

Characterization of a new mitovirus OMV1c in a Canadian isolate of the Dutch Elm
Disease pathogen *Ophiostoma novo-ulmi* 93-1224

by

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B.Sc., from Kiev State University, 1993

M.Sc., from Kiev State University, 1995

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Supervisory Committee

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Abstract

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The fungal pathogen *Ophiostoma novo-ulmi* is the causal agent of Dutch elm disease (DED) and has been responsible for the catastrophic decline of elms in North America and Europe. Double-stranded RNA (dsRNA) viruses are common to all fungal classes and although these viruses do not always cause disease symptoms, the presence of certain dsRNA viruses have been associated with reduced virulence (hypovirulence) in *O. novo-ulmi*. A new mitovirus was found in a Canadian isolate of *O. novo-ulmi* (93-1224) and has been named *Ophiostoma* mitovirus 1c (OMV1c). The positive strand of the dsRNA of OMV1c was 3,003 nucleotides in length and when the mitochondrial codon usage pattern was employed (mitochondria use UGA to encode tryptophan rather than as a chain terminator), a single large open reading frame (ORF) was found. This ORF had the potential to encode a protein of 784 amino acids, and revealed a high degree of nucleotide identity to genes encoding RNA-dependent RNA polymerase (RdRp) in other mitoviruses. The putative RdRp region of the newly characterized virus had the highest

sequence similarity to *Ophiostoma* mitovirus 1b. The 5'-terminal sequence of the positive strand could potentially be folded into a double-stranded stem-loop structure with a free energy of 16.6 kcal/mol. Attempts to cure the *O. novo-ulmi* isolate 93-1224 of virus were unsuccessful. Screening of the re-cultured isolates for the presence of OMV1c revealed that it was still present in the fungus despite repeated hyphal tip transfer, a method known to cure cytoplasmic but not mitochondrial viruses. Based on the genome size, phylogenetic analysis, and the observation that infected isolates could not be cured, it was surmised that the virus was a member of the genus *Mitovirus* (family *Narnaviridae*). To assess the distribution of the virus in *O. novo-ulmi* at the disease front in Winnipeg, a small sample of thirteen isolates were screened for the presence of the new mitovirus. All proved to be negative for OMV1c, which indicated this dsRNA virus was rare and that isolate 93-1224 was the only isolate identified to date infected with OMV1c.

It was also discovered that the isolate *O. novo-ulmi* 93-1224 potentially harboured more than one virus. Electron microscopy of fractionated cells revealed the presence of two flexuous rod-shaped particles that may represent additional novel viruses.

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List of Abbreviations

A	adenine
CAN	acetonitrile
ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
c	cysteine
cDNA	complementary DNA
CeMV	<i>Chlora elegans mitovirus</i>
DDT	dl-dithiothreitol
DED	Dutch elm disease
DMSO	dimethyl sulphoxide
dsRNA	double stranded RNA
DTT	dithiothreitol
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EAN	Eurasian aggressive race of <i>O. novo-ulmi</i>
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetic acid
EM	electron microscopy
Fwd	forward
G	guanine
kbp	kilo base-pair
kDa	kilo Dalton
LB	lysogeny broth
MALDI	matrix-assisted laser desorption/ionization
Met	methionine
mRNA	messenger RNA
MS	mass spectrometry
MS/MS	mass spectrometry tandem
m/z	mass-to-charge ratio
NAN	North America aggressive race of <i>O. novo-ulmi</i>
NCBI	National Center for Biotechnology Information
nm	nanometre
OCM	<i>Ophiostoma</i> complete medium
<i>O. himal-ulmi</i>	<i>Ophiostoma himal-ulmi</i>
OMV	<i>Ophiostoma</i> Mitovirus
OMV1a	<i>Ophiostoma</i> mitovirus 1a
OMV1b	<i>Ophiostoma</i> mitovirus 1b
OMV1c	<i>Ophiostoma novo-ulmi</i> mitovirus 1c
OMV3a	<i>Ophiostoma novo-ulmi</i> mitovirus 3a
<i>O. novo-ulmi</i>	<i>Ophiostoma novo-ulmi</i>
ORF	open reading frame
<i>O. ulmi</i>	<i>Ophiostoma ulmi</i>

RAPD	random amplified polymorphic DNA
PTA	phosphotungstic acid
PCR	polymerase chain reaction
PVC	putatively virus cured
RACE	Rapid Amplification of cDNA Ends
RdRp	RNA-dependent RNA polymerase
Rev	reverse
RNA	ribonucleic acid
<i>R. solani</i>	<i>Rhizoctonia solani</i>
RT	reverse transcription
SDS	sodium dodecyl sulphate
<i>S. homoeocarpa</i>	<i>Sclerotinia homoeocarpa</i>
SPAT	single primer amplification technique
ssRNA	single stranded RNA
STE	sodium-Tris-EDTA
T	thymine
TBE	Tris-borate-EDTA
TAE	Tris-acetate-EDTA
TEM	transmission electron microscopy
TFA	trifluoroacetic acid
Trp	tryptophan
U	uracil
UA	uranyl acetate
UTR	untranslated regions
VLP	virus like particle
vc	vegetative compatibility

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Chapter 1: General Introduction

1.1 The Elm

Elms are deciduous and semi-deciduous trees comprising the genus *Ulmus*, family Ulmaceae, Elms originated in central Asia in the Miocene period about 40 million years ago. During that time the tree flourished and established itself over most of the Northern Hemisphere. There are approximately 30 species of elm with six being endemic to North America, twenty to Asia, and only three to Europe (Collin, 2006).

The American elm (*Ulmus americana*), less commonly called white elm, is a species native to eastern North America, occurring from Nova Scotia to as far west as British Columbia, and from northern Alberta to Florida and central Texas (Plotnic and Arboretum, 2000). Historically, from the 18th century to the early 20th century, the American elm was a favoured urban tree for city planners and landscape architects as an ornamental and shade tree and was widely planted in Canadian cities. The elm is a very good choice in our northern climate. It is an extremely hardy tree that can survive winter temperatures as low as -42°C and is resistant to stresses such as high wind, and salt air. The American elm was also very popular for its other unique properties including rapid growth, adaptation to a broad range of climates and soils, strong wood, resistance to wind damage and air-pollution, the comparatively rapid decomposition of their leaf-litter in the fall, vase-like growth habit requiring minimal pruning, and long lifetime span (over 400 years) (Plotnic and Arboretum, 2000). Elm was also valued for its interlocking grain and resistance to splitting and decay when permanently wet. Elm was therefore highly used in carpentry and for city engineering. Hollowed elm trunks were used as water pipes during the medieval period in Europe. Elm was also used as piers in the construction of

the original London Bridge. Elms also have a long history of cultivation for animal feed, with the leafy branches cut for livestock. The practice continues today in the Himalaya mountains. Elm was even used as a food: its bark, cut into strips and boiled, sustained much of the rural population of Norway during the great famine of 1812. The seeds are particularly nutritious, comprising 45% crude protein by dry mass (Osborne, 1983).

1.2 Dutch Elm Disease

Unfortunately, both natural and planted populations of this tree have been devastated by pathogenic fungi of the genus *Ophiostoma* (Division Ascomycota, Class Sordariomycetes, Family Ophiostomataceae). This pathogen, which is the causal agent of Dutch elm disease (DED), spreads within the tree's vascular system and has been responsible for the catastrophic decline of elms in North America and Europe. The name of the disease comes from the fact that the elm pathogen was first isolated in the Netherlands in 1921 by the Dutch phytopathologist Bea Schwarz (Samson *et al.*, 2004).

Ophiostoma fungi grow and reproduce exclusively within elms. Sometimes they are parasitic, feeding on living tissue, while at other times they are saprophytic, gaining nourishment from dead elm tissue. Fungi spread within stems and roots by passive transport of spores and by mycelial growth of colonies that have been started by spores germinated in the xylem. The mycelium is composed of septate hyphae with haploid nuclei (Schreiber *et al.*, 1979).

