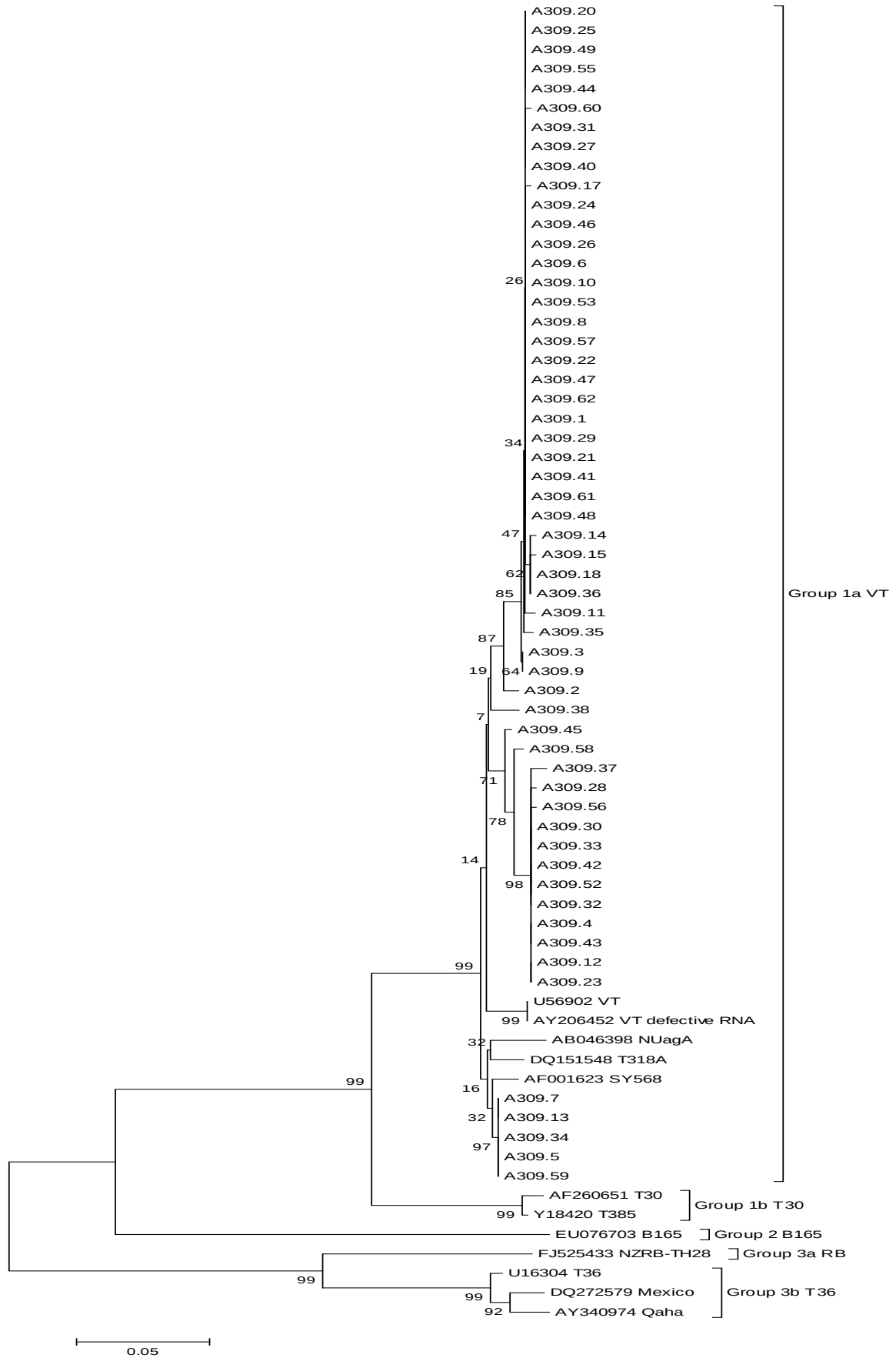


Figure 3.3: Phylogenetic tree of the A-fragment of sample replicate 3 of GFMS 35 on Star Ruby. All groups share a 95-99% intragroup sequence similarity. Group 1a contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the T30 reference contigs. Group 2 contains clones grouping with the B165 reference contig. Group 3a contains clones grouping with the New Zealand Resistance Breaking reference contig. Group 3b contains clones grouping with the T36 reference contigs. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

Appendix 4

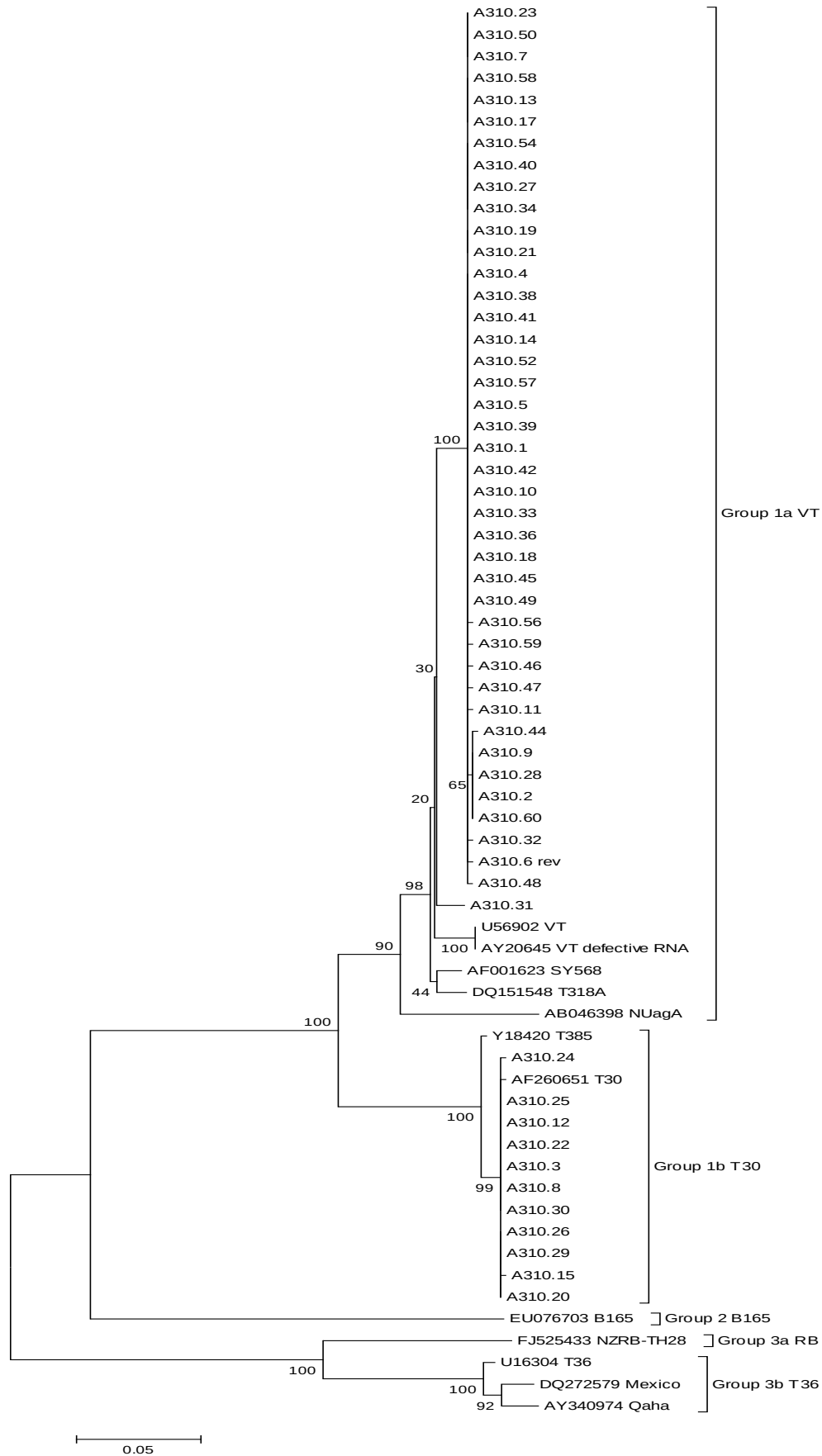


Figure 4.1: Phylogenetic tree of the A-fragment of sample replicate 1 of GFMS 35 on Flame. All groups share a 95-99% intragroup sequence similarity. Group 1a contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the T30 reference contigs. Group 2 contains clones grouping with the B165 reference contig. Group 3a contains clones grouping with the New Zealand Resistance Breaking reference contig. Group 3b contains clones grouping with the T36 reference contigs. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

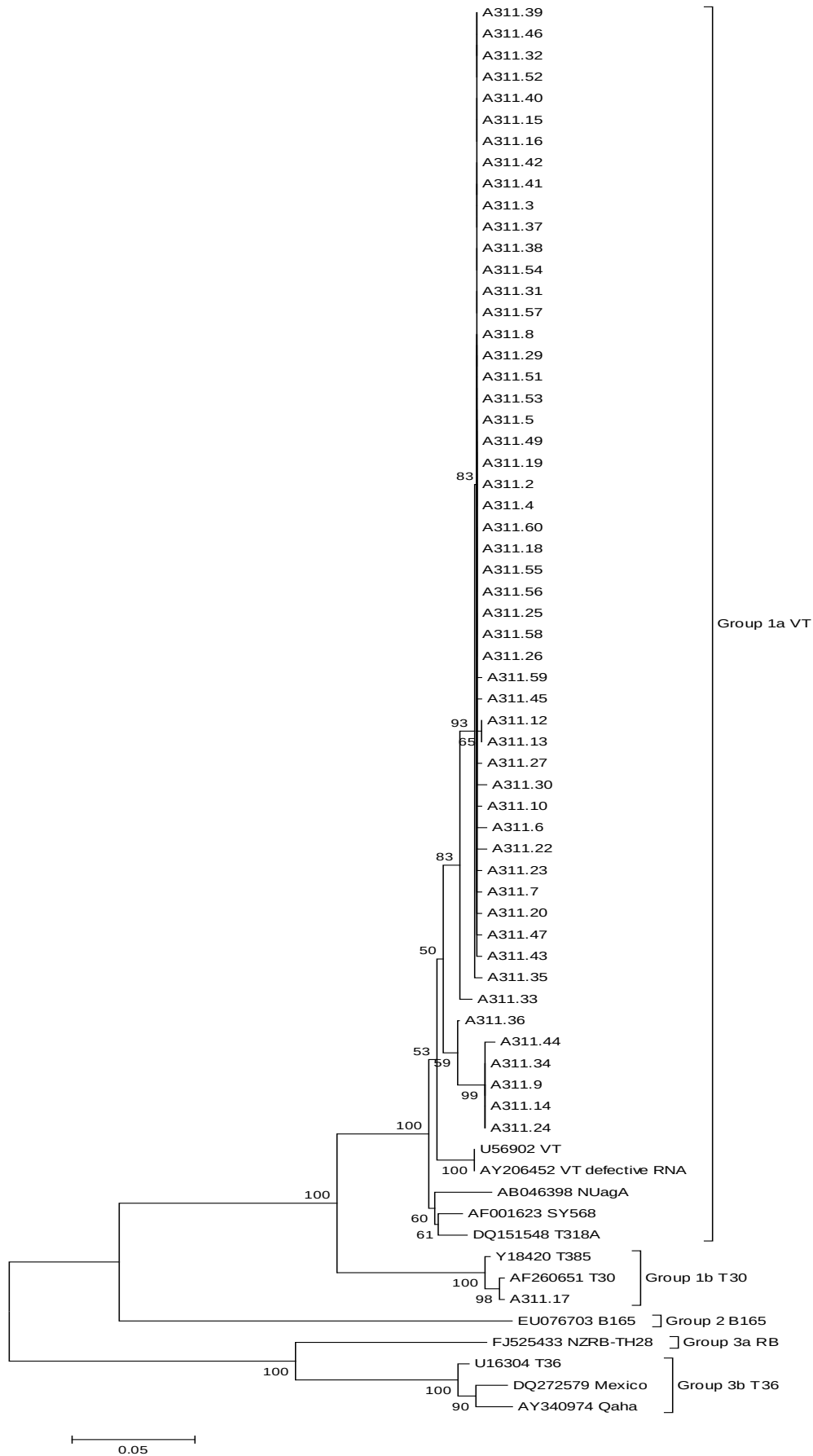


Figure 4.2: Phylogenetic tree of the A-fragment of sample replicate 2 of GFMS 35 on Flame. All groups share a 95-99% intragroup sequence similarity. Group 1a contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the T30 reference contigs. Group 2 contains clones grouping with the B165 reference contig. Group 3a contains clones grouping with the New Zealand Resistance Breaking reference contig. Group 3b contains clones grouping with the T36 reference contigs. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

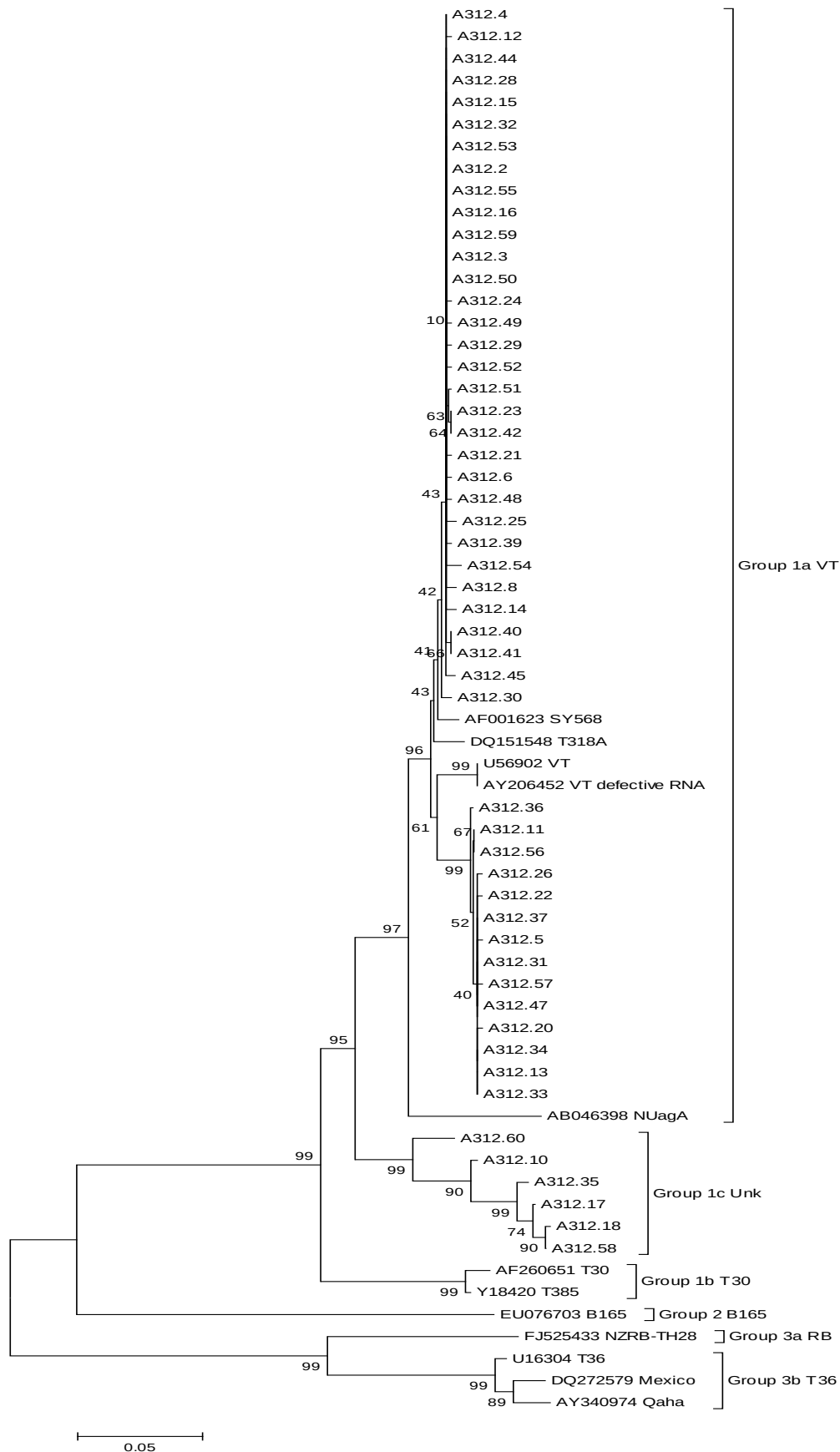


Figure 4.3: Phylogenetic tree of the A-fragment of sample replicate 3 of GFMS 35 on Flame. All groups share a 95-99% intragroup sequence similarity. Group 1a contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the T30 reference contigs. Group 1c contains clones grouping none of the available reference contigs. Group 2 contains clones grouping with the B165 reference contig. Group 3a contains clones grouping with the New Zealand Resistance Breaking reference contig. Group 3b contains clones grouping with the T36 reference contigs. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