Both *O. ulmi* and *O. novo-ulmi* have two asexual forms that produce asexual spores called conidia. The first type is produced in the xylem vessels of living trees. Small, white, oval conidia are formed on short mycelial branches. These conidia are carried in

the xylem vessels where they reproduce by budding, germinate to produce mycelium, and thus spread the disease throughout the tree. The second type is produced in dying or recently dead trees where conidia are produced by mycelium growing in tunnels created by beetles just beneath the bark. These sticky conidia are produced at the tips of 1-2 mm tall synnemata built of hyphae fused to form an erect, dark stalk with a round, colorless head of sticky spores. Beetle vectors carry the sticky spores to new elm trees and the pathogens overwinter in the bark and outer wood of dying or recently dead elm trees. All of the *Ophiostoma* fungi also have a sexual stage. When two mating types come into contact, ascospores are produced in spherical, black, long-necked perithecia whose development takes place in the feeding galleries under the bark. Ascospores are produced in asci that are formed inside the perithecia. The free ascospores are discharged at the opening of the perithecial neck, where they accumulate in sticky droplets that may become attached to passing beetles and hence be transported to new trees (Schreiber *et al.*, 1979).

In North America the disease is transmitted by two bark beetle species: American elm bark beetle *Hylurgopinus rufipes* and European elm bark beetle *Scolytus multistriatus* (Lanier, 1989). The adult female beetle bores through the bark of dead or dying elm trees and elm logs and creates a tunnel in the wood as she feeds. She lays eggs in the tunnel behind her. The eggs hatch into larvae and feed, creating tunnels, called galleries. If the fungi are present in the tree, the emerging adults pick up and carry thousands of sticky sexual or asexual spores on their bodies. When young beetles feed on a new tree, fungal spores are deposited. The spores move into feeding wounds, germinate and produce mycelium growing into the xylem. The mycelium produces millions of small, white, oval

conidia that in their turn spread through the xylem sap. Infections that take place in the spring or early summer involve “springwood” which has very long xylem vessels. In these vessels the fungus can spread rapidly throughout the tree, which then may die quickly. Later in the season, the fungus is restricted to the much shorter vessels of the “summerwood,” and spreads much more slowly in the tree. Localized infections often result, and the tree is likely to survive longer (Schumann, 1991).

In an attempt to block the spread of the fungus the elm tree responds by secreting gums into the xylem and developing tyloses which are outgrowths of the parenchyma cells of the xylem. When the plant is introduced to stress like drought or infection, tyloses will fall from the sides of the cells and block the vascular tissue to prevent further damage to the plant. Because the xylem delivers water and nutrients to the rest of the plant, these plugs prevent the rest of the tree from receiving water and nutrients, eventually killing the tree (Spooner *et al.*, 2005). The first symptom of infection is usually an upper branch of the tree with leaves starting to wither and yellow in summer, months before the normal autumnal leaf shedding. This progressively spreads to the rest of the tree, with further dying of branches. Eventually, the roots die, starved of nutrients from the leaves. Sometimes, the roots of some species put up suckers which grow for approximately fifteen years, after which they too die (Spooner *et al.*, 2005). Infection can also occur through root grafts. When elms are growing near each other, *Ophiostoma* may spread up to 15 m following root contact and grafting in the soil. Elms growing within 7 meters of infected elm have almost a 100% chance of becoming infected through root grafts (Schreiber *et al.*, 1979).

Populations of *Ophiostoma* have been separated on the basis of aggressiveness and phenotype characteristics. This segregation has resulted in the establishment of three distinct species, the less aggressive *O. ulmi* (formerly known as *Ceratocystis ulmi*), the highly aggressive *O. novo-ulmi* (Brasier, 1991) and *O. himal-ulmi*, a species endemic to the western Himalaya (Brasier and Mehotra, 1995).

There have been two destructive epidemics of the disease in Europe and North America during the last century, caused by successive introductions of this pathogen. The less aggressive *O. ulmi* was first introduced to Western Europe in 1918 and then arrived in America on imported timber around 1928. This first disease wave was relatively mild, and killed only a small proportion of elms, more often just causing dieback in select branches. The disease had largely decreased by 1940 possibly due to its susceptibility to viruses (Campana and Stipes, 1981). The second, more aggressive wave of the disease, caused by *O. novo-ulmi*, was first reported in the United States in 1930 (Campana and Stipes, 1981). It was believed that vectoring disease beetles arrived in a shipment of logs from the Netherlands destined for use as veneer in the Ohio furniture industry. The disease spread slowly from New England westward and southward, almost completely destroying the famous elms in the 'Elm City' of New Haven, reaching the Detroit area in 1950, the Chicago area by 1960, and Minneapolis by 1970.

In Canada *O. novo-ulmi* was first observed in Quebec in 1944, and then moved relentlessly through Ontario in 1946, New Brunswick in 1957, Nova Scotia in 1969, Manitoba in 1975, Saskatchewan in 1981, and finally in Alberta in 1998. Today the disease is migrating westward from Winnipeg, Manitoba and is threatening elm

populations in Saskatchewan and Alberta. *O. novo-ulmi* has spread as two distinct races, the Eurasian (EAN) and North American (NAN), now considered equivalent to be subspecies (Brasier, 1991). The loss of elms due to *O. novo-ulmi* is enormous. In England 17 million of the country's 23 million elm trees were dead within 20 years (Hubbes, 1999). In North America this disease destroyed over half the elm trees in eastern Canada and the United States of America, and by 1976, only 34 million elm trees were left (Hubbes, 1999). Winnipeg, which has the biggest urban elm population in Canada, has lost 40,000 trees during the last 20 years. The city's elm population is now numbering just 200,000 and the city continues to lose between 4000 to 5600 elm trees each year spending \$2.5 million a year on sanitation and pruning (Skerrit, 2011). Mature trees also make a contribution to property values. The estimated value of a mature elm for insurance purposes is \$3,600 per tree. With roughly 700 000 elms in cities in Canada, the total value is more than \$2.5 billion (Westwood, 1991).

China was long considered a possible site of origin for DED. A series of surveys were carried out by Brasier and his colleagues in 1986 in an attempt to find the location of the geographic origins of both *O. ulmi* and *O. novo-ulmi*, but this still remains elusive. A survey across Central China and Xinjiang Province on the Central Asian border as well as Japan revealed no evidence of the pathogen (Brasier, 1990). However a survey in the Western Himalayas revealed a new, endemic species of DED pathogen, later named *O. himal-ulmi*, which is aggressive towards European elms, but is in ecological balance with the native Himalayan elms. It is likely that *O. ulmi* and *O. novo-ulmi* were derived from Asian isolates. The two *Ophiostoma* subspecies might even derive from two separate

geographical origins. Future surveys are proposed for the Eastern Himalayas, Burma and Yunnan Province in China (Brasier and Mehrotra, 1995).

There are currently no effective methods to control the spread of Dutch elm disease. Traditionally the focus has been on fungicides to stem the growth of the fungus or pesticides to control the spread of the insect vector. Treatment with pesticides proved to be a very expensive option and was not very effective as the beetles simply moved to other tree species during fumigation. They rapidly returned to their favoured habitat, the elm. This approach was called into question by people concerned about the impact of those insecticides on wildlife and people (Carlile, 2006). More recently, several different fungicides have been used in attempts to control the pathogen, key amongst them being benomyl, but these too were deemed to be too expensive and not very effective as this treatment must be repeated every year resulting in damage to the tree (Packham *et al.*, 2001). The best practice to slow down the spread of the disease is a year-round sanitation program: removing infected trees, and promptly destroying the wood by burial or burning, so that it cannot provide a home for beetle vectors. Infected wood must also be debarked before being used as firewood. Because the fungus can spread from tree to tree via root grafts, it is also recommended to protect healthy trees growing near the infected ones by trenching to disrupt root grafts and to plant trees further apart or choose mixed tree species. Without the development of an effective control for Dutch elm disease the natural elm population in North America will be reduced to a scrub population of immature trees under intense infection by this fungus. Once these trees become mature they become targets for infestation by the beetles.

1.3 Hypovirulence

An attractive alternative to the use of chemical pesticides or fungicides is the development of a biological control for *O. novo-ulmi*. This requires, at minimum, an agent which is antagonistic to the fungus, is transmissible to extant populations of the fungus in the field, and is very specific to minimize off-target effects. One such agent might be found in the mycoviruses which have been reported in all classes of fungi. In many cases, these viral infections have not caused disease symptoms in their hosts however some mycoviruses reduce the ability of their hosts to cause disease in plants (Buck, 1986). This property, known as hypovirulence is very attractive as a control method due to the importance of fungal diseases in agriculture and forestry.

Generally, hypovirulence can result from several causes: mitochondrial DNA mutations (Mahanty and Fulbright, 1995); nuclear genome mutations (Anagnostakis, 1984); and the presence of mycoviruses (Boland, 2004). Currently, eight well-characterized examples of hypovirulence have been reported for fungal diseases of plants ranging from turf grass to trees (Nuss, 2005). The fungal pathogens are primarily members of the *Ascomycota*, with one example in the *Basidiomycota* (Nuss and Banerjee, 2005). The taxonomic classes of mycoviruses that cause hypovirulence vary even more than these of their hosts. All hypovirulence-associated mycoviruses have double-stranded (ds) or single-stranded (ss) RNA genomes and include representatives of the *Totiviridae*, *Chrysoviridae*, *Narnaviridae*, and *Reoviridae* (Nuss and Banerjee, 2005).

The use of virus-induced hypovirulence as a biological control relies on the ability to transfer the virus between isolates within a population of the target pathogen.

Unfortunately there are many restrictions to such transmission. RNA viruses that have been found in *O. novo-ulmi* to date are located in mitochondria and can only be transmitted during anastomosis between compatible hyphae, or induced forms of cytoplasmic mixing. There are no known secondary vectors for transmission between isolates. These viruses are transmitted only intracellularly: vertically during host cell division and sporogenesis and horizontally during cell fusion as a result of hyphal anastomosis. DsRNAs are not transmitted to the next generation through ascospores providing a mechanism for fungi to rid themselves of viral infection (Boland, 2003). Horizontal transmission is possible, but usually occurs only between individuals within the same species from the same or closely related vegetative compatibility (vc) groups (Rosewich and Kistler, 2000).

Anastomosis, the main mode of transmission of viruses, probably occurs in the saprotrophic (bark) stage. Within a species, anastomosis is controlled by vegetative incompatibility (*vic*) genes. In *O. novo-ulmi* there are at least seven different *vic* genes, some of which might be multi-allelic. Only when all *vic* genes of two genotypes are identical, are the genotypes vegetatively compatible and anastomosis can occur. If one or more *vic* genes are different, hyphal fusion results in the death of the fused cells (Buck and Brasier, 2002). One of the functions of *vic* systems is to restrict the spread of deleterious intrahyphally transmitted viruses (Caten, 1972).

In 1983 Brasier reported the discovery of a disease agent in *Ceratocystic ulmi* (now called *Ophiostoma novo-ulmi*) and named it as a 'd'-factor. The diseased isolate consistently caused the development of abnormal growth in 'recipient' cultures with which it was paired. D-infected isolates showed a much slower and sparser growth and

sometimes developed unstable 'amoeboid' colonies. Conidia showed a significant loss of viability (13-70%) in spore germination tests. When d-infected isolates were sexually crossed, all resulting progeny were healthy, indicating that the d-factor may be transmitted to the conidia but not to the sexual ascospores of the fungus. Transmission of the d-factor occurred in all pairings with isolates of the same *vic* group, but only in 10 % from different *vic* groups (mostly between isolates having a majority of *vic* genes in common). The abnormal sectors of these pairings were tested to determine whether the transforming factor was cytoplasmic or nuclear. They were found to be cytoplasmic (Brasier, 1983).

It appeared that whenever *O. novo-ulmi* arrived at a "new" locale in Europe, it first spread as a clone of a single *vic* type. These clones also had a uniform colony morphology and a single sexual mating type (Brasier, 1988). These d-factors, which later were determined to be viruses, also tended to spread abundantly in the expanding *vic* clones with the effect that within only a few years, multiple new *vic* types had arisen in the previously clonal population. When the new *vic* types appeared, the frequency of 'd-factors' in the population dropped rapidly. Studies on migrating *O. novo-ulmi* populations in North America showed that a similar change from a clonal population to multiple *vic* types had occurred, but that the rate of change has been much slower and so far only partial. In addition, virus pressure on the clones in North America is low (Milgroom and Brasier, 1997). In New Zealand, where *O. novo-ulmi* arrived in the late 1980s, an immigrant *vic* clone has continued to persist unchanged for over a decade, and no viruses have been detected in the clone (Brasier and Gadgil, 1992). It may be

significant that in New Zealand the weaker pathogen *O. ulmi* was not present prior to the arrival of *O. novo-ulmi*.

Two hypotheses may be drawn from the different outcomes in the European, American, and New Zealand scenarios. First, it may be that *O. novo-ulmi* vic clones diversify into new vic types only where *O. ulmi* was originally present presumably by limited recombination with the extant *O. ulmi*. Second, the clones diversify rapidly and extensively only where virus activity is very high, as in Europe. Also it is possible that the selection pressure exerted by the viruses favours the survival of novel vic types over the original vic clones. Results of a molecular study initiated to test the hypothesis that the novel vic genes come from *O. ulmi* are consistent with the hypothesis. Segments of *O. ulmi* DNA have been found flanking the novel vic genes in *O. novo-ulmi* (Brasier, 2001).

The very low genetic diversity among North American *O. novo-ulmi* isolates contrasts sharply with the very high diversity in current European populations. In North America, populations of *O. novo-ulmi* also have very low level of d-infection. From 112 samples of *O. novo-ulmi* in North America (from California to Nova Scotia), only 16 vic types were found with the first type present in 58 % of isolates while the second and third types were present in 20 % and 10 % of isolates respectively. The remaining 13 isolates had 13 unique vic types (Brasier, 1996). Very interesting questions as to the origins of these viruses arise by the fact that some disease fronts are virus free while others are virus infected. Because there were two waves of infection spread through Europe and North America, with the less aggressive *O. ulmi* being replaced by the more aggressive *O.*

novo-ulmi, it is hypothesised that *O. ulmi* served as a reservoir for the virus. Usually, when *O. novo-ulmi* was introduced to a “naive” site, *O. ulmi* was already resident, albeit at a low level. Shortly thereafter the more aggressive *O. novo-ulmi* rapidly replaced *O. ulmi* (Brasier, 1986-a) illustrating a classic case of a species having greater fitness replacing a less fit species. *Ophiostoma novo-ulmi* has several advantages over *O. ulmi*. Being more aggressive towards elm it can capture more of the host resource (the internal sapstream or xylem of the tree and the highly nutritious inner bark around the beetle breeding galleries) than *O. ulmi*. *Ophiostoma novo-ulmi* may also be better adapted to the temperate climate of Europe and North America, while *O. ulmi* may be disadvantaged through being a tropically or sub tropically adapted organism (Brasier and Mehrotra, 1995). During this replacement process, the close proximity of *O. ulmi* and *O. novo-ulmi* in the bark beetle galleries provided the physical opportunity not only for direct competition but also interspecific genetic exchange. Limited sexual hybridization is possible between these two species (Brasier, 1977; Kile and Brasier, 1990). Rare *O. ulmi*–*O. novo-ulmi* hybrids do occur in nature and appear to be less fit than the parental species, quickly disappearing in competition with the parent species (Brasier *et al.*, 1998). Despite being transient, the hybrids could act as “genetic bridges,” allowing gene flow from one species to the other. It is also possible that the deleterious viruses may also be acquired from *O. ulmi*. A preliminary comparison of viruses in *O. ulmi* and *O. novo-ulmi* isolates obtained from the same epidemic front site in Europe indicates very close similarity in their RNA sequences. Isolates of *O. novo-ulmi* from the xylem of infected trees in the pathogenic phase of DED are generally free from deleterious viruses, but almost 90% of isolates taken from the bark in the ensuing saprotrophic phase are virus

infected (Brasier, 1986-b). Together, these possibilities suggest that a remarkable series of events has occurred. *O. novo-ulmi* has apparently competitively eliminated *O. ulmi* across much of the Northern hemisphere, causing local extinctions of *O. ulmi*. At the same time, *O. novo-ulmi* may have acquired debilitating viral infections from *O. ulmi* (Abdelali *et al.*, 1999). Mycovirus infection probably exerts a strong selection pressure for virus-free vegetatively incompatible genotypes resistant to virus transmission that would be expected to outgrow the virus-infected individuals (Brasier and Buck, 2001).