Appendix 5

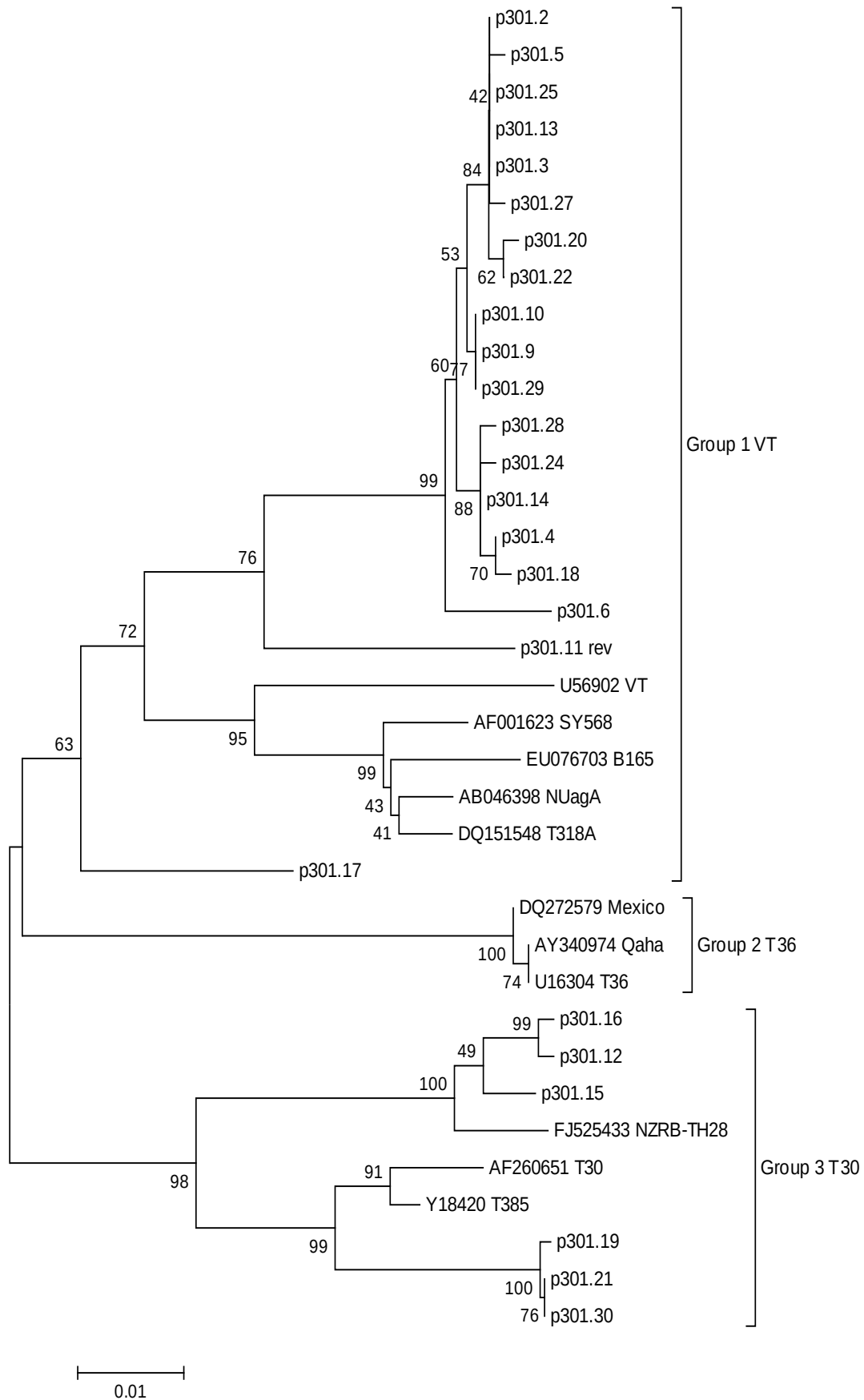


Figure 5.1: Phylogenetic tree of the p23 gene region of sample replicate 1 of GFMS 12 on Star Ruby. All groups share a 90-99% intragroup sequence similarity. Group 1 contains clones grouping with the VT reference contigs. Group 2 contains clones grouping with the T36 reference contigs. Group 3 contains clones grouping with the T30 reference contig. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

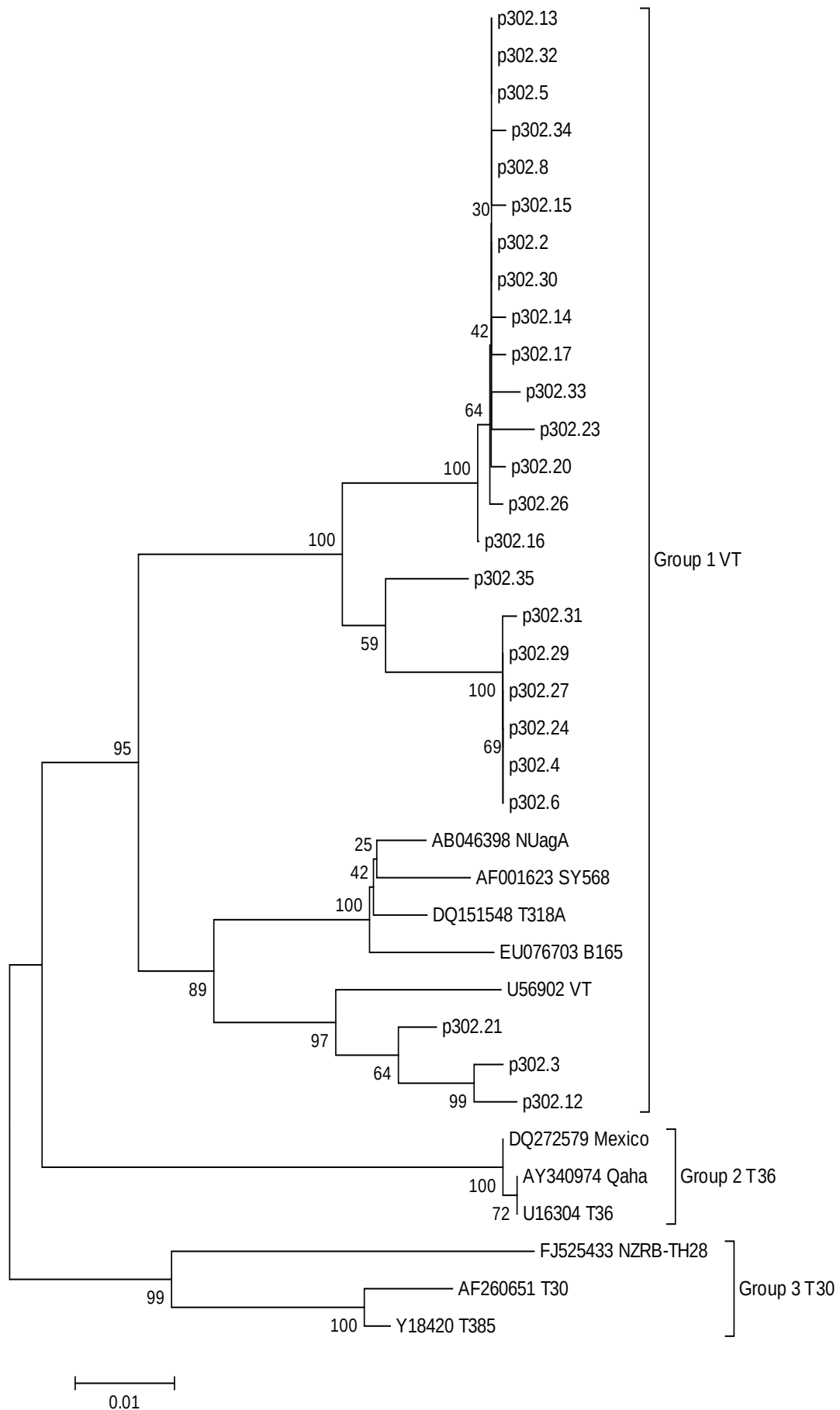


Figure 5.2: Phylogenetic tree of the p23 gene region of sample replicate 2 of GFMS 12 on Star Ruby. All groups share a 90-99% intragroup sequence similarity. Group 1 contains clones grouping with the VT reference contigs. Group 2 contains clones grouping with the T36 reference contigs. Group 3 contains clones grouping with the T30 reference contig. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

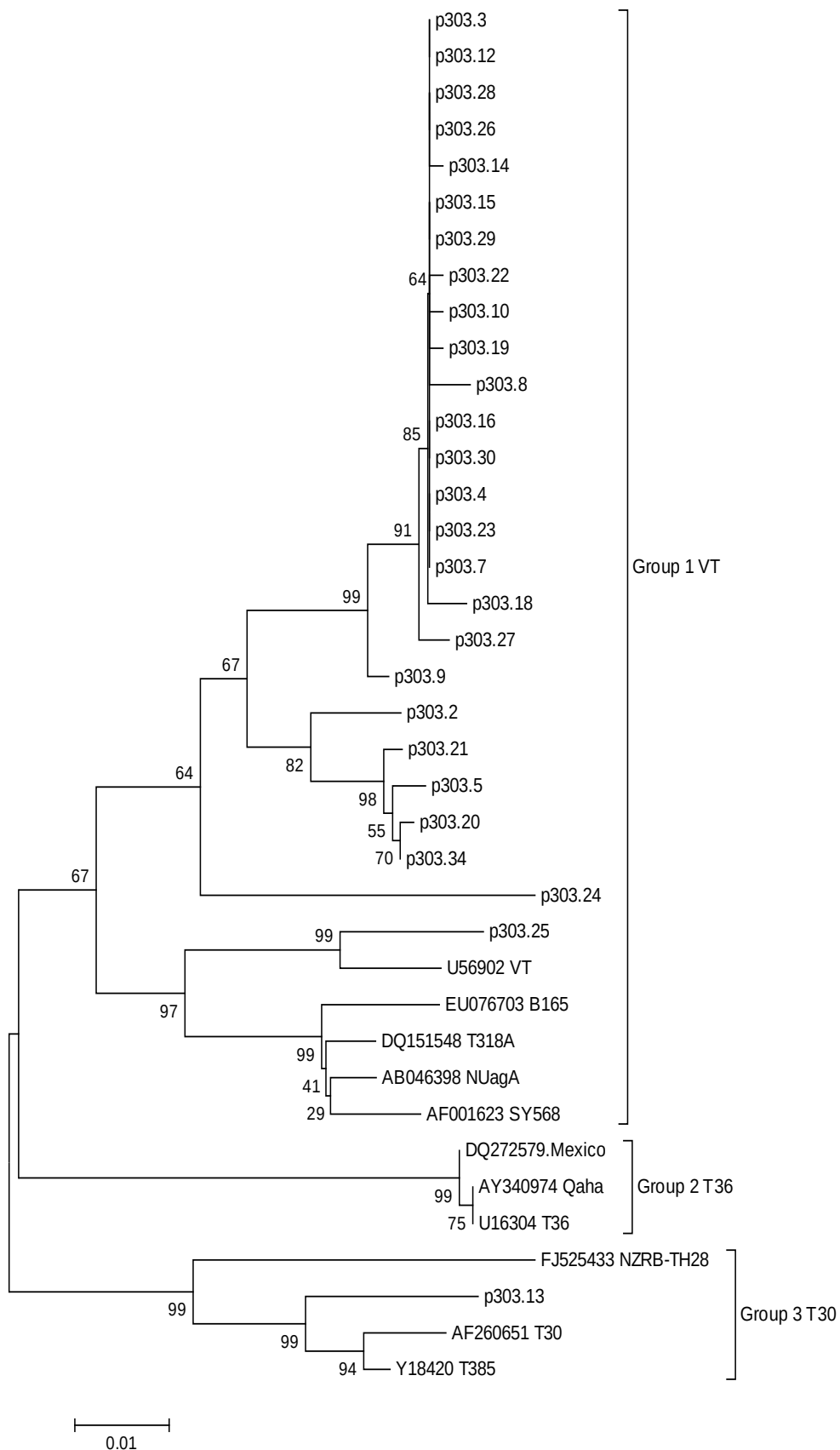


Figure 5.3: Phylogenetic tree of the p23 gene region of sample replicate 3 of GFMS 12 on Star Ruby. All groups share a 90-99% intragroup sequence similarity. Group 1 contains clones grouping with the VT reference contigs. Group 2 contains clones grouping with the T36 reference contigs. Group 3 contains clones grouping with the T30 reference contig. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

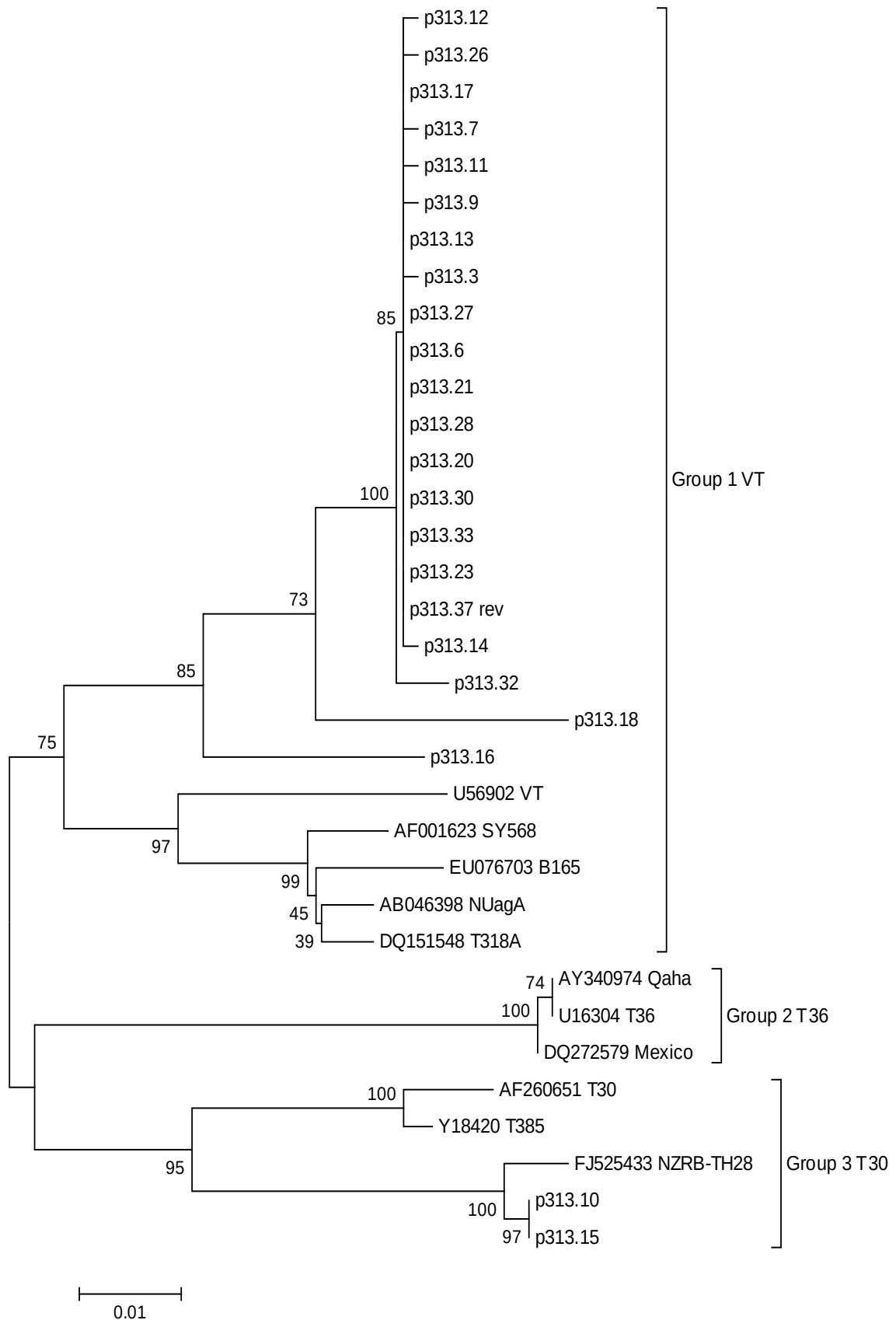


Figure 5.4: Phylogenetic tree of the p23 gene region of sample replicate 4 of GFMS 12 on Star Ruby. All groups share a 90-99% intragroup sequence similarity. Group 1 contains clones grouping with the VT reference contigs. Group 2 contains clones grouping with the T36 reference contigs. Group 3 contains clones grouping with the T30 reference contig. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

Appendix 6

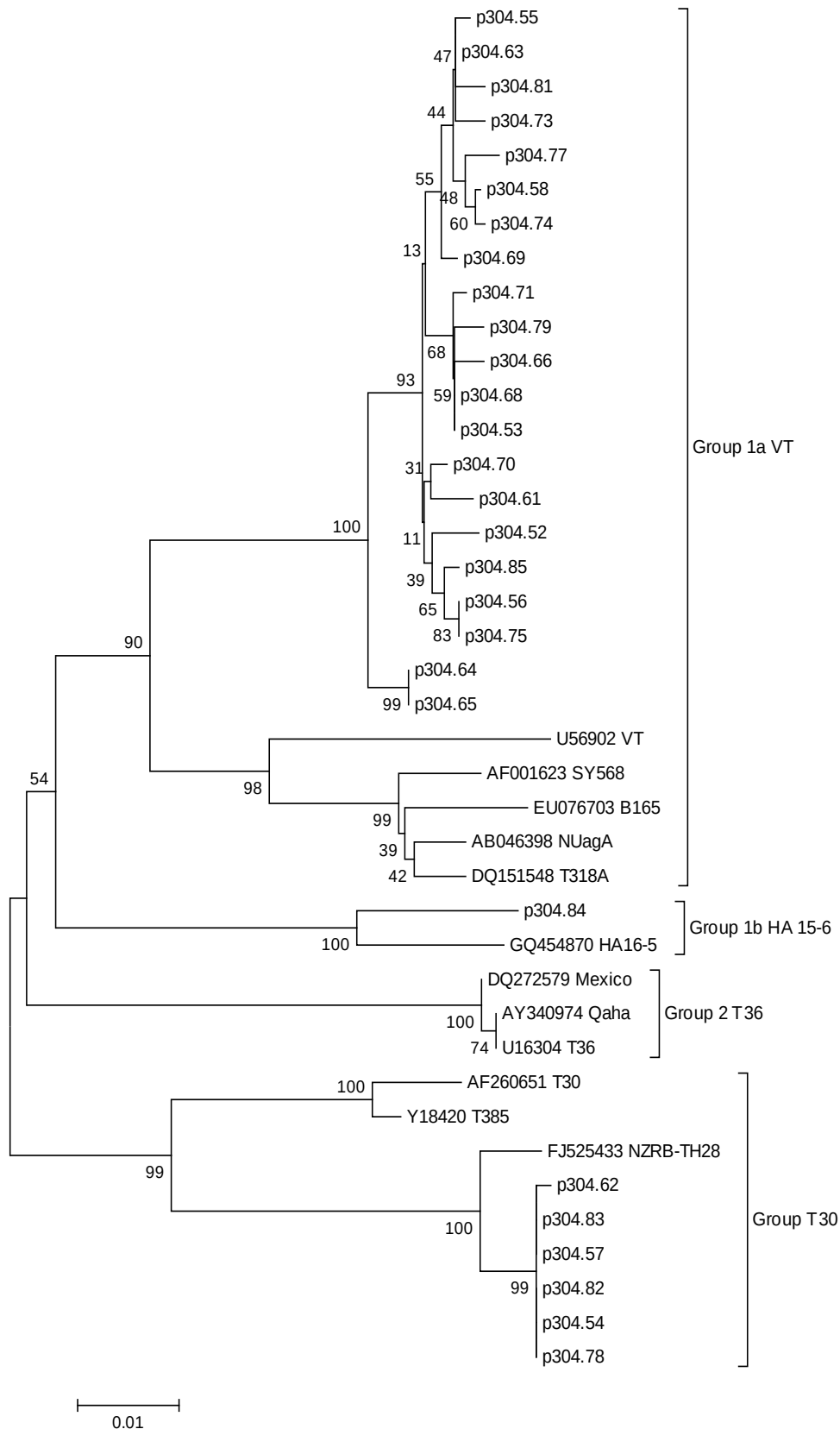


Figure 6.1: Phylogenetic tree of the p23 gene region of sample replicate 1 of GFMS 12 on Flame. All groups share a 90-99% intragroup sequence similarity. Group 1 contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the HA15-6 reference contig. Group 2 contains clones grouping with the T36 reference contigs. Group 3 contains clones grouping with the T30 reference contig. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

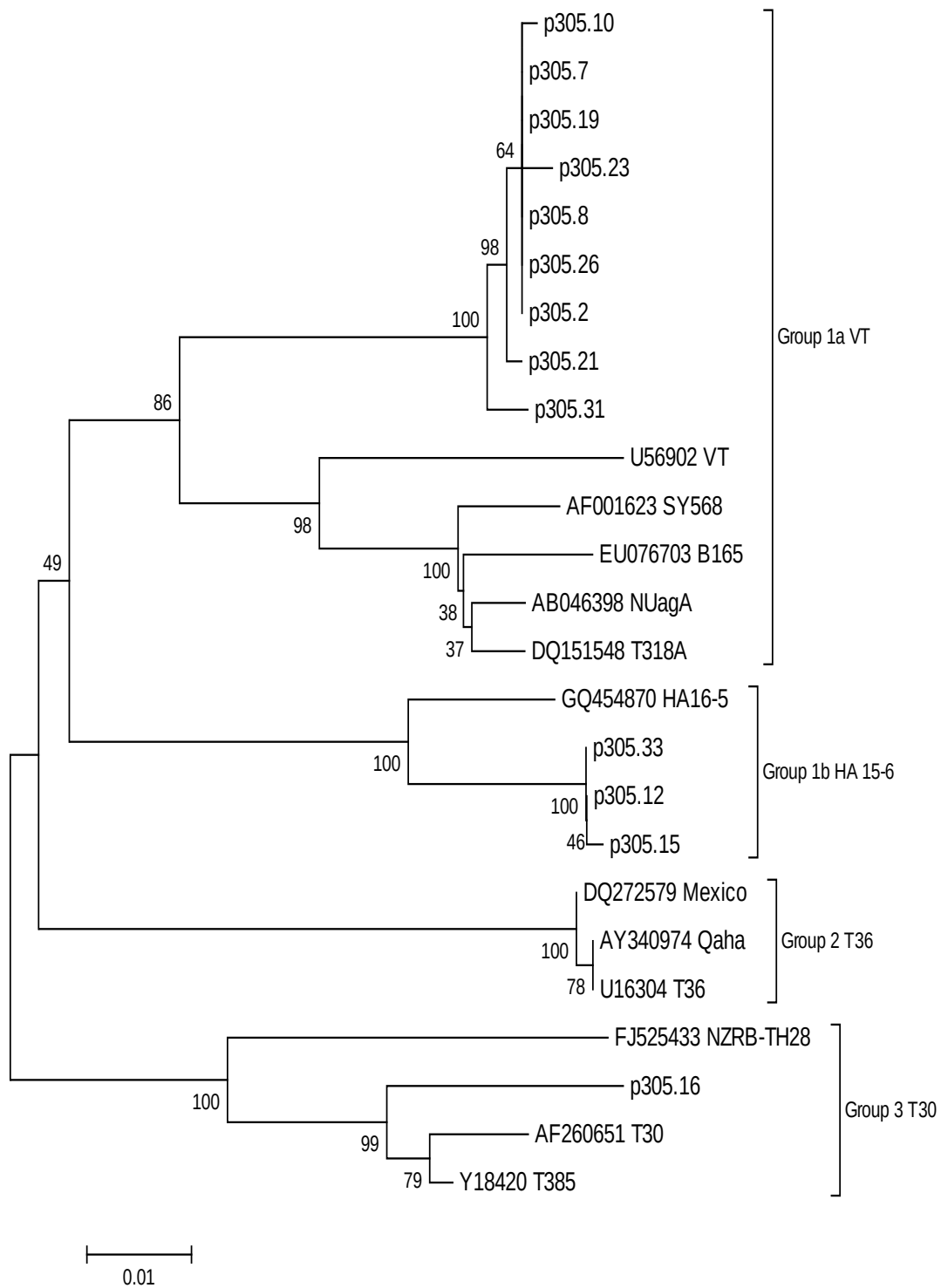


Figure 6.2: Phylogenetic tree of the p23 gene region of sample replicate 2 of GFMS 12 on Flame. All groups share a 90-99% intragroup sequence similarity. Group 1 contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the HA15-6 reference contig. Group 2 contains clones grouping with the T36 reference contigs. Group 3 contains clones grouping with the T30 reference contig. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

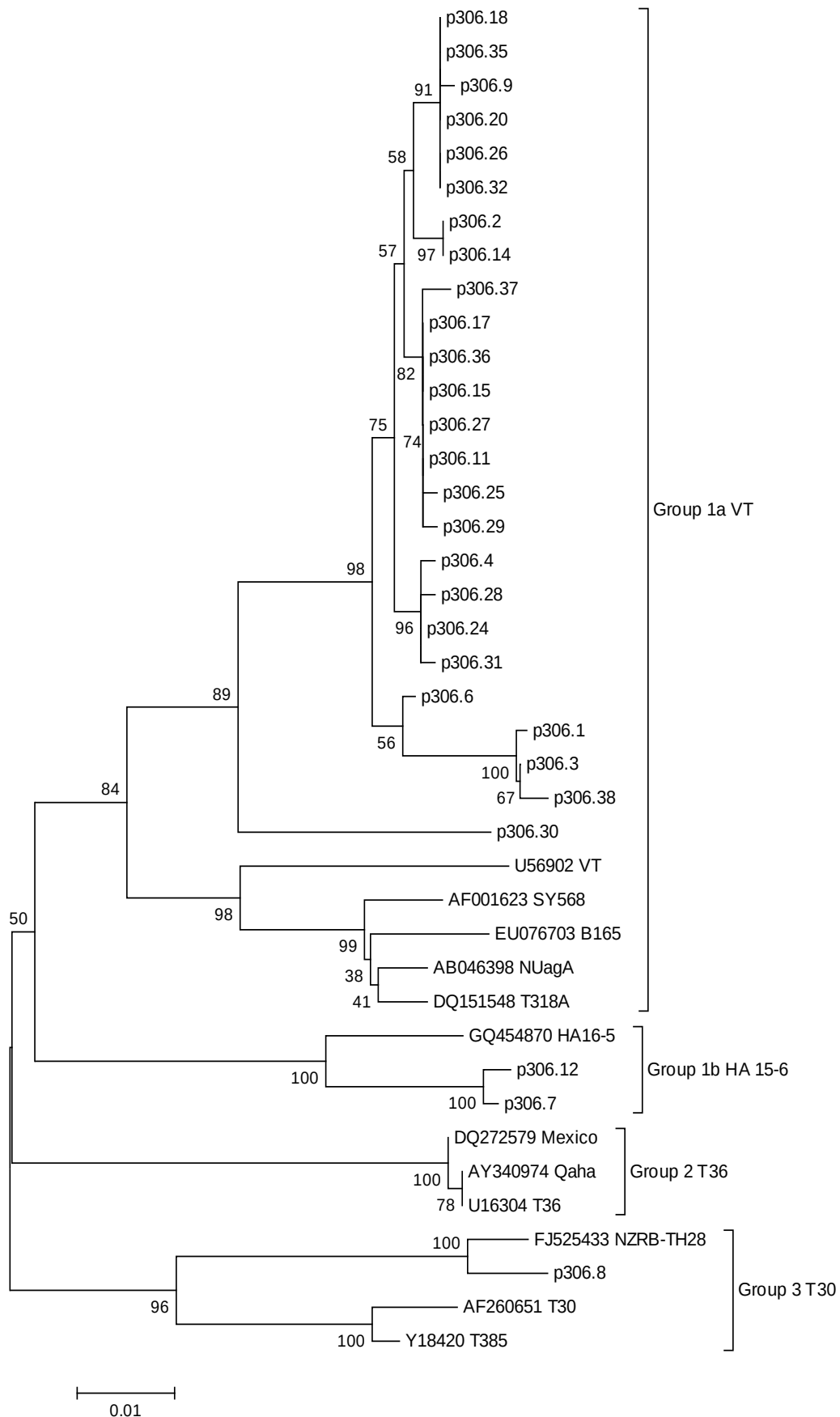


Figure 6.3: Phylogenetic tree of the p23 gene region of sample replicate 3 of GFMS 12 on Flame. All groups share a 90-99% intragroup sequence similarity. Group 1 contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the HA15-6 reference contig. Group 2 contains clones grouping with the T36 reference contigs. Group 3 contains clones grouping with the T30 reference contig. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

Appendix 7

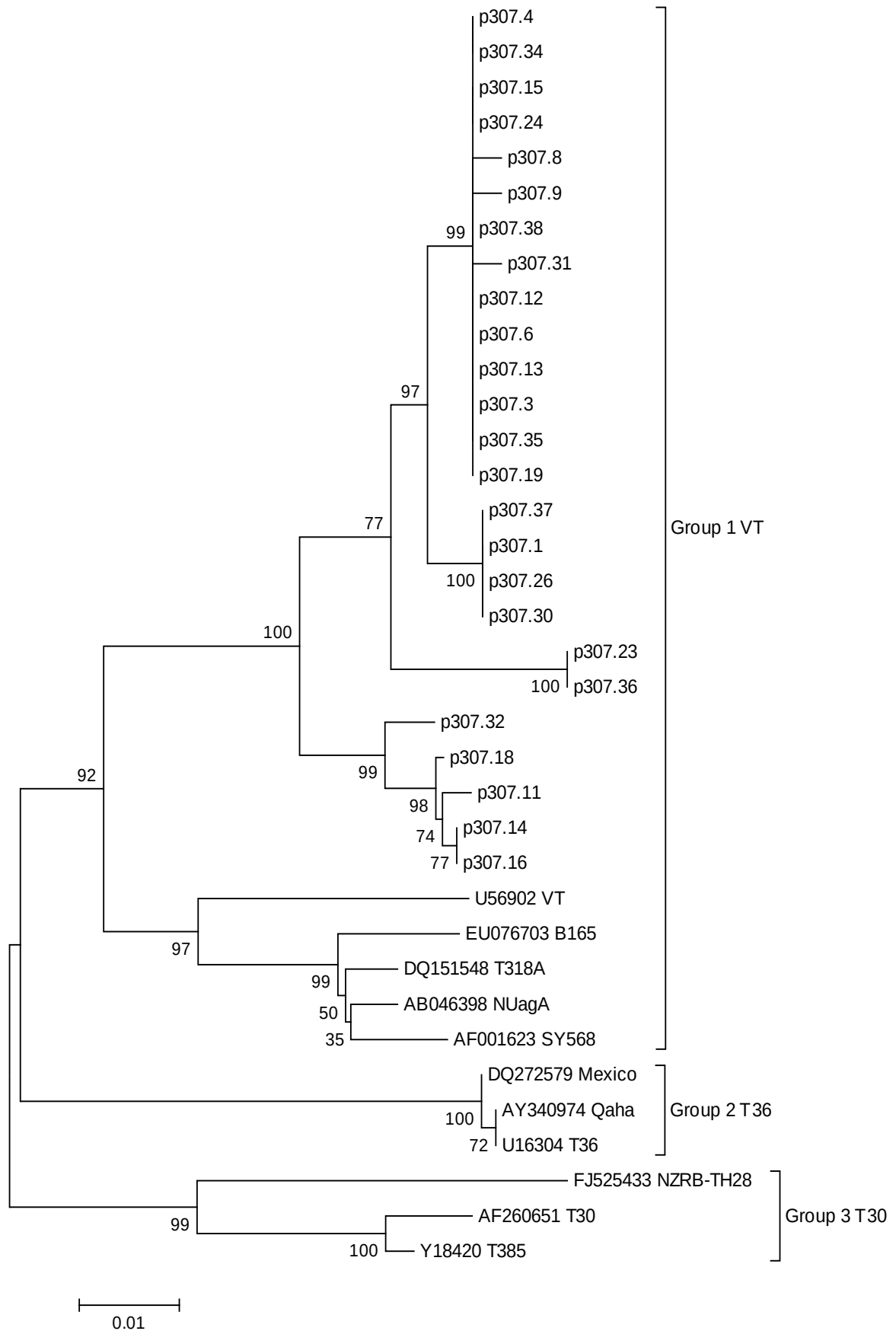


Figure 7.1: Phylogenetic tree of the p23 gene region of sample replicate 1 of GFMS 35 on Star Ruby. All groups share a 90-99% intragroup sequence similarity. Group 1 contains clones grouping with the VT reference contigs. Group 2 contains clones grouping with the T36 reference contigs. Group 3 contains clones grouping with the T30 reference contig. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

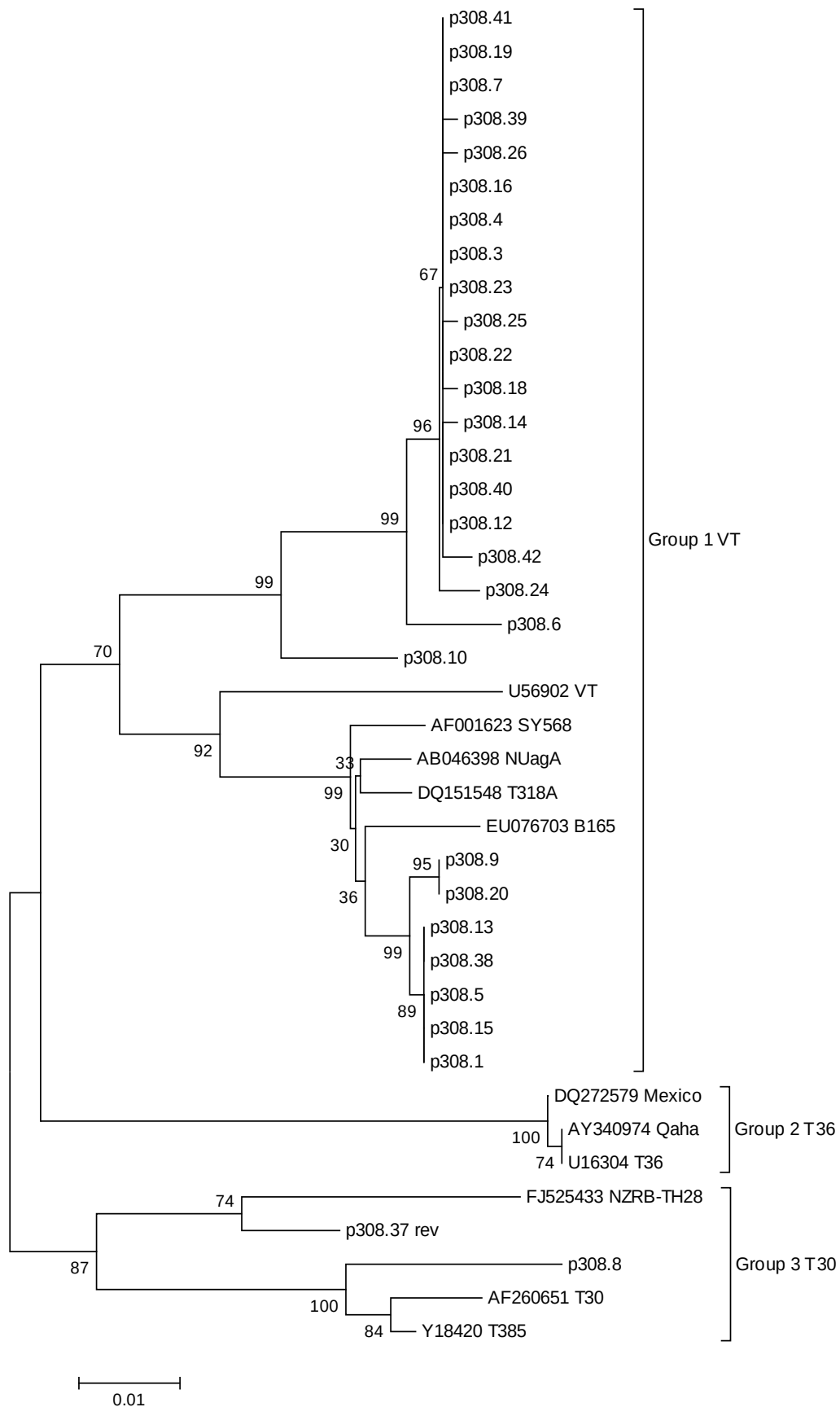


Figure 7.2: Phylogenetic tree of the p23 gene region of sample replicate 2 of GFMS 35 on Star Ruby. All groups share a 90-99% intragroup sequence similarity. Group 1 contains clones grouping with the VT reference contigs. Group 2 contains clones grouping with the T36 reference contigs. Group 3 contains clones grouping with the T30 reference contig. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