1.4 General Research Objective

Studies of fungal viruses and hypovirulence can increase our understanding of molecular mechanisms influencing the expression of virulence in these plant pathogens and broaden the potential of fungal viruses as a biological control. The hypothesis that we can use these fungal viruses as biological control agents came from both a scientific and disease-management perspective (Boland, 2003). My research objective is to understand the mechanism of hypovirulence sufficiently well to determine whether we might be able to develop effective biological controls for DED. My main objective was to explore potential diversity of viruses in a Canadian isolate of *O. novo-ulmi*, 93-1224 and to determine whether there were any phenotypic effects associated with viral infection.

Chapter 2: Characterization of a New Mitovirus in a Canadian Isolate of *Ophiostoma novo-ulmi* (Isolate 93-1224)

2.1 Introduction

2.1.1 Mitoviruses

Mycoviruses are usually located in the cytoplasm of the fungal host. Certain double-stranded RNA (dsRNA) viruses, however, are found exclusively in the mitochondria (Polashock and Hillman, 1994). This latter class, referred to as mitoviruses, have no capsid and consist of a single naked RNA encoding an RNA-dependent RNA polymerase (RdRp) that is required to duplicate the RNA (Doherty *et al.*, 2006; Ghabrial, 1998). Mitoviruses have been assigned to the family Narnaviridae which is composed of only two genera: *Narnavirus* and *Mitovirus*. The genus *Mitovirus* consists of positive single stranded RNA (ssRNA) viruses with no DNA stage. There are nineteen fully or partially characterized species of genus *Mitovirus* listed in the National Center for Biotechnology Information (NCBI) Genome database. Nine of these are found in the fungal genus *Ophiostoma* (Table 1). Mitoviruses lack structural proteins and consist of monopartite, linear, naked positive-stranded ssRNAs. A defining feature of mitoviruses is that they inhabit mitochondria and therefore utilize the mitochondrial codon preference as opposed to the cytoplasmic codon preference (universal). Many mitochondrial viruses have a single open reading frame (ORF) that encodes the RdRp and associates with its own RNA to form an RNA/RdRp complex that plays a key role in RNA replication in mitochondria of the host (Shackelton and Holmes, 2008). *Mitovirus* genomes vary in size from 2.3 to 3.5 kilo base-pairs (kbp) (Hong *et al.*, 1999; Polashock and Hillman,

Table 1. *Ophiostoma* genome sequences (from NCBI Genome Data Bank).

a)

Partial genome sequences:	Accession:
<i>Ophiostoma</i> mitovirus 1a RdRp gene	AM087548.1
<i>Ophiostoma</i> mitovirus 1b RdRp gene	AM087549.1
<i>Ophiostoma</i> mitovirus 3b RdRp gene	AM087550.1
<i>O. novo-ulmi</i> mitovirus 4-Ld repeat region, segment 7	AJ003120.1
<i>O. novo-ulmi</i> mitovirus 4-Ld repeat region, segment 10	AJ003121.1

b)

Complete genome sequences:	Accession:
<i>Ophiostoma</i> mitovirus 3a	NC_004049.1
<i>Ophiostoma</i> mitovirus 4	NC_004052.1
<i>Ophiostoma</i> mitovirus 5	NC_004053.1
<i>O. novo-ulmi</i> mitovirus 6-Ld	NC_004054.1

1994) and replication of virus RNA is catalyzed by the RdRp. This takes place in two stages: synthesis of a complementary negative-stranded RNA using the virus genomic RNA as a template, and synthesis of progeny virus genomic RNA using the negative-strand RNA as a template. Thus, both ssRNA and dsRNA forms are present in fungus and the ratio of ssRNA to dsRNA is approximately 5:1 with the positively stranded ssRNA predominating (Hayes and Buck, 1990; Polashock and Hillman, 1994). This pattern has been observed for mitoviruses 4, 5, and 6 isolated from *O. novo-ulmi* (Hong et al, 1999). Certain of the unencapsidated dsRNAs associated with the phenomenon known as hypovirulence have been characterized and classified into the genus *Mitovirus* (Hillman *et al.*, 2000; Wickner *et al.*, 2000).

2.1.2 Evolution of Mitoviruses

Given that there is limited sequence information for mitochondrial dsRNAs it is difficult to ascertain the evolutionary relationships and origins of dsRNA viruses (Attoui *et al.*, 2000). Nevertheless, there are a few hypotheses regarding the origin and evolution of mitoviruses. Key to this is the observation that mitoviruses utilize the mitochondrial codon usage pattern. Though most organisms use the “universal” or “standard” genetic code, a number of organisms and organelles have developed slightly different codon assignments. For example, the universal stop codon UGA encodes tryptophan in yeast (order *Saccharomycetales*) and in bacteria from the genus *Mycoplasma*, while universal stop codons, UAA and UAG, can code for glutamine in *Acetabularia acetabulum* (a unicellular marine algae that belongs to the family Dasycladaceae (Schneider *et al.*, 1989). In order for an exogenous genetic element, such as a virus, to be accurately

expressed within the cell's translation machinery it is extremely important to use the same genetic code as the host. This leads to the supposition that codon preference for both parasite and host must match fairly closely and that hosts could avoid infection by slight alterations to codon usage. Another hypothesis that logically arises from the use of the same code by a virus and its host is that viruses could have originated by the escape of genetic material from host genomes.

In fungal mitochondria the universal opal stop codon UGA encodes tryptophan. This creates a block to the translation of mitochondrial RNAs in the cytoplasm as each UGA encountered would result in translation termination. Mitoviruses of the family *Narnaviridae* that infect fungi appear to use the mitochondrial code of their hosts. Mitoviral RdRp ORFs contain a number of internal UGA codons that must be expressed as coding for tryptophan for the correct production of protein product (Shackelton and Holmes, 2008; Hong *et al.*, 1998). The mitoviral RdRps were found to be closely related to mitochondrial RdRp ORFs in plants (*Brassica napus*, *Arabidopsis thaliana*) (Marienfeld *et al.*, 1997; Osaki *et al.*, 2005). Based on this homology, Marienfeld *et al.*, (1997) suggested that their unexpectedly high sequence similarity was the result of horizontal gene transfer and integration into the plant mitochondrial genome. A different scenario was proposed by Hong *et al.*(1998), who proposed that the RdRp sequence was present in the plant/fungus common ancestor, and, only after the divergence of plants and fungi, integrated into the plant mitochondrial genome. It is also possible that the ancestral sequence was present in the common ancestor and escaped from the fungal mitochondrial genome following the plant/fungus divergence. This idea is supported by the observation that sequences identical to the mitoviruses of the phytopathogen

Thanatephorus cucumeris, the causal agent of rice sheath blight, were also found encoded by the DNA genome of its fungal host (Lakshman *et al.*, 1998). As a competing theory, Sexton *et al.* (2006) suggested that the genetic elements in plants were horizontally transferred to fungi. It may also be the case that emerging viruses provide a selection pressure leading to the use of alternative codes in potential hosts. It has been hypothesized that alternative codes appeared because they provided a more immediate benefit, protection against viral infection from other host species. Mutant hosts with alternative codons, which would be removed from the population under normal conditions, may have a selective advantage when a new virus is introduced (Shackelton and Holmes, 2008). Viruses would therefore be less likely to cross a host-species barrier when the barrier included codon differences.

2.1.3 Mitoviruses in *Ophiostoma novo-ulmi*

Studies on the diversity of mitoviruses of European isolates of *O. novo-ulmi* demonstrated that there were a variety of novel viruses within populations of *O. novo-ulmi* at the disease fronts. A total of thirteen d-factors were isolated from several isolates (Sutherland and Brasier, 1997), and one diseased isolate, *O. novo-ulmi* Ld, was reported to be multiplicatively infected with twelve distinct mitoviruses: 1a, 1b, 2, 3a, 3b, 4, 5, 6, 7, 8, 9, and 10 (Cole *et al.*, 1998). The complete nucleotide sequence was determined for five of these *Ophiostoma* mitoviruses by Hong *et al.* (1998, 1999) corresponding to mitovirus RNAs 4, 5, 6, 7 and 10. The sequences of three additional mitoviruses RdRp, 1a, 1b, and 3a, were determined by Doherty *et al.* in 2006.

In 1993 and 2002, the genetic diversity of the population of *O. novo-ulmi* in Winnipeg, Manitoba, Western Canada was surveyed using RAPD marker analysis, vegetative compatibility tests and screened for the presence of dsRNA. In 1993, the pathogen at the disease front was mostly clonal with three quarters of the isolates belonging to genotype I and one quarter belonging to genotype II. If the dynamics of the NAN (North America aggressive race of *O. novo-ulmi*) populations in North America were similar to what was observed for the EAN (Eurasian aggressive race of *O. novo-ulmi*) populations in Europe, the genetic diversity would be expected to increase over time. Data from the 2002 survey showed that over a nine-year span in the same geographic area there was apparently no increase in genetic diversity and that the population of the pathogen had remained largely clonal (Temple *et al.*, 2006). These results are different from results previously obtained in Europe by Brasier (1988), who showed that the pathogen very quickly established a variety of vic types behind the disease front within a period of six to ten years. It was hypothesized that one of the major drivers for this diversification was the presence of deleterious mitoviruses (Brasier and Kirk, 2000). It would therefore be anticipated that the largely clonal populations in western Canada, demonstrating a low diversity of vic types, would be relatively free of dsRNA viruses. When samples of this pathogen collected from the Winnipeg disease front in both 1993 and 2002 were screened for the presence of dsRNA viruses, only one isolate (isolate *O. novo-ulmi* 93-1224) was found to be obviously infected with a dsRNA. Purification of dsRNAs demonstrated the presence of two RNA molecules which migrated at ~ 2.1 and ~ 2.3 kbp on an agarose gel (Temple *et al.*, 2006). These RNAs were confirmed to be dsRNAs as they were resistant to DNase and S1 nuclease while

susceptible to degradation by RNase and they were transmissible by hyphal anastomosis (Temple *et al.*, 2006).

2.1.4 Current Study and Research Objectives

We were interested in determining whether this element had similarity to other viruses characterized in *Ophiostoma*. Since there was a possibility that there was more than one type of virus, we also sought to determine how many viruses might be harboured in the Western Canadian disease front and whether any of them belong to the genus *Mitovirus*. This would be expected since all of the viruses previously characterized in *O. novo-ulmi* were mitoviruses.

We describe here the isolation and characterization of a new mitovirus from isolate 93-1224 collected at the disease front in Winnipeg. We have named this virus OMV1c (*Ophiostoma novo-ulmi* mitovirus 1c) based on sequence similarity to other well-characterized *Ophiostoma* mitoviruses. In order to better understand the nature of this new virus we also tested whether we could cure *O. novo-ulmi* 93-1224 of this dsRNA element by repeated hyphal tip transfer. While the titre of the dsRNA element was reduced to the point where it was no longer visible by agarose gel electrophoresis, it was still possible to detect the presence of mitovirus OMV1c using a combination of reverse transcription and PCR amplification. To determine the range and natural occurrence of this new mitovirus we screened additional isolates from the western Canadian disease front as well as two other *O. novo-ulmi* isolates: VA30 from Virginia (USA) and H327 from Bratislava (Slovakia, former Czechoslovakia) for the presence of OMV1c. We found that OMV1c was unique to isolate 93-1224.

2.2 Materials and Methods

2.2.1 Fungal Growth and Culture Maintenance

Ophiostoma novo-ulmi isolate VA30 was collected by L. Schreiber and A. Townsend in Virginia, USA (Temple *et al.*, 2006). The isolated culture of *O. novo-ulmi* 93-1224 was collected by Philip Pines from an infected elm in Winnipeg in 1993, while culture H327 was collected by J. Jamnicky from Brezno-Nizke, Tatry, Bratislava, Slovakia in 1979 (Konrad *et al.*, 2002). The other *Ophiostoma novo-ulmi* isolates were collected by Joyce Carneiro from the disease front in Winnipeg in 2010. All isolates were grown in laboratory conditions on solid *Ophiostoma* complete media (OCM) (Bernier and Hubbes, 1990) at 23° C and kept at 4° C for short-term storage. Stock cultures were maintained at -196° C in liquid nitrogen, after infusion with 15% glycerol (Temple *et al.*, 2006).

Cultures *O. novo-ulmi* 93-1224 and *O. novo-ulmi* VA30 were prepared for RNA extraction and virus purification as described by Temple *et al.* (2006). Flasks containing 50 ml of liquid OCM medium were inoculated with 0.5 cm² of fungus from solid media and incubated 7 days at 23°C. Mycelia from twenty flasks was harvested onto sterile Miracloth, washed 3 times with sterile distilled water, pelleted by centrifugation at 2000 g for 10 minutes and used for RNA extraction or virus purification.

2.2.2 RNA Extraction

Total RNA extraction was performed according to the protocol “Purification of total RNA from plant cells and tissues and filamentous fungi” using an RNeasy Mini Kit, (Qiagen, Toronto, ON). Mycelium (1-3g freshweight) was harvested by centrifugation at 2000 g for 10 minutes, flash frozen in liquid nitrogen and crushed to a fine powder using

a chilled mortar and pestle. The dsRNA was extracted as described by Temple *et al.* (2006). The dsRNA was visualized following electrophoresis on 1.0 % agarose in 1x TAE buffer (0.04 M Tris-acetate; 1 mM EDTA) at 100 V for 60 minutes followed by staining with GelRed stain (Biotium Inc., Burlington, ON.)

2.2.3 Complementary DNA (cDNA) Synthesis

We used the single-primer amplification technique (SPAT) as described by Attoui *et al.* (2000) to synthesize cDNA using a viral RNA template. The basic step in the SPAT was the ligation of a 3' blocked DNA oligonucleotide (5'-PO₄-AGGTCTCGTAGACCGTGCACC-NH₂-3') to both 3' ends of the dsRNA. This key step was achieved using the DNA-RNA ligation properties of the T4 RNA ligase. A double-stranded complementary DNA (cDNA) was synthesised from the tailed dsRNA using a complementary primer (5'-GGTGCACGGTCTACGAGACCT-3'). A second cDNA synthesis was performed for further gene walking using the protocol for first-strand cDNA synthesis from Omniscript Reverse Transcription Kit (Qiagen, Toronto, ON). For this reverse transcription (RT)-PCR genome specific primers for both strands were designed on the basis of the first sequenced clone. The forward primer (TGCAATTTGTTGCTAGTGGA) was used for making cDNA from the negative (3'-5') strand, and the reverse primer (TGCAATTTGTTGCTAGTGGA) was used for cDNA synthesis from positive (5'-3') strand of viral dsRNA.