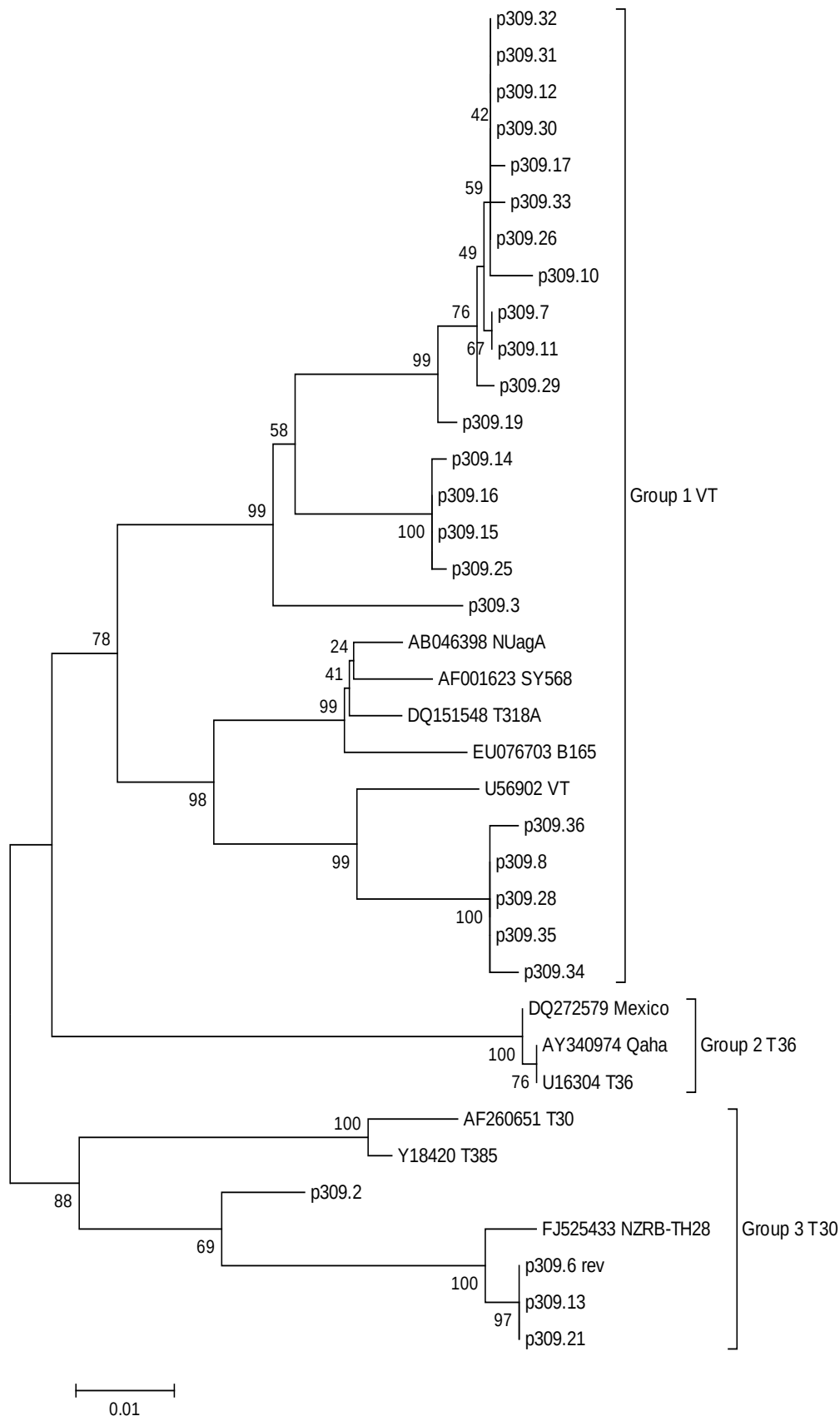


Figure 7.3: Phylogenetic tree of the p23 gene region of sample replicate 3 of GFMS 35 on Star Ruby. All groups share a 90-99% intragroup sequence similarity. Group 1 contains clones grouping with the VT reference contigs. Group 2 contains clones grouping with the T36 reference contigs. Group 3 contains clones grouping with the T30 reference contig. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

Appendix 8

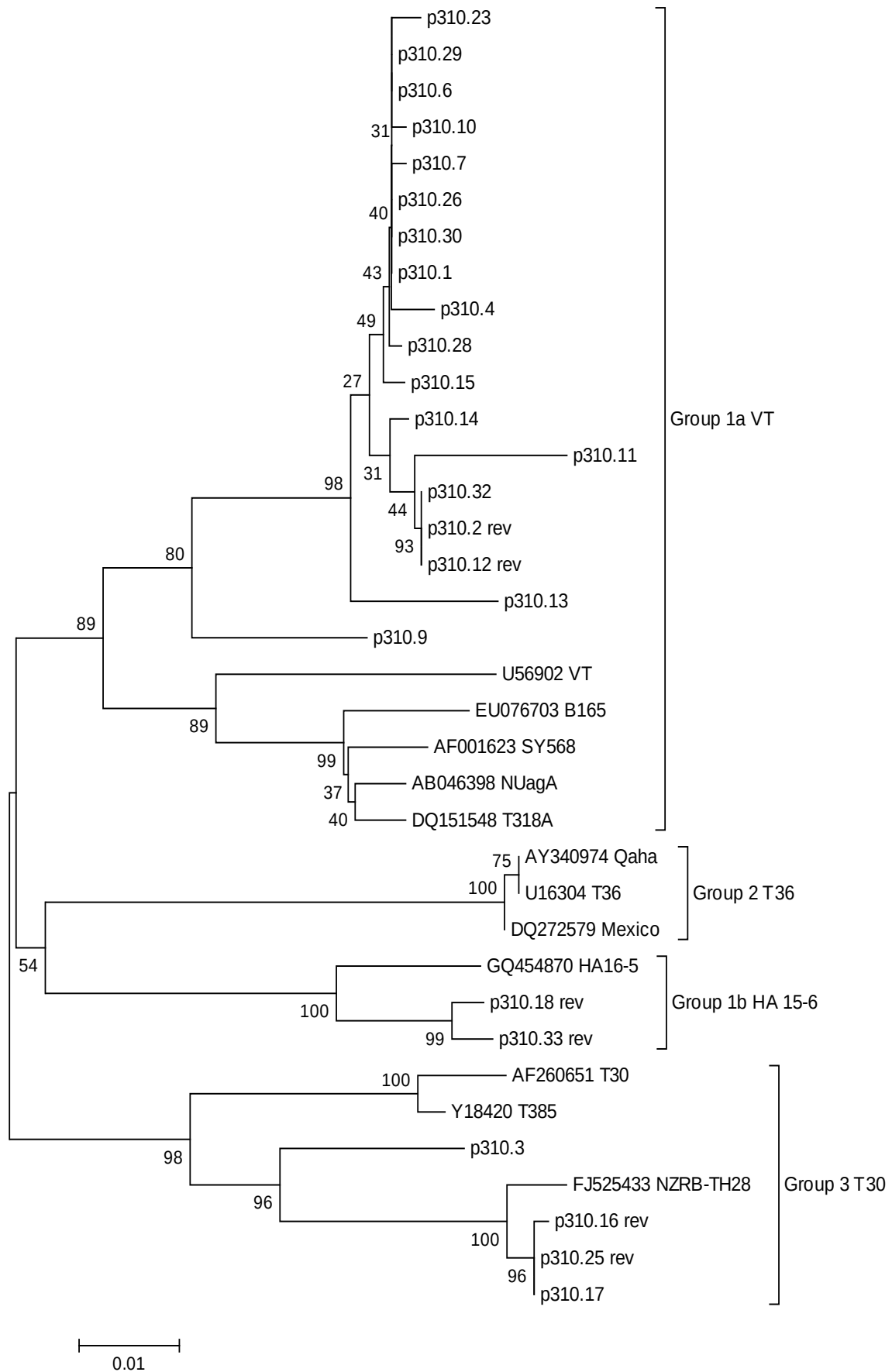


Figure 8.1: Phylogenetic tree of the p23 gene region of sample replicate 1 of GFMS 35 on Flame. All groups share a 90-99% intragroup sequence similarity. Group 1 contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the HA15-6 reference contig. Group 2 contains clones grouping with the T36 reference contigs. Group 3 contains clones grouping with the T30 reference contig. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

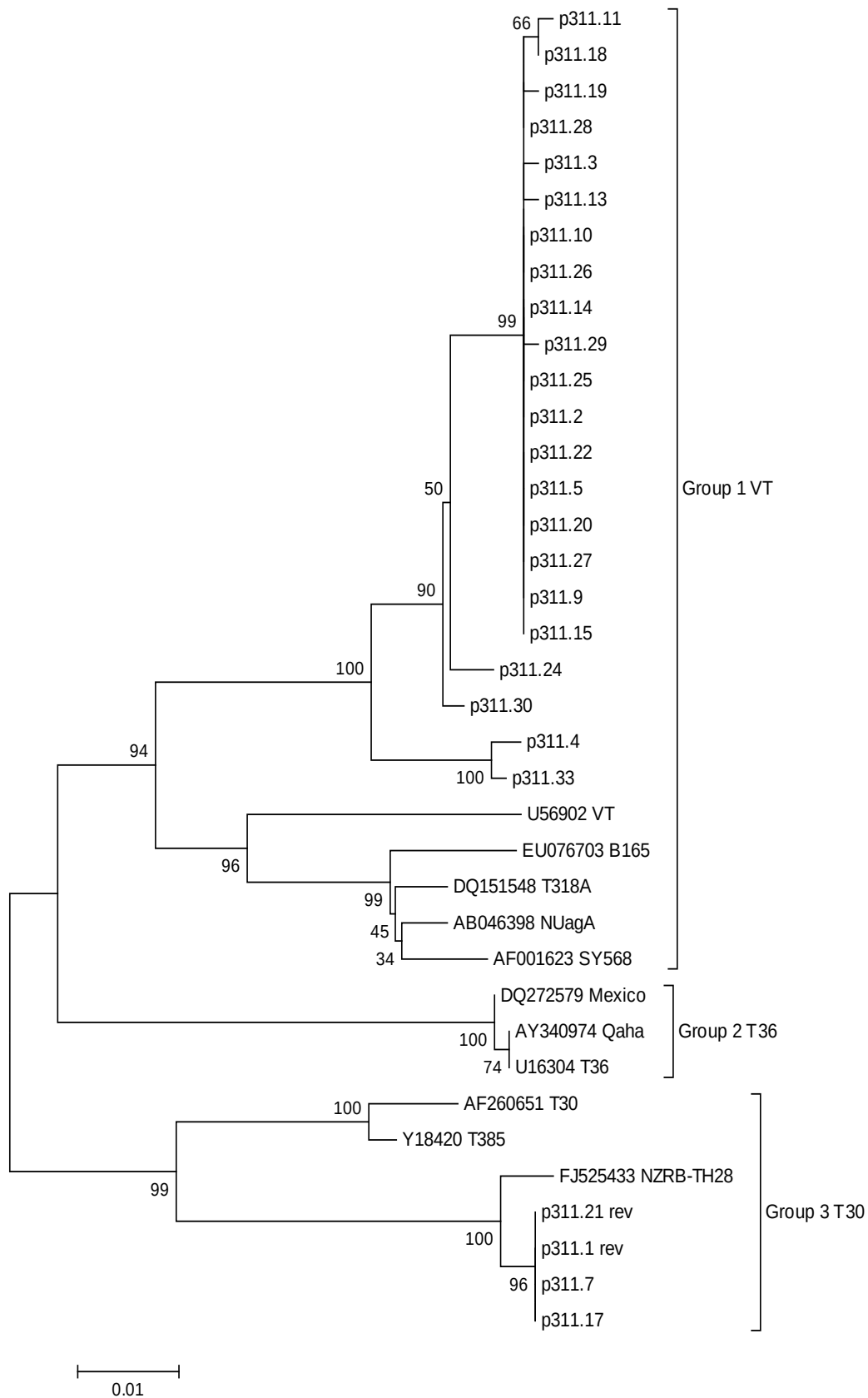


Figure 8.2: Phylogenetic tree of the p23 gene region of sample replicate 2 of GFMS 35 on Flame. All groups share a 90-99% intragroup sequence similarity. Group 1 contains clones grouping with the VT reference contigs. Group 2 contains clones grouping with the T36 reference contigs. Group 3 contains clones grouping with the T30 reference contig. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

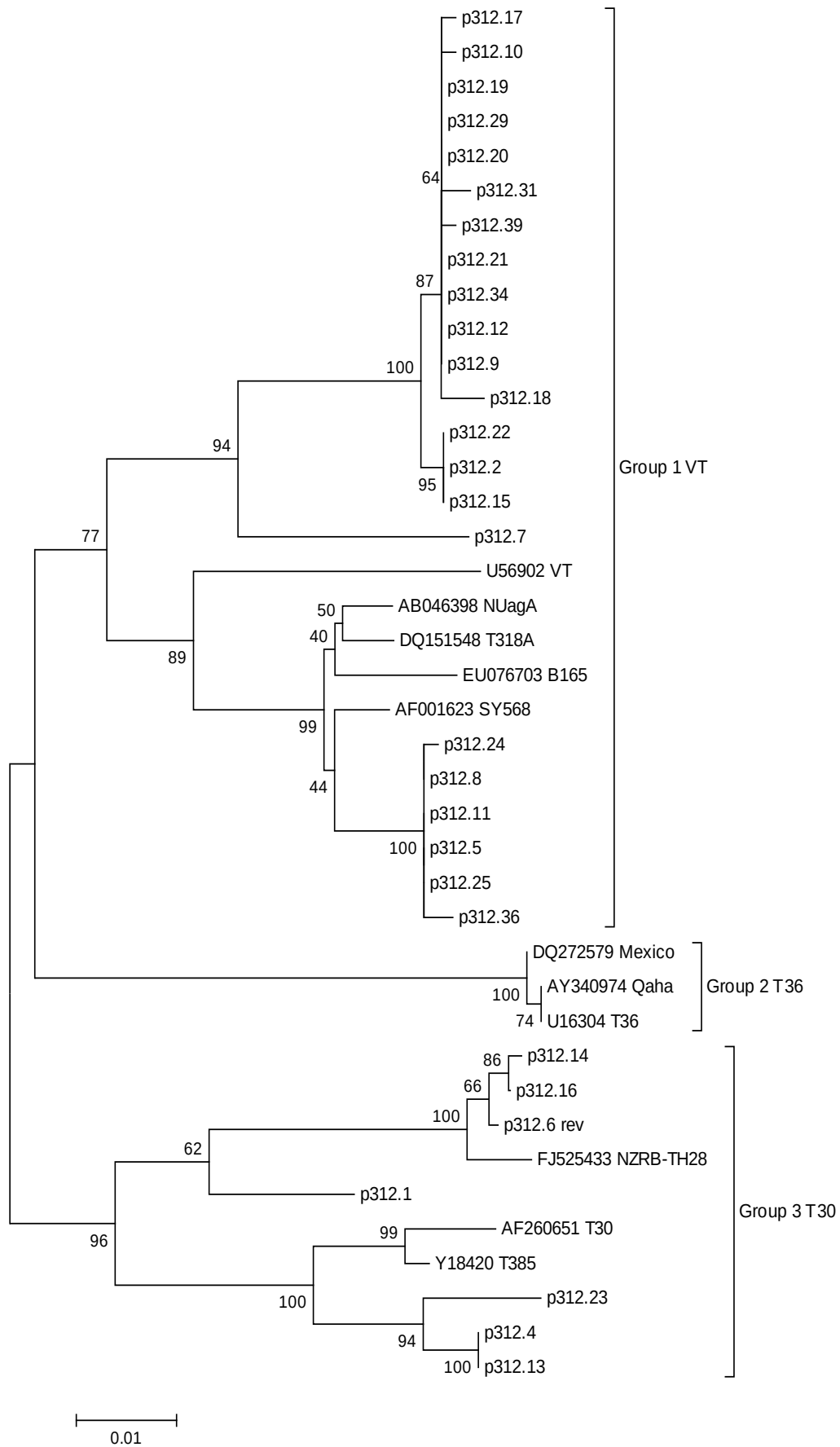


Figure 8.3: Phylogenetic tree of the p23 gene region of sample replicate 3 of GFMS 35 on Flame. All groups share a 90-99% intragroup sequence similarity. Group 1 contains clones grouping with the VT reference contigs. Group 2 contains clones grouping with the T36 reference contigs. Group 3 contains clones grouping with the T30 reference contig. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

Appendix 9

Table 9: Nucleotide diversities within groups for the A-region of the field samples. The values within brackets represent the percentage nucleotide distance.

Sample number	Group 1a VT	Group 1b T30	Group 1c Unk	Group 2 B165	Group 3a RB	Group 3b T36
301	0.013 (1.3%)	0.012 (1.2%)	0.003 (0.3%)	n/c	n/c	0.027 (2.7%)
302	0.015 (1.5%)	0.01 (1%)	-	n/c	n/c	0.026 (2.6%)
303	0.021 (2.1%)	0.008 (0.8%)	-	n/c	n/c	0.026 (2.6%)
304	0.009 (0.9%)	0.007 (0.7%)	-	n/c	n/c	0.026 (2.6%)
305	0.008 (0.8%)	0.006 (0.6%)	-	n/c	n/c	0.027 (2.7%)
306	0.008 (0.8%)	0.007 (0.7%)	0.005 (0.5%)	n/c	n/c	0.026 (2.6%)
307	0.015 (1.5%)	0.007 (0.7%)	n/c	n/c	n/c	0.026 (2.6%)
308	0.011 (1.1%)	0.01 (1%)	-	n/c	n/c	0.026 (2.6%)
309	0.018 (1.8%)	0.01 (1%)	-	n/c	n/c	0.026 (2.6%)
310	0.01 (1%)	0.002 (0.2%)	-	n/c	n/c	0.026 (2.6%)
311	0.013 (1.3%)	0.008 (0.8%)	-	n/c	n/c	0.026 (2.6%)
312	0.018 (1.8%)	0.012 (1.2%)	0.027 (2.7%)	n/c	n/c	0.026 (2.6%)
313	0.01 (1%)	0.01 (1%)	0.007 (0.7%)	n/c	n/c	0.026 (2.6%)

Table 10: Nucleotide diversities within groups for the p23 gene region of the field samples. The values within brackets represent the percentage nucleotide distance.