2.2.4 PCR Amplification and Cloning

PCR mixtures (25 µl of total volume) were made using Taq DNA Polymerase Kit from Invitrogen and cDNA from RT-PCR. The reaction mixture was subjected to 35 cycles of denaturation at 94° C for 1 min, primer annealing at 35° C - 63° C (depending on the primer's melting temperature) for 1-2 min, and extension at 72° C for 1 min. A final 5 min elongation was done at 72° C. Amplifications were performed in a Perkin Elmer DNA thermal cycler (Norwalk, CT). The amplified DNA products were separated by electrophoresis on 1% agarose gels and visualized by staining with GelRed (Biotium, Hayward, CA). Amplification products were excised from the gel and extracted with the "MinElute Gel" Extraction Kit (Qiagen, Valencia, CA). The double-stranded cDNA fragments were ligated into the pGEM -T vector (Promega, Madison, WI), and then transformed into electrocompetent *E. coli*™ (Lucigen, Middleton, WI) using an Electro Cell Manipulator - 600 (LabCommerce, Inc., Santa Clara, CA). The plasmid-bearing *E. coli*™ strain was cultured on solid Lysogeny broth (LB) medium (10 g/L- tryptone, 5 g/L- yeast extract and 10 g/L - NaCl, 15 g/L - agar, which was supplemented with ampicillin (100 µg/mL). Colonies showing the presence of plasmid DNA were selected and cultured in liquid LB medium (10 g/L- tryptone, 5 g/L- yeast extract and 10 g/L- NaCl, also supplemented with ampicillin 100 µg/mL) by incubating at 37° C for 24 h with shaking at 250 rpm in an incubator shaker (New Brunswick Scientific Excella E24 Incubator Shaker Series).

Plasmid DNA was purified by standard protocols using the QIAprep Miniprep Kit from Qiagen (Toronto, ON) and analyzed by restriction enzyme digestion with *Sac*1 and *Sac*2 (New England Biolabs, Hitchin, UK). Agarose gel (1% in TBE buffer) electrophoresis was carried out at 100 V for 60 min. Cloned cDNAs, putatively corresponding to dsRNA

sequences, were screened for similarity to characterized mitovirus sequences from the GenBank database using the Basic Local Alignment Search Tool (BLAST) available online at <http://blast.ncbi.nlm.nih.gov/Blast.cgi> (Altschul *et al.*, 1990). Multiple sequence alignments were done using CLUSTAL W2 program, version 2.0 available online at <http://www.ebi.ac.uk/Tools/msa/clustalw2/> (Larkin *et al.*, 2007). Open reading frames were found by Open Reading Frame Finder (ORF Finder) available online at <http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi>.

2.2.5 Gene Walking

Following the initial identification of an RdRp gene fragment from our dsRNA preparations the full-length sequence of the target virus was obtained by primer walking. The target two cDNA libraries (one for each strain) were constructed with genome specific primers (Fwd: TGCAATTTGTTGCTAGTGGA; Rev: TGCAATTTGTTGCTAGTGGA) designed according to the novel RdRp gene fragment. These target cDNAs were then amplified using a combination of genome specific primers paired with random or degenerate primers to extend the characterized sequence and this process was repeated to extend the characterized novel sequence. Genome specific primers were designed based on the sequence obtained. Degenerate primers were also designed based on well conserved regions of the other mitoviruses' genomes from GenBank. This provided overlapping sequences of both strands of the virus genome, which permitted the determination of a consensus sequence for the new virus. All clones were sequenced in both directions.

To determine the terminal sequences of the linear dsRNA molecule, cDNA clones of the 3'-end and the 5'-end were obtained using the 5' Rapid Amplification of cDNA Ends (RACE) Kit (Invitrogen, Grand Island, NY). This procedure was used for the 3'-end as well as for 5'-end. This was possible because even though mitoviruses are predominantly regarded as having a single stranded RNA genome, there is a double stranded RNA stage in their replication cycle (Hayes and Buck, 1990; Polashock and Hillman, 1994) which permitted the use of the negative strand to obtain the sequence of the 3' end using 5'RACE. The nucleotide and deduced amino acid sequences were compiled and analyzed using Gene Runner (Hastings Software, Inc.). The RNA secondary structures were analyzed and visualized by using the program RNAfold (available online at <http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>) (Hofacker *et al.*, 1994; Zuker and Stiegler, 1981; and McCaskill, 1990).

2.2.6 Phylogenetic Analysis

Phylogenetic analysis was performed for the eight complete genomes and for twenty three RdRp genes of mitoviruses on the Phylogeny.fr platform available online at <http://www.phylogeny.fr/> (Dereeper *et al.*, 2008). Sequences were aligned with MUSCLE (v3.7) configured for highest accuracy (MUSCLE with default settings; maximum number of iteration 16) (Edgar, 2004). The alignment curation was done with Gblocks parsing with conservative settings (maximum number of contiguous nonconserved positions allowed was 4; minimum length of a block allowed was 10) (Castresana, 2000). The phylogenetic tree was reconstructed using the maximum likelihood method with the approximate Likelihood-Ratio Test (aLRT) implemented in

the PhyML program (v3.0 aLRT). The Whelan and Goldman (WAG) model (Whelan and Goldman, 2001) substitution model was selected assuming an estimated proportion of invariant sites (of 0.000) and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (gamma=1.534). Reliability for internal branch was assessed using the aLRT test (SH-Like). Graphical representation and editing of the phylogenetic trees were performed with TreeDyn (v198.3) (Chevenet *et al.*, 2006).

2.2.7 Screening *O. novo-ulmi* 93-1224 for the Presence of *Ophiostoma* Mitoviruses: 1a, 1b, 3a, 3v, 4LD, 5LD, and 6LD

To screen isolate 93-1224 for the presence of previously characterized *Ophiostoma* mitoviruses a series of PCR amplifications were performed using genome specific primers designed according to mitoviruses 1a, 1b, 3a, 3b, 4LD, 5LD, and 6LD (Tables 1 and 2). These primers were first used to make a complementary DNA followed by PCR amplification of a diagnostic portion of the mitovirus cDNA. cDNA synthesis was performed using mitovirus-specific reverse primers (Table 2). PCR was done with mitovirus-specific forward and reverse primers as outlined in the above section (2.2.4).

2.2.8 Attempts to Cure *O. novo-ulmi* Isolate 93-1224 of dsRNA Virus

In order to confirm the mitochondrial location of OMV1c and to understand the association between the OMV1c and the double band observed following agarose gel electrophoresis, we attempted to cure *O. novo-ulmi* 93-1224 of virus infection by hyphal tip re-culturing. Mycelium of *O. novo-ulmi* 93-1224 was grown on solid OCM at 23° C. Following five days of growth on solid medium the apical tips of the hyphae, corresponding to the first 50 – 100 µm were micro-dissected with the help of a light

microscope and re-cultured on a new Petri dish using the same media. This procedure was repeated a total of ten times and the cultures were examined for the presence of mitoviruses as described in 2.2.2 (Buck, 1979).

2.2.9 Screening *O. novo-ulmi* H327, VA30, and the 13 New Isolates from Winnipeg for the Presence of New Sequenced Mitovirus

To determine the prevalence of the novel mitovirus at the Western Canadian disease front, a total of thirteen isolates of *O. novo-ulmi* collected in the city of Winnipeg during the summer of 2010 were screened for the presence of the new virus of *O. novo-ulmi* 93-1224. Negative controls included isolates VA30 and H327. For each isolate assayed, total RNA was extracted and first strand cDNA synthesized using the primer (Rev:

Table 2. Primers for screening *O. novo-ulmi* 93-1224, VA30, and 93-1224-PVC for other known *Ophiostoma* mitoviruses. F – forward, R – reverse.

Ophiostoma mitovirus 1a:

Primer1a-F ggatcatcaaggtacggcact

Primer1a-R gcgggaccatctcttaacaa

Ophiostoma mitovirus 1b:

Primer1b-F cgtggctaaggatcccaata

Primer1b- R actcccgggtattccagaac

Ophiostoma mitovirus 3a:

Primer3a-F tgcgatggtttaatggaaca

Primer3a- R ggattctagtcgcccctac

Ophiostoma mitovirus 3b:

Primer3b-F agcaggtcctaattggtggtg

Primer3b- R ctggtaaaccggagccataa

Ophiostoma mitovirus 4LD:

Primer4LD-F tttaccaggatccatttca

Primer4LD- R atacgtcccattggttgc

Ophiostoma mitovirus 5LD:

Primer5LD-F ccattcgcaattgttctca

Primer5LD- R gtaggtcaatgggaaacga

Ophiostoma novo-ulmi mitovirus 6LD:

Primer6LD-F tgacattatcagcagcaatgg

Primer6LD- R agctcttgcctttgtcca

TTGAGCCACTCGCTGATATG). The individual cDNAs were then used as amplification targets using mitovirus specific primers designed according to the sequence of the new virus (Fwd: CCTCCGTACGATGAGGAAGA and Rev: TTGAGCCACTCGCTGATATGPCR) (Carneiro, J., unpublished). PCR amplification and product visualization were done as previously described.

2.3 Results

2.3.1 Production of cDNA Clones and Sequencing dsRNA

The nucleotide sequences of several cDNA clones obtained following the SPAT procedure were compared to sequences in the GenBank database using Basic Local Alignment Search Tool (BLAST) (Altschul *et. al.*1990) (Tables 3, 4). One clone demonstrated a 70% similarity to the RNA dependent RNA polymerase (RdRp) of the *Ophiostoma* mitovirus 1a (OMV1a) (accession number at GenBank: AM087548.1) and a lower similarity to other mitovirus sequences (Doherty *et. al.*, 2006). This clone was used for designing genome specific primers for the process of recovering the entire mitovirus genome. The complete sequence was obtained in seven steps of gene walking (Figure 1, 2).

2.3.2 Sequence Analysis and Genome Organization of a New Virus

The complete dsRNA sequence of the mitovirus from *O. novo-ulmi*, isolate 93-1224, measured 3,003 nucleotides in length, and was relatively rich in A and U (63.4%). This RNA molecule was not polyadenylated.

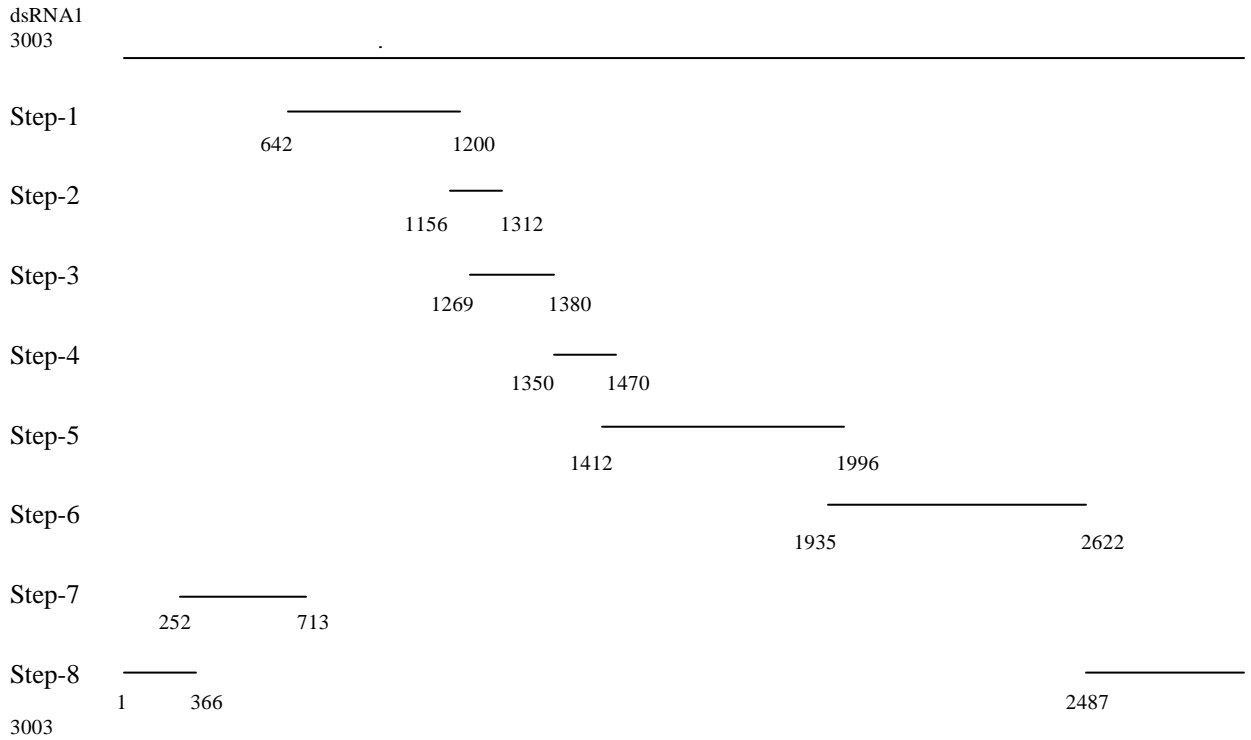


Figure 1. Map of primer walking. Steps 1- 4 represented extensions using newly characterized sequence paired with random primers. Steps 5, 6, 7 represented extensions using newly characterized sequence paired with degenerate primer designed according to other mitovirus sequences. Step 8 represented rapid amplification of cDNA ends (5' RACE).

2.3.3 Open Reading Frame and Codon Usage Analysis

Because both strands of a dsRNA could potentially encode proteins, the nucleotide sequence of the new mitovirus was examined for the presence of open reading frames (ORFs) in all six reading frames. When the universal codon usage for cytoplasmically translated proteins was applied, there were no long open reading frames however shorter segments of the RdRp gene could be recognized. Because of the high similarity of these RdRp fragments to mitoviruses, a mitochondrial-specific codon usage pattern was applied to the new sequence. When the genetic code for Mold, Protozoan, and Coelenterate Mitochondrial and the Mycoplasma Code (code 4) was employed, a single large ORF was found on the positive strand. This ORF, which started with the AUG - start codon, and terminated with the UAG – stop codon, had the potential to encode a protein of 784 amino acids. This ORF had sixteen AUG codons, the first of which served as a start codon while the rest encoded methionine (Nakamoto, 2009) as well as fifteen AUA codons which in the mitochondrial codon usage, also, encode methionine (Met). Also, there were 12 UGA codons, which in mitochondrial codon usage were used to encode tryptophan (Trp) rather than acting as a chain terminator (Figure 2). The genome showed a high (~ 78%) preference for using adenine and uracil in the third position of codons.

The predicted molecular mass of the putative RNA-dependent RNA polymerase was 77.23 kDa, which is within the range of RdRp proteins of other mitoviruses (Table 3).

Table 3. List of protein predicted molecular masses of select mitoviruses.