Sample number	Group 1 VT	Group 1b HA 15-6	Group 2 T36	Group 3 T30
301	0.033 (3.3%)	-	0.001 (0.1%)	0.042 (4.2%)
302	0.039 (3.9%)	-	0.001 (0.1%)	0.045 (4.5%)
303	0.036 (3.6%)	-	0.001 (0.1%)	0.043 (4.3%)
304	0.028 (2.8%)	0.03 (3%)	0.001 (0.1%)	0.026 (2.6%)
305	0.039 (3.9%)	0.016 (1.6%)	0.001 (0.1%)	0.046 (4.6%)
306	0.028 (2.8%)	0.023 (2.3%)	0.001 (0.1%)	0.046 (4.6%)
307	0.033 (3.3%)	-	0.001 (0.1%)	0.045 (4.5%)
308	0.035 (3.5%)	-	0.001 (0.1%)	0.055 (5.5%)
309	0.047 (4.7%)	-	0.001 (0.1%)	0.04 (4%)
310	0.031 (3.1%)	0.022 (2.2%)	0.001 (0.1%)	0.039 (3.9%)
311	0.027 (2.7%)	-	0.001 (0.1%)	0.031 (3.1%)
312	0.039 (3.9%)	-	0.001 (0.1%)	0.047 (4.7%)
313	0.028 (2.8%)	-	0.001 (0.1%)	0.04 (4%)

Appendix 10

Table 11: Nucleotide diversities between groups for the A-region of the field sample 301. The values within brackets represent the percentage nucleotide distance.

	1	2	3	4	5	6
1 Group 1a VT						
2 Group 1b T30	0.113 (11.3%)					
3 Group 1c Unk	0.128 (12.8%)	0.14 (14%)				
4 Group 2 B165	0.313 (31.3%)	0.317 (31.7%)	0.327 (32.7%)			
Group 3a RB	0.391 (39.1%)	0.399 (39.9%)	0.406 (40.6%)	0.397 (39.7%)		
Group 3b T36	0.389 (38.9%)	0.401 (40.1%)	0.406 (40.6%)	0.404 (40.4%)	0.155 (15.5%)	

Table 12: Nucleotide diversities between groups for the A-region of the field sample 302. The values within brackets represent the percentage nucleotide distance.

	1	2	3	4	5
1 Group 1a VT					
2 Group 1b T30	0.121 (12.1%)				
4 Group 2 B165	0.307 (30.7%)	0.309 (30.9%)			
Group 3a RB	0.391 (39.1%)	0.402 (40.2%)	0.396 (39.6%)		
Group 3b T36	0.39 (39%)	0.402 (40.2%)	0.403 (40.3%)	0.158 (15.8%)	

Table 13: Nucleotide diversities between groups for the A-region of the field sample 303. The values within brackets represent the percentage nucleotide distance.

	1	2	3	4	5
1 Group 1a VT					
2 Group 1b T30	0.119 (11.9%)				
4 Group 2 B165	0.308 (30.8%)	0.31 (31%)			
Group 3a RB	0.389 (38.9%)	0.403 (40.3%)	0.395 (39.5%)		
Group 3b T36	0.392 (39.2%)	0.403 (40.3%)	0.395 (39.5%)	0.159 (15.9%)	

Table 14: Nucleotide diversities between groups for the A-region of the field sample 304. The values within brackets represent the percentage nucleotide distance.

	1	2	3	4	5
1 Group 1a VT					
2 Group 1b T30	0.11 (11%)				
4 Group 2 B165	0.307 (30.7%)	0.31 (31%)			
Group 3a RB	0.382 (38.2%)	0.391 (39.1%)	0.397 (39.7%)		
Group 3b T36	0.38 (38%)	0.397 (39.7%)	0.41 (41%)	0.156 (15.6%)	

Table 15: Nucleotide diversities between groups for the A-region of the field sample 305. The values within brackets represent the percentage nucleotide distance.

	1	2	3	4	5
1 Group 1a VT					
2 Group 1b T30	0.117 (11.7%)				
4 Group 2 B165	0.319 (31.9%)	0.316 (31.6%)			
Group 3a RB	0.39 (39%)	0.402 (40.2%)	0.396 (39.6%)		
Group 3b T36	0.39 (39%)	0.403 (40.3%)	0.41 (41%)	0.159 (15.9%)	

Table 16: Nucleotide diversities between groups for the A-region of the field sample 306. The values within brackets represent the percentage nucleotide distance.

	1	2	3	4	5	6
1 Group 1a VT						
2 Group 1b T30	0.113 (11.3%)					
3 Group 1c Unk	0.125 (12.5%)	0.134 (13.4%)				
4 Group 2 B165	0.306 (30.6%)	0.308 (30.8%)	0.324 (32.4%)			
Group 3a RB	0.386 (38.6%)	0.398 (39.8%)	0.408 (40.8%)	0.398 (39.8%)		
Group 3b T36	0.39 (39%)	0.403 (40.3%)	0.417 (41.7%)	0.402 (40.2%)	0.159 (15.9%)	

Table 17: Nucleotide diversities between groups for the A-region of the field sample 307. The values within brackets represent the percentage nucleotide distance.

	1	2	3	4	5	6
1 Group 1a VT						
2 Group 1b T30	0.123 (12.3%)					
3 Group 1c Unk	0.128 (12.8%)	0.119 (11.9%)				
4 Group 2 B165	0.33 (33%)	0.326 (32.6%)	0.341 (34.1%)			
Group 3a RB	0.388 (38.8%)	0.4 (40%)	0.403 (40.3%)	0.388 (38.8%)		
Group 3b T36	0.394 (39.4%)	0.402 (40.2%)	0.406 (40.6%)	0.404 (40.4%)	0.157 (15.7%)	

Table 18: Nucleotide diversities between groups for the A-region of the field sample 308. The values within brackets represent the percentage nucleotide distance.

	1	2	3	4	5
1 Group 1a VT					
2 Group 1b T30	0.113 (11.3%)				
4 Group 2 B165	0.317 (31.7%)	0.313 (31.3%)			
Group 3a RB	0.389 (38.9%)	0.4 (40.%)	0.393 (39.3%)		
Group 3b T36	0.387 (38.7%)	0.4 (40.%)	0.407 (40.7%)	0.158 (15.8%)	

Table 19: Nucleotide diversities between groups for the A-region of the field sample 309. The values within brackets represent the percentage nucleotide distance.

	1	2	3	4	5
1 Group 1a VT					
2 Group 1b T30	0.118 (11.8%)				
3 Group 2 B165	0.316 (31.6%)	0.314 (31.4%)			
4 Group 3a RB	0.389 (38.9%)	0.402 (40.2%)	0.392 (39.2%)		
5 Group 3b T36	0.389 (38.9%)	0.401 (40.1%)	0.392 (39.2%)	0.157 (15.7%)	

Table 20: Nucleotide diversities between groups for the A-region of the field sample 310. The values within brackets represent the percentage nucleotide distance.

	1	2	3	4	5
1 Group 1a VT					
2 Group 1b T30	0.119 (11.9%)				
3 Group 2 B165	0.321 (32.1%)	0.322 (32.2%)			
4 Group 3a RB	0.384 (38.4%)	0.402 (40.2%)	0.396 (39.6%)		
5 Group 3b T36	0.388 (38.8%)	0.408 (40.8%)	0.405 (40.5%)	0.156 (15.6%)	

Table 21: Nucleotide diversities between groups for the A-region of the field sample 311. The values within brackets represent the percentage nucleotide distance.

	1	2	3	4	5
1 Group 1a VT					
2 Group 1b T30	0.123 (12.3%)				
3 Group 2 B165	0.306 (30.6%)	0.309 (30.9%)			
4 Group 3a RB	0.384 (38.4%)	0.398 (39.8%)	0.398 (39.8%)		
5 Group 3b T36	0.388 (38.8%)	0.402 (40.2%)	0.402 (40.2%)	0.159 (15.9%)	

Table 22: Nucleotide diversities between groups for the A-region of the field sample 312. The values within brackets represent the percentage nucleotide distance.

	1	2	3	4	5	6
1 Group 1a VT						
2 Group 1b T30	0.12 (12%)					
3 Group 1c Unk	0.107 (10.7%)	0.132 (13.2%)				
4 Group 2 B165	0.321 (32.1%)	0.32 (32%)	0.334 (33.4%)			
Group 3a RB	0.383 (38.3%)	0.4 (40%)	0.399 (39.9%)	0.393 (39.3%)		
Group 3b T36	0.389 (38.9%)	0.405 (40.5%)	0.399 (39.9%)	0.405 (40.5%)	0.159 (15.9%)	

Table 23: Nucleotide diversities between groups for the A-region of the field sample 313. The values within brackets represent the percentage nucleotide distance.

	1	2	3	4	5	6
1 Group 1a VT						
2 Group 1b T30	0.115 (11.5%)					
3 Group 1c Unk	0.137 (13.7%)	0.15 (15%)				
4 Group 2 B165	0.316 (31.6%)	0.322 (32.2%)	0.345 (34.5)			
Group 3a RB	0.384 (38.4%)	0.397 (39.7%)	0.397 (39.7%)	0.389 (38.9%)		
Group 3b T36	0.393 (39.3%)	0.397 (39.7%)	0.412 (41.2%)	0.402 (40.2%)	0.159 (15.9%)	

Appendix 11

Table 24: Nucleotide diversities between groups for the p23 gene region of the field sample 301. The values within brackets represent the percentage nucleotide distance.

	1	2	3
1 Group 1 VT			
2 Group 2 T36	0.09 (9%)		
3 Group 3 T30	0.094 (9.4%)	0.096 (9.6%)	

Table 25: Nucleotide diversities between groups for the p23 gene region of the field sample 302. The values within brackets represent the percentage nucleotide distance.

	1	2	3
1 Group 1 VT			
2 Group 2 T36	0.092 (9.2%)		
3 Group 3 T30	0.093 (9.3%)	0.094 (9.4%)	

Table 26: Nucleotide diversities between groups for the p23 gene region of the field sample 303. The values within brackets represent the percentage nucleotide distance.

	1	2	3
1 Group 1 VT			
2 Group 2 T36	0.092 (9.2%)		
3 Group 3 T30	0.094 (9.4%)	0.094 (9.4%)	

Table 27: Nucleotide diversities between groups for the p23 gene region of the field sample 304. The values within brackets represent the percentage nucleotide distance.

	1	2	3	4
1 Group 1a VT				
2 Group 1b HA 16-5	0.087 (8.7%)			
3 Group 2 T36	0.088 (8.8%)	0.091 (9.1%)		
4 Group 3 T30	0.095 (9.5%)	0.103 (10.3%)	0.098 (9.8%)	

Table 28: Nucleotide diversities between groups for the p23 gene region of the field sample 305. The values within brackets represent the percentage nucleotide distance.

	1	2	3	4
1 Group 1a VT				
2 Group 1b HA 16-5	0.098 (9.8%)			
3 Group 2 T36	0.103 (10.3%)	0.103 (10.3%)		
4 Group 3 T30	0.096 (9.6%)	0.101 (10.1%)	0.106 (10.6%)	

Table 29: Nucleotide diversities between groups for the p23 gene region of the field sample 306. The values within brackets represent the percentage nucleotide distance.

	1	2	3	4
1 Group 1 VT				
2 Group 1b HA 16-5	0.089 (8.9%)			
3 Group 2 T36	0.089 (8.9%)	0.092 (9.2%)		
4 Group 3 T30	0.093 (9.3%)	0.1 (10%)	0.094 (9.4%)	

Table 30: Nucleotide diversities between groups for the p23 gene region of the field sample 307. The values within brackets represent the percentage nucleotide distance.

	1	2	3
1 Group 1 VT			
2 Group 2 T36	0.092 (9.2%)		
3 Group 3 T30	0.094 (9.4%)	0.094 (9.4%)	

Table 31: Nucleotide diversities between groups for the p23 gene region of the field sample 308. The values within brackets represent the percentage nucleotide distance.

	1	2	3
1 Group 1 VT			
2 Group 2 T36	0.089 (8.9%)		
3 Group 3 T30	0.091 (9.1%)	0.097 (9.7%)	

Table 32: Nucleotide diversities between groups for the p23 gene region of the field sample 309. The values within brackets represent the percentage nucleotide distance.

	1	2	3
1 Group 1 VT			
2 Group 2 T36	0.092 (9.2%)		
3 Group 3 T30	0.094 (9.4%)	0.099 (9.9%)	

Table 33: Nucleotide diversities between groups for the p23 gene region of the field sample 310. The values within brackets represent the percentage nucleotide distance.

	1	2	3	4
1 Group 1 VT				
2 Group 1b HA 15-6	0.088 (8.8%)			
2 Group 2 T36	0.088 (8.8%)	0.09 (9%)		
3 Group 3 T30	0.092 (9.2%)	0.102 (10.2%)	0.099 (9.9%)	

Table 34: Nucleotide diversities between groups for the p23 gene region of the field sample 311. The values within brackets represent the percentage nucleotide distance.

	1	2	3
1 Group 1 VT			
2 Group 2 T36	0.089 (8.9%)		
3 Group 3 T30	0.099 (9.9%)	0.099 (9.9%)	

Table 35: Nucleotide diversities between groups for the p23 gene region of the field sample 312. The values within brackets represent the percentage nucleotide distance.

	1	2	3
1 Group 1 VT			
2 Group 2 T36	0.089 (8.9%)		
3 Group 3 T30	0.091 (9.1%)	0.099 (9.9%)	

Table 36: Nucleotide diversities between groups for the p23 gene region of the field sample 313. The values within brackets represent the percentage nucleotide distance.

	1	2	3
1 Group 1 VT			
2 Group 2 T36	0.091 (9.1%)		
3 Group 3 T30	0.09 (9%)	0.098 (9.8%)	

Appendix 12

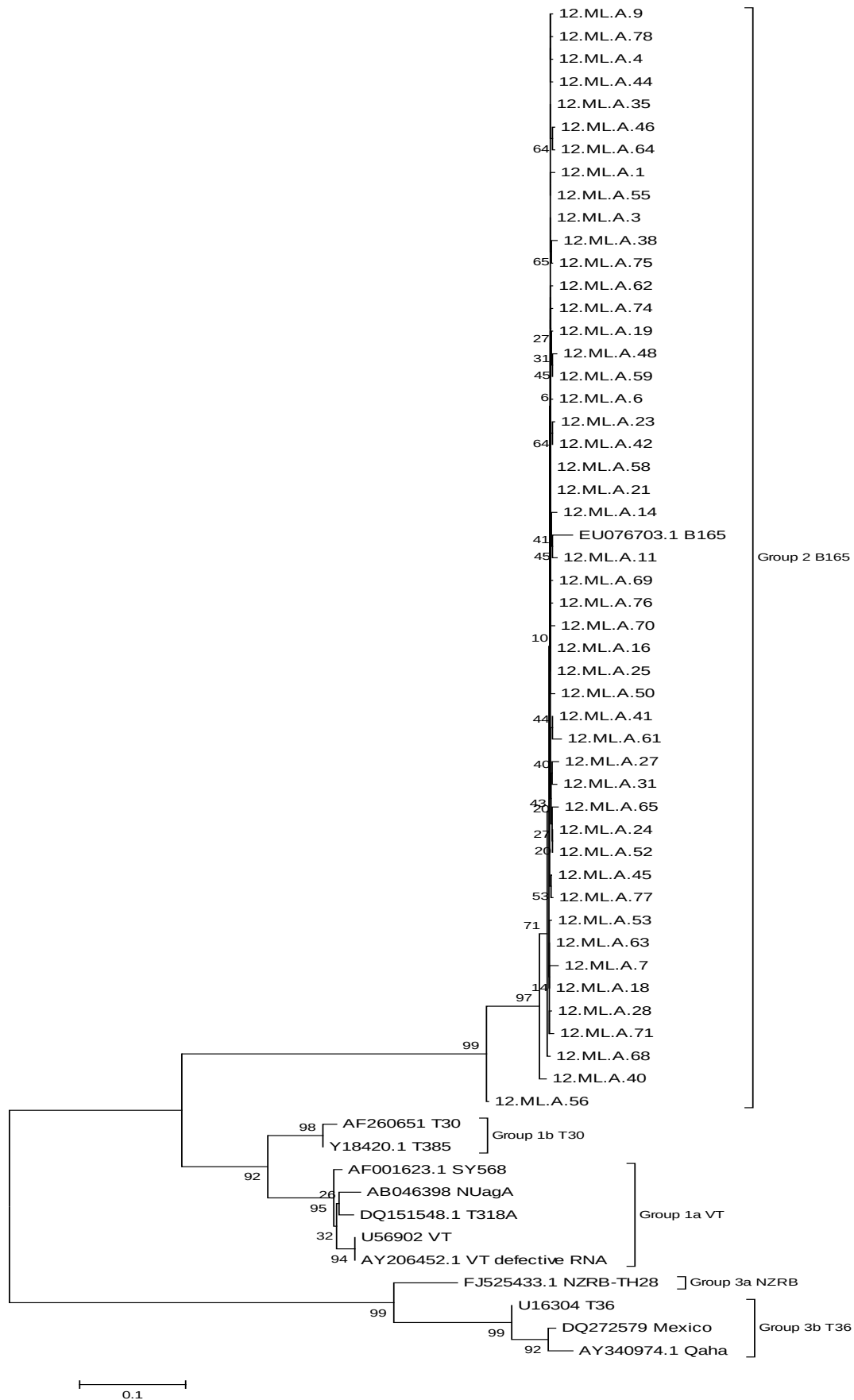


Figure 12.1: Phylogenetic tree of the A-fragment of sample MS12.ML.A from the greenhouse. All groups share a 95-99% intragroup sequence similarity. Group 1a contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the T30 reference contigs. Group 2 contains clones grouping with the B165 reference contig. Group 3a contains clones grouping with the New Zealand Resistance Breaking reference contig. Group 3b contains clones grouping with the T36 reference contigs. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

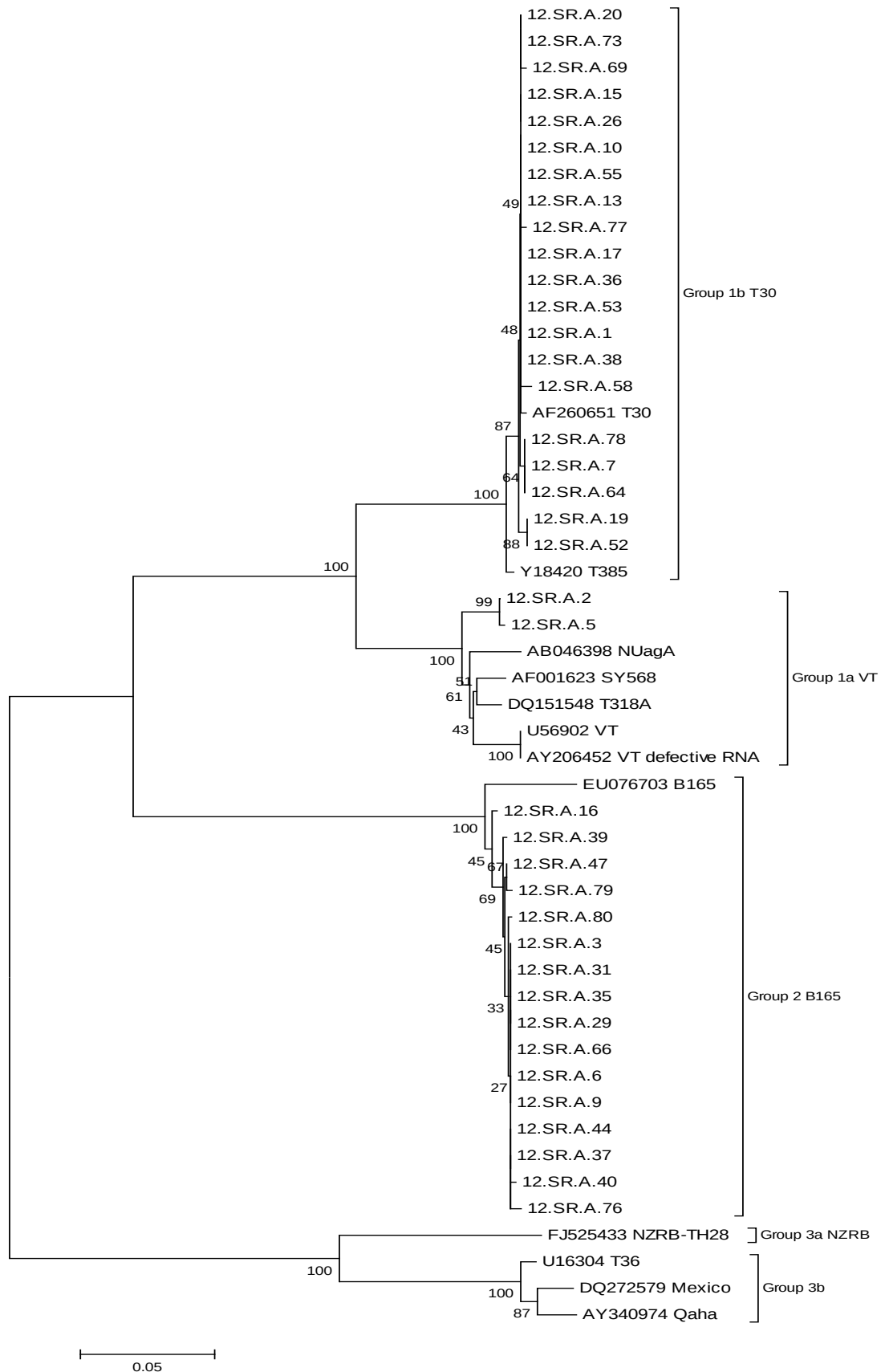


Figure 12.2: Phylogenetic tree of the A-fragment of sample MS12.SR.A from the greenhouse. All groups share a 95-99% intragroup sequence similarity. Group 1a contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the T30 reference contigs. Group 2 contains clones grouping with the B165 reference contig. Group 3a contains clones grouping with the New Zealand Resistance Breaking reference contig. Group 3b contains clones grouping with the T36 reference contigs. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

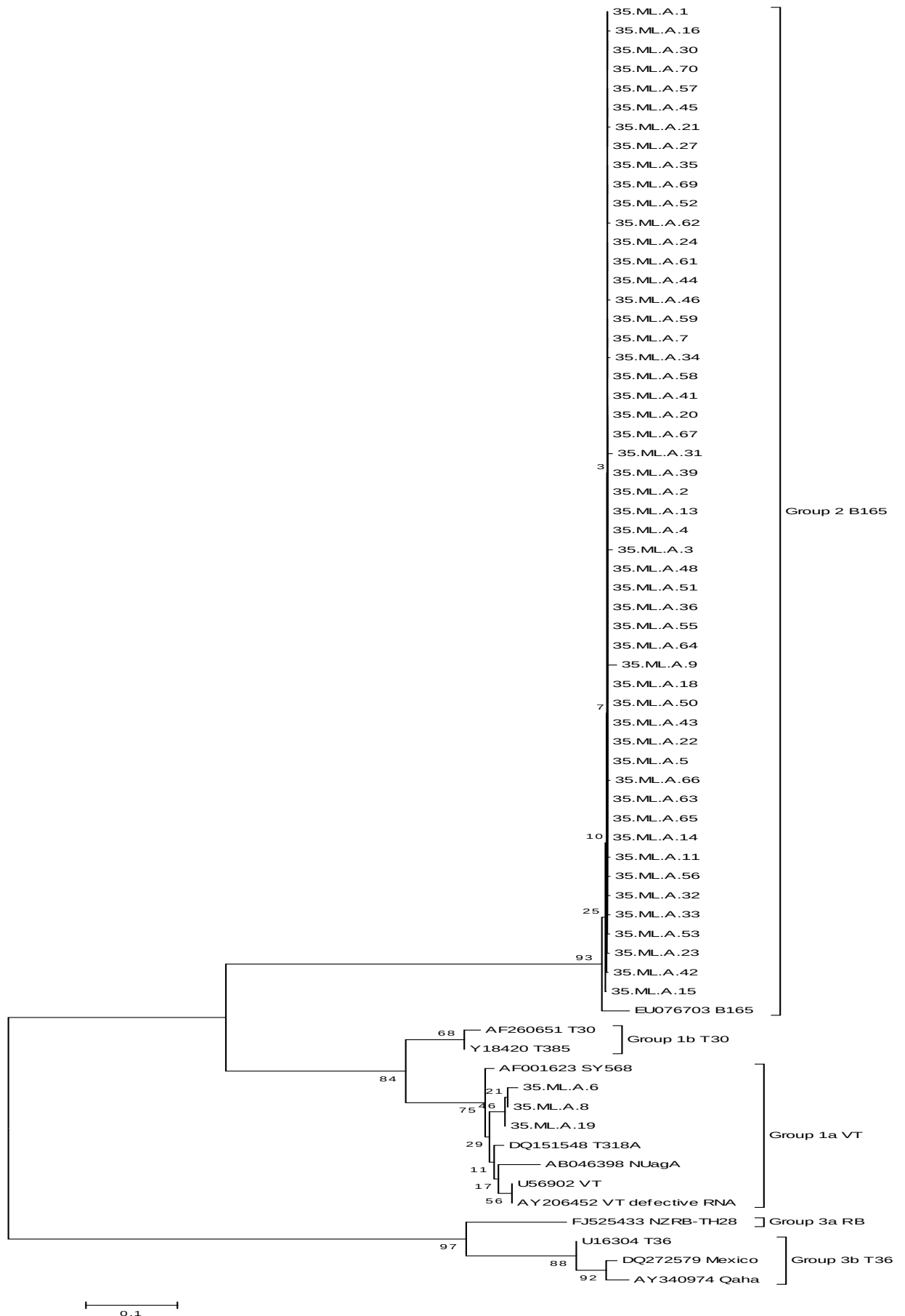


Figure 12.3: Phylogenetic tree of the A-fragment of sample MS35.ML.A from the greenhouse. All groups share a 95-99% intragroup sequence similarity. Group 1a contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the T30 reference contigs. Group 2 contains clones grouping with the B165 reference contig. Group 3a contains clones grouping with the New Zealand Resistance Breaking reference contig. Group 3b contains clones grouping with the T36 reference contigs. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

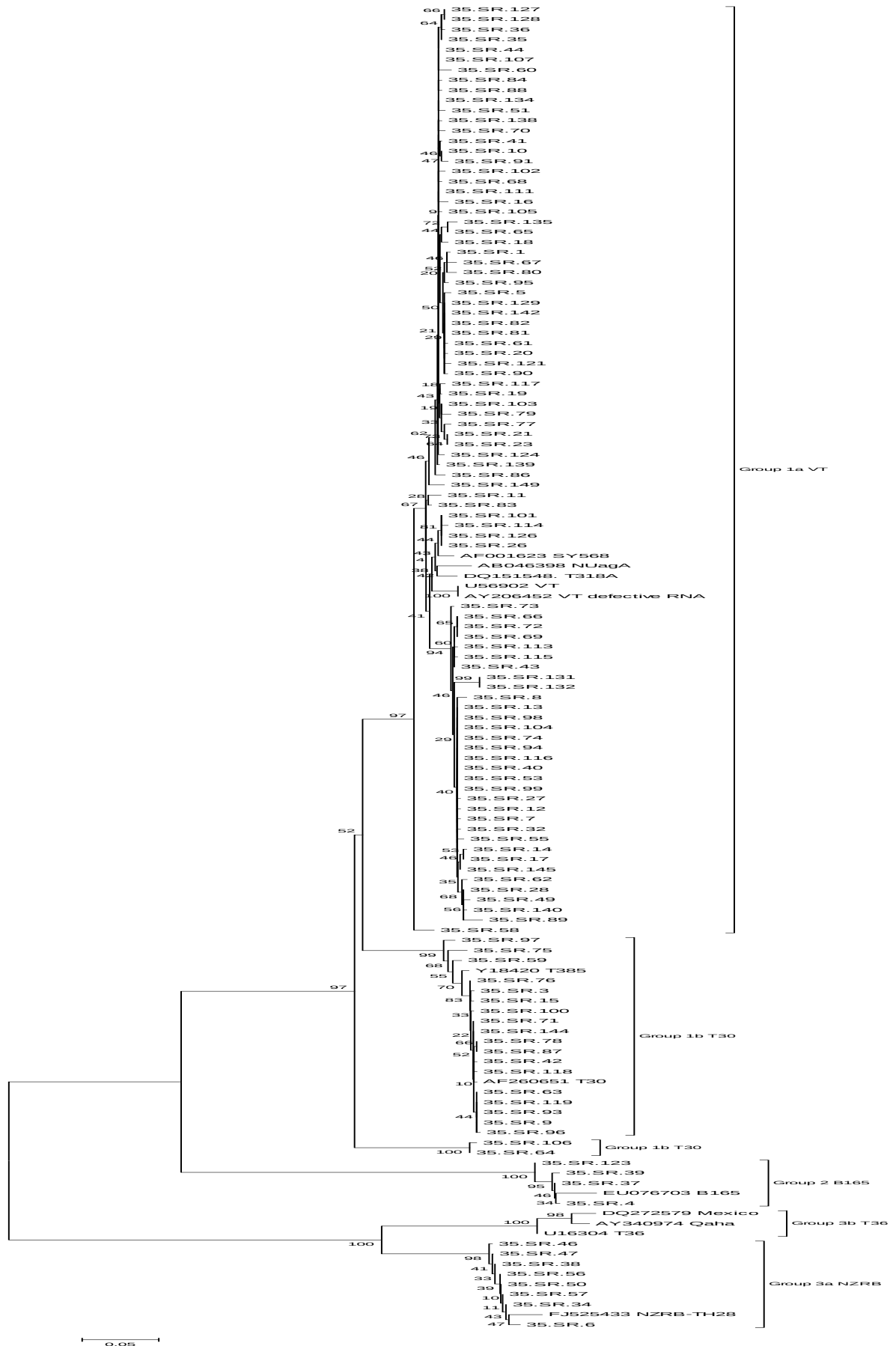


Figure 12.4: Phylogenetic tree of the A-fragment of sample MS35.SR.A from the greenhouse. All groups share a 95-99% intragroup sequence similarity. Group 1a contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the T30 reference contigs. Group 2 contains clones grouping with the B165 reference contig. Group 3a contains clones grouping with the New Zealand Resistance Breaking reference contig. Group 3b contains clones grouping with the T36 reference contigs. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

Appendix 13

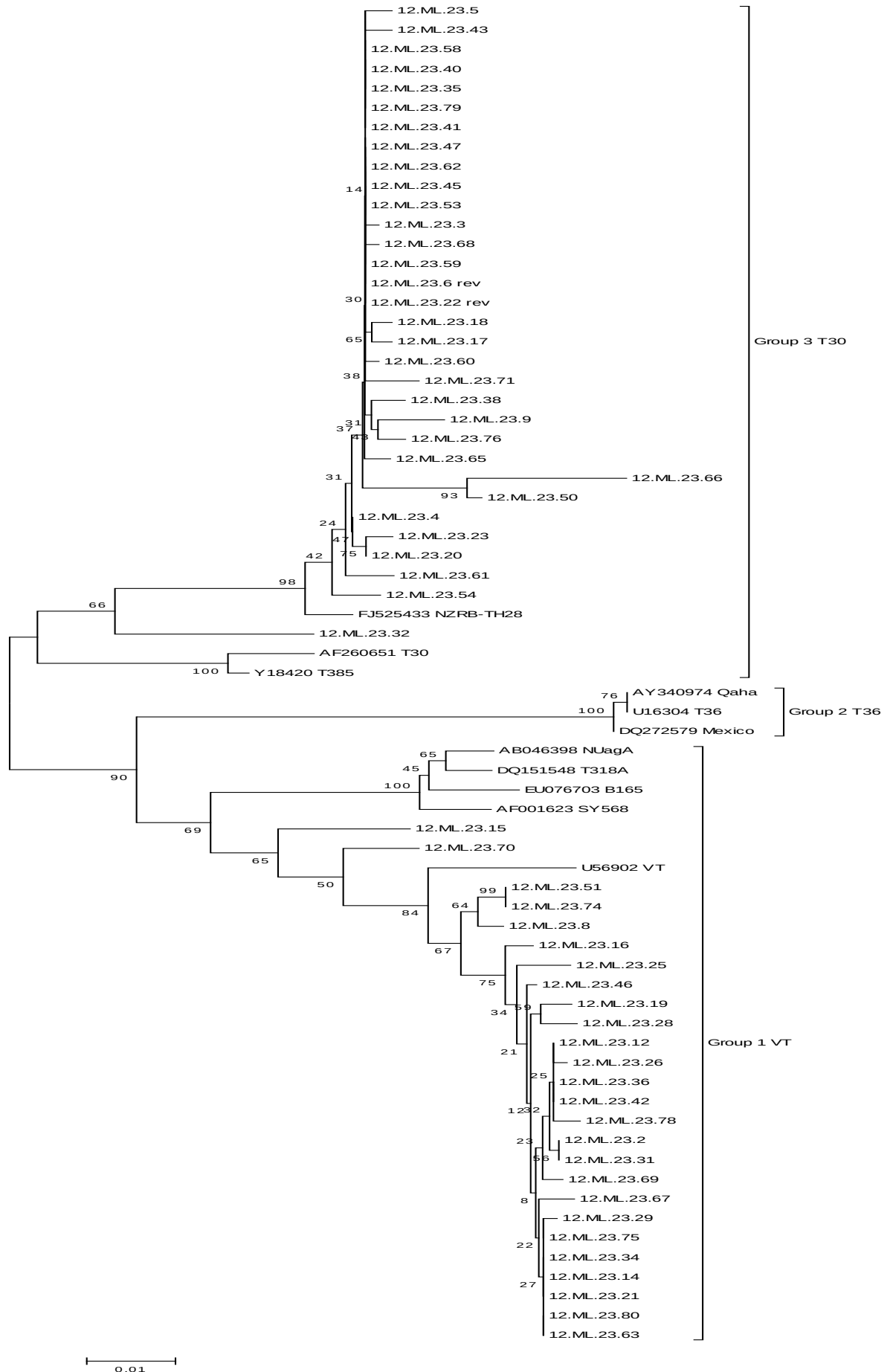


Figure 13.1: Phylogenetic tree of the p23 gene region of sample MS12.ML.p23 from the greenhouse. All groups share a 90-99% intragroup sequence similarity. Group 1 contains clones grouping with the VT reference contigs. Group 2 contains clones grouping with the T36 reference contigs. Group 3 contains clones grouping with the T30 reference contig. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

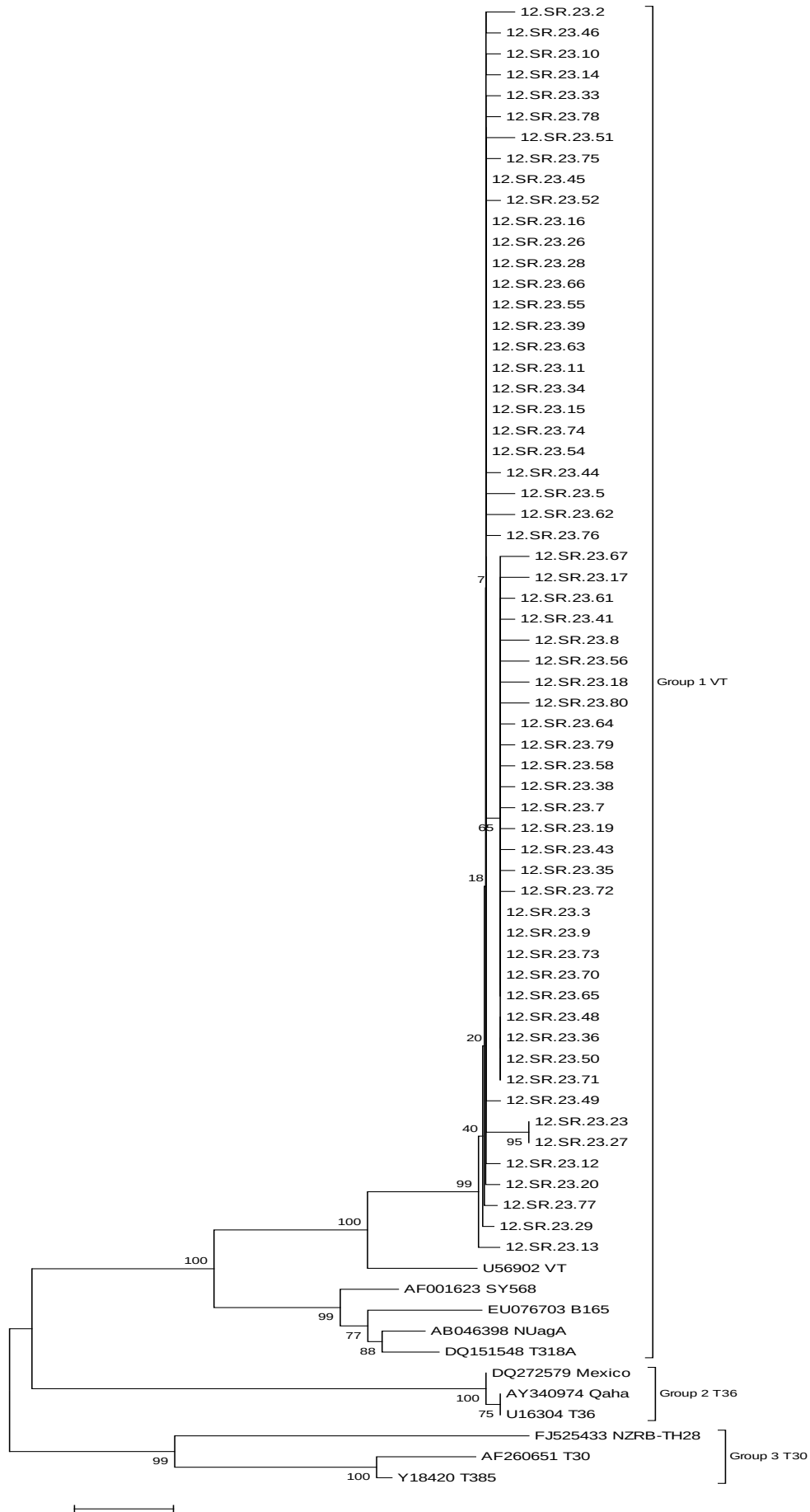


Figure 13.2: Phylogenetic tree of the p23 gene region of sample MS12.SR.p23 from the greenhouse. All groups share a 90-99% intragroup sequence similarity. Group 1 contains clones grouping with the VT reference contigs. Group 2 contains clones grouping with the T36 reference contigs. Group 3 contains clones grouping with the T30 reference contig. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).



Figure 13.3: Phylogenetic tree of the p23 gene region of sample MS35.ML.p23 from the greenhouse. All groups share a 90-99% intragroup sequence similarity. Group 1 contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the HA15-6 reference contig. Group 2 contains clones grouping with the T36 reference contigs. Group 3 contains clones grouping with the T30 reference contig. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

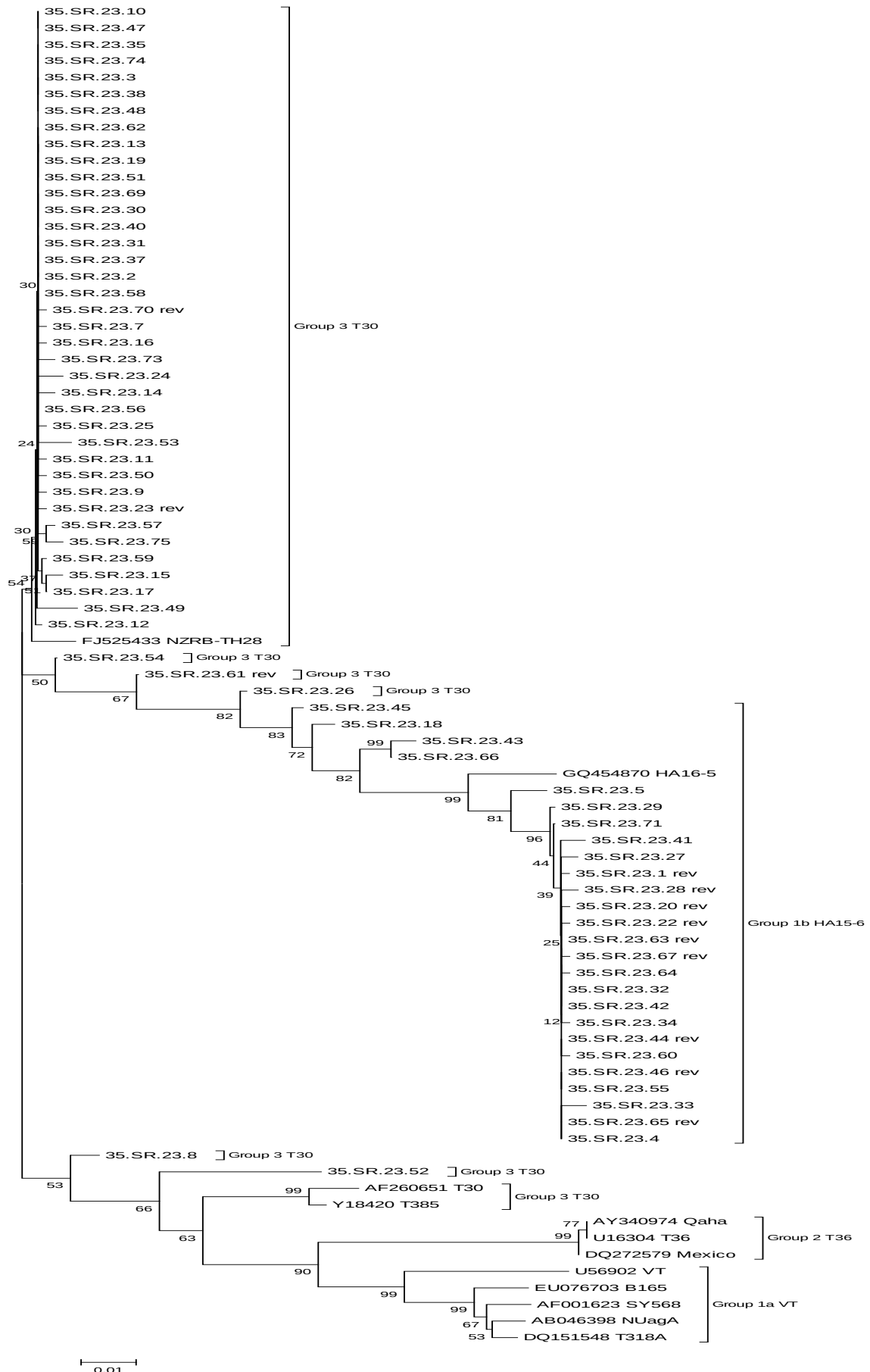


Figure 13.4: Phylogenetic tree of the p23 gene region of sample MS35.SR.p23 from the greenhouse. All groups share a 90-99% intragroup sequence similarity. Group 1 contains clones grouping with the VT reference contigs. Group 1b contains clones grouping with the HA15-6 reference contig. Group 2 contains clones grouping with the T36 reference contigs. Group 3 contains clones grouping with the T30 reference contig. The bootstrap values are shown below the branches and the scale indicates genetic distance (in nucleotide substitutions per position in the sequence).

Appendix 14

Table 37: Nucleotide diversities within groups for the A-region of the greenhouse source samples. The values within brackets represent the percentage nucleotide distance.

Sample number	Group 1a VT	Group 1b T30	Group 2 B165	Group 3a RB	Group 3b T36
MS12.ML.A	0.028 (2.8%)	0.010 (1.0%)	0.010 (1.0%)	n/c	0.027 (2.7%)
MS12.SR.A	0.029 (2.9%)	0.003 (0.3%)	0.007 (0.7%)	n/c	0.026 (2.6%)
MS35.ML.A	0.030 (3%)	0.010 (1%)	0.003 (0.3%)	n/c	0.027 (2.7%)
MS35.SR.A	0.024 (2.4%)	0.029 (2.9%)	0.016 (1.6%)	0.008 (0.8%)	0.025 (2.5%)

Table 38: Nucleotide diversities within groups for the p23 gene region of the greenhouse source samples. The values within brackets represent the percentage nucleotide distance.

Sample number	Group 1 VT	Group 1b HA 15-6	Group 2 T36	Group 3 T30
MS12.ML.p23	0.020 (2%)	-	0.001 (0.1%)	0.017 (1.7%)
MS12.SR.p23	0.010 (1%)	-	0.001 (0.1%)	0.045 (4.5%)
MS35.ML.p23	0.029 (2.9%)	0.007 (0.7%)	0.001 (0.1%)	0.009 (0.9%)
MS35.SR.p23	0.030 (3%)	0.017 (1.7%)	0.001 (0.1%)	0.014 (1.4%)

Appendix 15

Table 39: Nucleotide diversities between groups for the A-region of the greenhouse sample MS12.ML.A. The values within brackets represent the percentage nucleotide distance.

	1	2	3	4	5
1 Group 1a VT					
2 Group 1b T30	0.118 (11.8%)				
4 Group 2 B165	0.307 (30.7%)	0.293 (29.3%)			
Group 3a RB	0.370 (37%)	0.382 (38.2%)	0.406 (40.6%)		
Group 3b T36	0.372 (37.2%)	0.386 (38.6%)	0.411 (41.1%)	0.154 (15.4%)	

Table 40: Nucleotide diversities between groups for the A-region of the greenhouse sample MS12.SR.A. The values within brackets represent the percentage nucleotide distance.

	1	2	3	4	5
1 Group 1a VT					
2 Group 1b T30	0.120 (12%)				
3 Group 2 B165	0.294 (29.4%)	0.283 (28.3%)			
4 Group 3a RB	0.378 (37.8%)	0.393 (39.3%)	0.393 (39.3%)		
5 Group 3b T36	0.385 (38.5%)	0.398 (39.8%)	0.404 (40.4%)	0.159 (15.9%)	

Table 41: Nucleotide diversities between groups for the A-region of the greenhouse sample MS35.ML.A. The values within brackets represent the percentage nucleotide distance.

	1	2	3	4	5
1 Group 1a VT					
2 Group 1b T30	0.119 (11.9%)				
3 Group 2 B165	0.301 (30.1%)	0.294 (29.4%)			
4 Group 3a RB	0.381 (38.1%)	0.392 (39.2%)	0.393 (39.3%)		
5 Group 3b T36	0.392 (39.2%)	0.403 (40.3%)	0.404 (40.4%)	0.166 (16.6%)	

Table 42: Nucleotide diversities between groups for the A-region of the greenhouse sample MS35.SR.A . The values within brackets represent the percentage nucleotide distance.

	1	2	3	4	5
1 Group 1a VT					
2 Group 1b T30	0.121 (12.1%)				
3 Group 2 B165	0.302 (30.2%)	0.290 (29%)			
4 Group 3a RB	0.397 (38.7%)	0.400 (40%)	0.403 (40.3%)		
5 Group 3b T36	0.391 (39.1%)	0.401 (40.1%)	0.414 (41.4%)	0.157 (15.7%)	

Appendix 16

Table 43: Nucleotide diversities between groups for the p23 gene region of the greenhouse sample MS12.ML.p23. The values within brackets represent the percentage nucleotide distance.

	1	2	3
1 Group 1 VT			
2 Group 2 T36	0.096 (9.6%)		
3 Group 3 T30	0.103 (10.3%)	0.110 (11%)	

Table 44: Nucleotide diversities between groups for the p23 gene region of the greenhouse sample MS12.SR.p23. The values within brackets represent the percentage nucleotide distance.

	1	2	3
1 Group 1 VT			
2 Group 2 T36	0.094 (9.4%)		
3 Group 3 T30	0.096 (9.6%)	0.093 (9.3%)	

Table 45: Nucleotide diversities between groups for the p23 gene region of the greenhouse sample MS35.ML.p23. The values within brackets represent the percentage nucleotide distance.

	1	1b	2	3
1 Group 1 VT				
1b Group 1b HA 15-6	0.093 (9.3%)			
2 Group 2 T36	0.084 (8.4%)	0.087 (8.7%)		
3 Group 3 T30	0.094 (9.4%)	0.105 (10.5%)	0.104 (10.4%)	

Table 46: Nucleotide diversities between groups for the p23 gene region of the greenhouse sample MS35.SR.p23. The values within brackets represent the percentage nucleotide distance.

	1	1b	2	3
1 Group 1 VT				
1b Group 1b HA 15-6	0.096 (9.6%)			
2 Group 2 T36	0.087 (8.7%)	0.093 (9.3%)		
3 Group 3 T30	0.097 (9.7%)	0.100 (10%)	0.107 (10.7%)	

Appendix 17

Table 47: Predicted amino acid sequences of the p23 protein of the cloned sequences from the field collected sample replicate 1 of GFMS 12 on Star Ruby (301). Abbreviations at the top of each column represents the at amino acid sequence at positions 78-80 of the p23 protein.

GSR	ALK	Anomalous
p301.10(VT); p301.14(VT); p301.2(VT); p301.20(VT); p301.24(VT); p301.25(VT); p301.4(VT); p301.5(VT); p301.6(VT); p301.9(VT); p301.13(VT); p301.18(VT); p301.22(VT); p301.27(VT); p301.28(VT); p301.29(VT); p301.3(VT)	p301.21(T30); p301.30(T30); p301.11(T30); p301.17(VT); p301.16(T30); p301.15(T30); p301.19(T30)	p301.12 (RSR)

Table 48: Predicted amino acid sequences of the p23 protein of the cloned sequences from the field collected sample replicate 2 of GFMS 12 on Star Ruby (302). Abbreviations at the top of each column represents the at amino acid sequence at positions 78-80 of the p23 protein.

GSR	ASR
p302.13(VT); p302.16(VT); p302.2(VT); p302.26(VT); p302.30(VT); p302.32(VT); p302.34(VT); p302.6(VT); p302.15(VT); p302.24(VT); p302.35(VT)	p302.14(VT); p302.17(VT); p302.20(VT); p302.27(VT); p302.31(VT); p302.33(VT); p302.4(VT); p302.8(VT); p302.23(VT); p302.29(VT); p302.12(VT); p302.21(VT)

Table 49: Predicted amino acid sequences of the p23 protein of the cloned sequences from the field collected sample replicate 3 of GFMS 12 on Star Ruby (303). Abbreviations at the top of each column represents the at amino acid sequence at positions 78-80 of the p23 protein.

GSR		ASR	ALK	Anomalous
p303.10(VT); p303.14(VT); p303.16(VT); p303.19(VT); p303.21(VT); p303.24(VT); p303.28(VT); p303.3(VT); p303.9(VT); p303.23(VT); p303.30(VT); p303.4(VT);	p303.12(VT); p303.15(VT); p303.18(VT); p303.20(VT); p303.22(VT); p303.27(VT); p303.29(VT); p303.5(VT); p303.2(VT); p303.26(VT); p303.34(VT); p303.7(VT)	p303.25(VT)	p303.13(T30)	p303.8 (GSG)(VT)

Table 50: Predicted amino acid sequences of the p23 protein of the cloned sequences from the field collected sample replicate 4 of GFMS 12 on Star Ruby (313). Abbreviations at the top of each column represents the at amino acid sequence at positions 78-80 of the p23 protein.

GSR	ALK	Anomalous
p313.12(VT); p313.14(VT); p313.16(VT); p313.17(VT); p313.18(VT); p313.21(VT); p313.26(VT); p313.3(VT); p313.30(VT); p313.33(VT); p313.6(VT); p313.9(VT); p313.11(VT); p313.13(VT); p313.20(VT); p313.23(VT); p313.27(VT); p313.32(VT); p313.37(VT); p313.7(VT)	p313.10(T30); p313.15(T30);	p313.28 (GRG with stops in frame)

Table 51: Predicted amino acid sequences of the p23 protein of the cloned sequences from the field collected sample replicate 1 of GFMS 12 on Flame (304). Abbreviations at the top of each column represents the at amino acid sequence at positions 78-80 of the p23 protein.

GSR	GLK	ALK	Anomalous
p304.64 (VT); p304.65(VT); p304.52(VT); p304.55(VT); p304.66(VT); p304.68(VT); p304.70(VT); p304.71(VT); p304.73(VT); p304.79(VT); p304.81(VT); p304.53(VT); p304.61(VT); p304.63(VT); p304.69(VT); p304.77(VT); p304.85(VT)	p304.84 (HA15-6)	p304.54(T30); p304.78(T30); p304.82(T30); p304.57(T30); p304.62(T30)	p304.83 (RSR) (T30); p304.58 (GRG, with stops in frame) (VT); p304.74 (GRG, with stops in frame) (VT); p304.75 (GVE, with stops in frame) (VT)

Table 52: Predicted amino acid sequences of the p23 protein of the cloned sequences from the field collected sample replicate 2 of GFMS 12 on Flame (305). Abbreviations at the top of each column represents the at amino acid sequence at positions 78-80 of the p23 protein.

GSR	GLK	ALK
p305.19(VT); p305.21(VT); p305.23(VT); p305.7(VT); p305.8(VT); p305.10(VT); p305.2(VT); p305.26(VT); p305.31(VT)	p305.33(HA15-6); p305.12(HA15-6); p305.15(HA15-6)	p305.16(T30)

Table 53: Predicted amino acid sequences of the p23 protein of the cloned sequences from the field collected sample replicate 3 of GFMS 12 on Flame (306). Abbreviations at the top of each column represents the at amino acid sequence at positions 78-80 of the p23 protein.

GSR	GLK	ALK
p306.11(VT); p306.15(VT); p306.17(VT); p306.18(VT); p306.24(VT); p306.26(VT); p306.27(VT); p306.30(VT); p306.31(VT); p306.32(VT); p306.35(VT); p306.38(VT); p306.9(VT); p306.1(VT); p306.14(VT); p306.2(VT); p306.20(VT); p306.25(VT); p306.28(VT); p306.29(VT); p306.3(VT); p306.36(VT); p306.37(VT); p306.4(VT); p306.6(VT)	p306.7(HA15-6); p306.12(HA15-6)	p306.8(T30)

Table 54: Predicted amino acid sequences of the p23 protein of the cloned sequences from the field collected sample replicate 1 of GFMS 35 on Star Ruby (307). Abbreviations at the top of each column represents the at amino acid sequence at positions 78-80 of the p23 protein.

GSR
p307.11(VT); p307.12(VT); p307.13(VT); p307.14(VT); p307.15(VT); p307.16(VT); p307.18(VT); p307.19(VT); p307.24(VT); p307.3(VT); p307.31(VT); p307.32(VT); p307.34(VT); p307.35(VT); p307.4(VT); p307.6(VT); p307.1(VT); p307.26(VT); p307.30(VT); p307.37(VT); p307.38(VT); p307.8(VT); p307.9(VT)

Table 55: Predicted amino acid sequences of the p23 protein of the cloned sequences from the field collected sample replicate 2 of GFMS 35 on Star Ruby (308). Abbreviations at the top of each column represents the at amino acid sequence at positions 78-80 of the p23 protein.

GSR		ASR	ALK
p308.12(VT); p308.16(VT); p308.19(VT); p308.26(VT); p308.6(VT); p308.10(VT); p308.22(VT); p308.25(VT); p308.40(VT); p308.42(VT)	p308.14(VT); p308.18(VT); p308.24(VT); p308.3(VT); p308.7(VT); p308.21(VT); p308.23(VT); p308.39(VT); p308.41(VT);	p308.38(VT); p308.5(VT); p308.20(VT); p308.9(VT); p308.1(VT); p308.13(VT); p308.15(VT) (These clones formed a separate branch on the phylogenetic tree within the VT-like group)	p308.8(T30); p308.37(T30)

Table 56: Predicted amino acid sequences of the p23 protein of the cloned sequences from the field collected sample replicate 3 of GFMS 35 on Star Ruby (309). Abbreviations at the top of each column represents the at amino acid sequence at positions 78-80 of the p23 protein.

GSR		ASR	ALK
p309.10(VT); p309.19(VT); p309.31(VT); p309.12(VT); p309.16(VT); p309.25(VT); p309.30(VT); p309.33(VT);	p309.14(VT); p309.29(VT); p309.11(VT); p309.15(VT); p309.17(VT); p309.26(VT); p309.32(VT); p309.7(VT)	p309.28(VT); p309.3(VT); p309.34(VT); p309.35(VT); p309.36(VT); p309.8(VT) (These clones formed separate branches on the phylogenetic tree within the VT-like group)	p309.21(T30); p309.2(T30); p309.6(T30); p309.13(T30)

Table 57: Predicted amino acid sequences of the p23 protein of the cloned sequences from the field collected sample replicate 1 of GFMS 35 on Flame (310). Abbreviations at the top of each column represents the at amino acid sequence at positions 78-80 of the p23 protein.

GSR	ASR	GLK	ALK
p310.10(VT); p310.11(VT); p310.13(VT); p310.15(VT); p310.26(VT); p310.29(VT); p310.30(VT); p310.32(VT); p310.4(VT); p310.7(VT); p310.1(VT); p310.12(VT); p310.14(VT); p310.2(VT); p310.23(VT); p310.28(VT); p310.6(VT)	p310.9(VT)	p310.18(HA15-6); p310.33(HA15-6)	p310.17(T30) p310.3(T30); p310.16(T30); p310.25(T30)

Table 58: Predicted amino acid sequences of the p23 protein of the cloned sequences from the field collected sample replicate 2 of GFMS 35 on Flame (311). Abbreviations at the top of each column represents the at amino acid sequence at positions 78-80 of the p23 protein.

GSR	ALK
p311.13(VT); p311.15(VT); p311.2(VT); p311.22(VT); p311.27(VT); p311.29(VT); p311.30(VT); p311.5(VT); p311.10(VT); p311.19(VT); p311.26(VT);	p311.14(VT); p311.18(VT); p311.20(VT); p311.25(VT); p311.28(VT); p311.3(VT); p311.33(VT); p311.9(VT); p311.11(VT); p311.24(VT); p311.4(VT); p311.17(T30); p311.1(T30); p311.21(T30); p311.7(T30)

Table 59: Predicted amino acid sequences of the p23 protein of the cloned sequences from the field collected sample replicate 3 of GFMS 35 on Flame (312). Abbreviations at the top of each column represents the amino acid sequence at positions 78-80 of the p23 protein.

GSR	ASR	ALK
p312.10(VT); p312.15(VT); p312.19(VT); p312.22(VT); p312.29(VT); p312.34(VT); p312.39(VT); p312.12(VT); p312.17(VT); p312.18(VT); p312.2(VT); p312.20(VT); p312.21(VT); p312.31(VT); p312.9(VT)	p312.25(VT); p312.36(VT); p312.8(VT); p312.11(VT); p312.24(VT); p312.5(VT) (These clones formed a separate branch on the phylogenetic tree within the VT-like group)	p312.1(T30); p312.13(T30); p312.14(T30); p312.16(T30); p312.4(T30); p312.7(T30); p312.6(T30); p312.23(T30)

Table 60: Predicted amino acid sequences of the p23 protein of the cloned sequences from the greenhouse collected reference sample GFMS 12 on Mexican Lime. Abbreviations at the top of each column represents the amino acid sequence at positions 78-80 of the p23 protein.

ASR	ALK	Anomalous
MS12.ML.14(VT); MS12.ML.16(VT); MS12.ML.25(VT); MS12.ML.26(VT); MS12.ML.29(VT); MS12.ML.3(T30); MS12.ML.42(VT); MS12.ML.46(VT); MS12.ML.50(T30); MS12.ML.63(VT); MS12.ML.70(VT); MS12.ML.78(VT); MS12.ML.12(VT); MS12.ML.15(VT); MS12.ML.19(VT); MS12.ML.28(VT); MS12.ML.34(VT); MS12.ML.36(VT); MS12.ML.51(VT); MS12.ML.66(T30); MS12.ML.69(VT); MS12.ML.74(VT); MS12.ML.75(VT); MS12.ML.8(VT); MS12.ML.80(VT)	MS12.ML.17(T30); MS12.ML.21(VT); MS12.ML.31(VT); MS12.ML.35(T30); MS12.ML.38(T30); MS12.ML.40(T30); MS12.ML.41(T30); MS12.ML.43(T30); MS12.ML.45(T30); MS12.ML.47(T30); MS12.ML.5(T30); MS12.ML.53(T30); MS12.ML.58(T30); MS12.ML.59(T30); MS12.ML.61(T30); MS12.ML.62(T30); MS12.ML.65(T30); MS12.ML.76(T30); MS12.ML.79(T30); MS12.ML.9(T30); MS12.ML.18(T30); MS12.ML.22(T30); MS12.ML.23(T30); MS12.ML.4(T30); MS12.ML.54(T30); MS12.ML.6(T30); MS12.ML.60(T30); MS12.ML.68(T30); MS12.ML.71(T30);	MS12.ML.2 (ATR)(VT); MS12.ML.32 (ATR) (T30); MS12.ML.67 (ATR)(VT)

Table 61: Predicted amino acid sequences of the p23 protein of the cloned sequences from the greenhouse collected reference sample GFMS 12 on Star Ruby. Abbreviations at the top of each column represents the amino acid sequence at positions 78-80 of the p23 protein.

ASR			
MS12.SR.10(VT);	MS12.SR.14(VT);	MS12.SR.15(VT);	MS12.SR.16(VT);
MS12.SR.18(VT);	MS12.SR.23(VT);	MS12.SR.26(VT);	MS12.SR.27(VT);
MS12.SR.28(VT);	MS12.SR.3(VT);	MS12.SR.34(VT);	MS12.SR.35(VT);
MS12.SR.39(VT);	MS12.SR.43(VT);	MS12.SR.44(VT);	MS12.SR.45(VT);
MS12.SR.5(VT);	MS12.SR.51(VT);	MS12.SR.52(VT);	MS12.SR.54(VT);
MS12.SR.56(VT);	MS12.SR.62(VT);	MS12.SR.63(VT);	MS12.SR.64(VT);
MS12.SR.65(VT);	MS12.SR.71(VT);	MS12.SR.72(VT);	MS12.SR.73(VT);
MS12.SR.75(VT);	MS12.SR.77(VT);	MS12.SR.79(VT);	MS12.SR.8(VT);
MS12.SR.11(VT);	MS12.SR.12(VT);	MS12.SR.13(VT);	MS12.SR.17(VT);
MS12.SR.19(VT);	MS12.SR.2(VT);	MS12.SR.20(VT);	MS12.SR.29(VT);
MS12.SR.33(VT);	MS12.SR.36(VT);	MS12.SR.38(VT);	MS12.SR.41(VT);
MS12.SR.46(VT);	MS12.SR.48(VT);	MS12.SR.49(VT);	MS12.SR.50(VT);
MS12.SR.55(VT);	MS12.SR.58(VT);	MS12.SR.61(VT);	MS12.SR.66(VT);
MS12.SR.67(VT);	MS12.SR.7(VT);	MS12.SR.70(VT);	MS12.SR.74(VT);
MS12.SR.76(VT);	MS12.SR.78(VT);	MS12.SR.80(VT);	MS12.SR.9(VT)

Table 62: Predicted amino acid sequences of the p23 protein of the cloned sequences from the greenhouse collected reference sample GFMS 35 on Mexican Lime. Abbreviations at the top of each column represents the at amino acid sequence at positions 78-80 of the p23 protein.

GLK	ALK	Anomalous
MS35.ML.117(HA15-6); MS35.ML.14(HA15-6); MS35.ML.15(HA15-6); MS35.ML.16(HA15-6); MS35.ML.25(T30); MS35.ML.3(HA15-6); MS35.ML.30(HA15-6); MS35.ML.36(HA15-6); MS35.ML.38(HA15-6); MS35.ML.41(HA15-6); MS35.ML.43(HA15-6); MS35.ML.54(HA15-6); MS35.ML.6(HA15-6); MS35.ML.64(HA15-6); MS35.ML.70(HA15-6); MS35.ML.92(HA15-6); MS35.ML.101(HA15-6); MS35.ML.112(HA15-6); MS35.ML.58(HA15-6); MS35.ML.75(HA15-6); MS35.ML.85(HA15-6); MS35.ML.93(HA15-6); MS35.ML.12(HA15-6); MS35.ML.18(HA15-6); MS35.ML.24(HA15-6); MS35.ML.48(HA15-6); MS35.ML.62(HA15-6); MS35.ML.66(HA15-6); MS35.ML.7(HA15-6); MS35.ML.77(HA15-6); MS35.ML.82(HA15-6); MS35.ML.102(HA15-6); MS35.ML.103(HA15-6); MS35.ML.2(HA15-6); MS35.ML.21(HA15-6); MS35.ML.37(HA15-6); MS35.ML.45(HA15-6); MS35.ML.46(HA15-6); MS35.ML.60(HA15-6); MS35.ML.61(HA15-6); MS35.ML.78(HA15-6)	MS35.ML.10(T30); MS35.ML.104(T30); MS35.ML.107(T30); MS35.ML.108(T30); MS35.ML.111(T30); MS35.ML.115(T30); MS35.ML.120(T30); MS35.ML.19(T30); MS35.ML.31(T30); MS35.ML.32(T30); MS35.ML.33(T30); MS35.ML.34(T30); MS35.ML.39(T30); MS35.ML.42(T30); MS35.ML.50(T30); MS35.ML.52(T30); MS35.ML.59(T30); MS35.ML.68(T30); MS35.ML.8(T30); MS35.ML.83(T30); MS35.ML.88(T30); MS35.ML.97(T30); MS35.ML.1(T30); MS35.ML.100(T30); MS35.ML.113(T30); MS35.ML.116(T30); MS35.ML.118(T30); MS35.ML.119(T30); MS35.ML.13(T30); MS35.ML.17(T30); MS35.ML.23(T30); MS35.ML.26(T30); MS35.ML.35(T30); MS35.ML.40(T30); MS35.ML.44(T30); MS35.ML.51(T30); MS35.ML.56(T30); MS35.ML.69(T30); MS35.ML.80(T30); MS35.ML.81(T30); MS35.ML.90(T30); MS35.ML.96(T30); MS35.ML.98(T30)	MS35.ML.20 (G*R, with stops in frame)(HA15-6); MS35.ML.22 (G*R, with stops in frame)(HA15-6); MS35.ML.4 (G*R, with stops in frame)(HA15-6); MS35.ML.74 (GVE, with stops in frame)(HA15-6); MS35.ML.95 (RSR, with stops in frame) (T30)

Table 63: Predicted amino acid sequences of the p23 protein of the cloned sequences from the greenhouse collected reference sample GFMS 35 on Star Ruby. Abbreviations at the top of each column represents the at amino acid sequence at positions 78-80 of the p23 protein.

GLK	ALK	Anomalous
MS35.SR.1(HA15-6); MS35.SR.20(HA15-6); MS35.SR.22(HA15-6); MS35.SR.28(HA15-6); MS35.SR.44(HA15-6); MS35.SR.46(HA15-6); MS35.SR.63(HA15-6); MS35.SR.65(HA15-6); MS35.SR.67(HA15-6); MS35.SR.27(HA15-6); MS35.SR.29(HA15-6); MS35.SR.32(HA15-6); MS35.SR.33(HA15-6); MS35.SR.34(HA15-6); MS35.SR.41(HA15-6); MS35.SR.42(HA15-6); MS35.SR.55(HA15-6); MS35.SR.60(HA15-6); MS35.SR.64(HA15-6); MS35.SR.71(HA15-6); MS35.SR.18(HA15-6); MS35.SR.43(HA15-6); MS35.SR.45(HA15-6); MS35.SR.5(HA15-6); MS35.SR.66(HA15-6);	MS35.SR.23(T30); MS35.SR.61(T30); MS35.SR.70(T30); MS35.SR.52(T30); MS35.SR.8(T30); MS35.SR.12(T30); MS35.SR.15(T30); MS35.SR.17(T30); MS35.SR.26(T30); MS35.SR.3(T30); MS35.SR.31(T30); MS35.SR.35(T30); MS35.SR.47(T30); MS35.SR.49(T30); MS35.SR.50(T30); MS35.SR.54(T30); MS35.SR.57(T30); MS35.SR.59(T30); MS35.SR.62(T30); MS35.SR.75(T30); MS35.SR.9(T30); MS35.SR.10(T30); MS35.SR.11(T30); MS35.SR.13(T30); MS35.SR.14(T30); MS35.SR.16(T30); MS35.SR.19(T30); MS35.SR.2(T30); MS35.SR.24(T30); MS35.SR.25(T30); MS35.SR.30(T30); MS35.SR.37(T30); MS35.SR.38(T30); MS35.SR.40(T30); MS35.SR.48(T30); MS35.SR.51(T30); MS35.SR.53(T30); MS35.SR.56(T30); MS35.SR.58(T30); MS35.SR.69(T30); MS35.SR.7(T30); MS35.SR.73(T30); MS35.SR.74(T30)	MS35.SR.4 (G*R, with stops in frame) (HA15- 6)

LIST OF ABBREVIATIONS

µg	Microgram
µl	Microlitre
µM	Micromolar
AMV-RT	<i>Avian myeloblastosis virus</i> reverse transcriptase
bp	Base pair
BChV	<i>Beet chlorosis virus</i>
BMVYV	<i>Beet mild yellowing virus</i>
BLAST	Basic Local Alignment Search Tool
BrCA	Brown citrus aphid
BrYV	<i>Brassica yellows virus</i>
BSA	Bovine serum albumin
CaMV	<i>Cauliflower mosaic virus</i>
cDNA	Complementary deoxyribonucleic acid
CIP	Southern African Citrus Improvement Scheme
CP	Coat protein
CRI	Citrus Research International
CSD	Citrus sudden death disease
CTV	<i>Citrus tristeza virus</i>
dNTP	Deoxyribonucleotide triphosphate
dsRNA	Double stranded ribonucleic acid
ELISA	Enzyme linked immunosorbent assay
GFMS	Grapefruit mild strain
gRNA	Genomic ribonucleic acid

HSP	Heat shock protein
IPTG	Isopropyl β -D-1 thiogalactopyranoside
kb	Kilobase/s
kDa	Kilodalton
LB	Luria-Bertani
MgCl ₂	Magnesium choride
ML	Mexican lime
mM	Millimolar
NCBI	National Centre for Biotechnology Information
nm	Nanometer
nt	Nucleotide
NZRB	New Zealand Resistance Breaking strain
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
pmol	Picomol
RdRp	RNA dependant RNA polymerase
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNAi	RNA interference
RT	Reverse transcriptase
sgRNA	Subgenomic ribonucleic acid
SP	Stem-pitting

SR	Star Ruby
SSCP	Single-stranded conformation polymorphism
THRIP	Technology and Human Resources for Industry Program
U	Unit
UP	University of Pretoria
UTR	Untranslated region
X-gal	Bromo-chloro-indolyl- galactopyranoside