Mitovirus	Protein predicted molecular mass (kDa)
<i>Arabidopsis thaliana</i> Mitovirus	27.48
<i>Cryphonectria cubensis</i> mitovirus 2c	57.48
<i>Botrytis cinerea</i> mitovirus 1 S	60.29
<i>Ophiostoma novo-ulmi</i> mitovirus 1c	77.23
<i>Sclerotinia homoeocarpa</i> mitovirus	80.62
<i>Tuber aestivum</i> mitovirus	89.4

1 ccgatatggggtcgcctgactttcgcgagtcaga
33 aacctccgtacgatgaggaagagtccttctcttatgcttaattg
78 catgtaattcctaataataggtcgtgtaagagtgtcgtccaagggc
123 tgattccttctaattaaagttagttagatcttgtctgtcgttcct
168 catcataagggaggttaaagctcggggttgaccctctttcttt
213 taacagacagaacttttactaaccgagattagtcggtggggcac
258 aatggaaagcgattaatctcgttctgttgaacccaagggtta
303 accatacctgtttaatcaaactgcctgcagattcctcattagagg
348 gaaaactgaagaaggaaatggtattaaattaataacttgcaac
393 taacaactttttacataaattatgatcatgtatcaacgttgtag
438 atgtacattttcttggcacaaggtctaaaaatcccttgacttatta
M Y I F L A Q G T K S T D L L
483 gtctgaccacttttacaagtataagactagtaaggggtgttta
V W P T L Q V M R T V R G C L
528 agcccagatttaatacaagcagatattgttttcgtgaaaagatca
S P D L I K A V F V F V K R S
573 agttctcttcagaaaactggagggttaaagttcgttgctttatat
S S T Q K T G G L K F V A L Y
618 tataaagcttgcataatctacactatgcaatttgttgctagtgg
Y K A C H I Y T M Q F V A S G
663 ggtgtagacaatccttcataacatcaacatggttaggagtaaac
G V R Q S F M T S T C Y G V N
708 gtatctctaactccggtggtctaccgagaattcttctctatttat
V S T T S G G T P R I T P I Y
753 ttaagaagattggttacttcacagaacaaagaaggtatcaaaatt
L R R L V T S Q N K E G I K I
798 gttttaactttatttaacctatagggcttaccctatccagga
V L T L F N T Y R V L P Y P G
843 aaagtaaaattatcaaccataactgataaatgatccggatcatat
K V K L S T M T D K W S G S Y
888 cccttagatatgatctcattcattcctaagtttgggtgcttcta
P L D M I S F I P K F W L T T
933 agatcccaaggaaagatagctccttttacctttattcgatcacca
R S Q G K M A P F T F I R S P
978 ttcgccatatacagcagtggtcaattacagggttttgggaaacat
F A M S A S G S I T G F G K H
1023 ctttctccatgctctggtttctttaagctcttctattcctaaga
T S S M S G F F K A T T F T R
1068 agagaggattctctgtgacaatcacttcaatgattttttacagag
R E D S T W Q S T Q W F F T E
1113 gccctttaagaaggggatgagcttctagaactctacgatgttga
A P L R R G W A S R T T R C W
1158 caatctatggattttatctcagattggtactgttgagggtcaa
Q S M D F I S R L L T V A G Q
1203 aaagtcgtatcttctcctttaggaaggttagcctttaagaggaa
K V V S S P L G K L A F K E E
1248 ccgggaaaagttagggtattcgctatggcggactgtataactcaa
P G K V R V F A M A D C M T Q
1293 tgagtattacatcctctccaccagtatttattttcaatcttgaaa
W V L H P T H Q Y L F S I L K
1338 caaataagcatcgttgatgcaacatttgatcaagaagaaggggna
Q M S I V D A T F D Q E E G X
1383 agaaccctttctcagaagataaaatctggttaagaagattgtattc
R T T S Q K M K S G K K I V F
1428 tccttggatttatcagccgctacagacagattaccactaacaatt
S L D L S A A T D R L P T T I
1473 caagcacagatcctgaatcatatagttccaaagttaggggaccac
Q A Q I T N H M V P K L G D H
1518 tggccaatcttctggttaacagagattactcagtgccaacat
W A N T T V N R D Y S V P N H
1563 attactctaccagttaatcctggtactgtagatagggagcgggc

I T T P V N P G T V R Y G A G
 1608 caacctatgggagcttatagttcatgagctatgcttgcccttaca
 Q P M G A Y S S W A M T A T T
 1653 catcacttcatagtacagtattgtgctttcaaagtctatcattct
 H H F M V Q Y C A F K V Y H S
 1698 aattcattcttcaaagattatctaacttaggagatgatttatta
 N S F F K D Y T I L G D D L L
 1743 ttgtagacgagaggttgcaaacagtagtcttaaagtcagagc
 L L D A R V A K Q Y L K V M S
 1788 cagctggatgtaggagtgaaactagcaaaatccttaactctctgtt
 Q T D V G V N T A K S L I S V
 1833 agaggttatggagaatttgctaaacaattcctatctccagagga
 R G Y G E F A K Q F T S P E G
 1878 cctttacaaggggtcagttttaaagaatcttctgtaaagat
 P L Q G V S L K E F S S L K D
 1923 ggaatgtctaacattctatccttatcggttaaagtgctctttaa
 G M S N I T S L S V K M S T K
 1968 ccttcttattgttaaggctgtttgggtttggttcaaatcagtt
 P S L L L R T F G F G S K S V
 2013 ggtcattcaactttacccttttctagatactcattgagatccagt
 G H S T L P F S R Y S L R S S
 2058 cttgatcatctattcttgagtcgcttgcaactctctgaatagt
 T D H T F L S P T A N S T N S
 2103 tgactagattatttctcggtattagacattcaaaattcttattg
 W T D Y F S C I R H S K F L L
 2148 attagtaagcttttattggcttactttctatgaggttttgaattt
 I S K S L L A Y F T W G F E F
 2193 atcaaatcaaatgagttttcaagaaggttagggagaatgagcaa
 I K Y K W V F K K V R E N E Q
 2238 ctgatgttgcgacatggaacacatcgctaccatcaattggaagt
 T M L R H G T H R Y H Q L V S
 2283 gagctttactgagttactgatgtccttctccatcagaagaggta
 E T L T S Y W C P S P S E E V
 2328 ttgaaattaccagaagttgctgattcatcagctaacttcttcttt
 L K L P E V A D S S A N F F F
 2373 tcaactcctcctccggtgaataaggcatcattcaagttattgatg
 S T P P P V N K A S F K L L M
 2418 ataatcatatcaaatatctgttctcatgtgggtctctcttttca
 I N H I N I L F S C G S T F S
 2463 gaattgatgaatggaggtgcaatgaatctagtccctaaattctta
 E L M N G G A M N T V P K F L
 2508 acatttgagttgtcctttgaagaggttactcagatcttgagaaa
 T F E L S F E E G Y S D F E K
 2553 gtggagaatgatcacttatgatcagtcacacaagggatccaaaa
 V E N D H L W S V I T R D P K
 2598 agttgggttaaaccataatatttgatattgttaatttatgagc
 S W V K P N N F D I V K F M S
 2643 cagcaaacatctgtagagtttcacaaaccaaggactcttaaggaa
 Q Q T S V E F H K P R T T K E
 2688 atttcttagctttacaactaaagaaggatttctttaatagtagt
 I S L A L Q T K K D F F N S S
 2733 ttggtaaatggatctcaaacatactagatgtgcctcttggtaaa
 L V N G S Q T M T D V P T G K
 2778 ccaaaaggcTaggtgactcattagcaatcatagtgatctctgggt
 P K G stop
 2823 gactcattagcaatcatagtgatctctggttgagaataactcagtt
 2868 aaagtgatcgagaattatgaaattattcatttcttttcgaaag
 2913 accctttcgtgtagtatttataaccagtagatcagagatctatta
 2958 ttgtagctaaatgtttcattcctcataaaggagatgttccaaaat
 3003 aatctgagctatctccccggttaagggaaaacaa

Figure 2. Open reading frame of the positive strand in the whole genome of OMV1c. Shaded + underlined – start (atg)codon and stop (tag) codon. Lower case letters indicate nucleotides, upper case letter indicates amino acids.

2.3.4 Sequence Similarities between OMV1c and Other Mitoviruses

Database searches with the nucleotide and amino acid sequences revealed that the ORF region of OMV1c had a very high sequence similarity to the *Ophiostoma* RdRp for mitovirus 1b (OMV1b), (82% of maximum nucleotide identity for 66% of query coverage (Figure 3) and 68% of max amino acid identity for 76% of query coverage (Figure 4). Less significant but obvious identity existed in nucleotide and amino acid sequences with other previously sequenced mitoviruses. The highest sequence identities of the putative RdRp region of OMV1c to other previously characterized mitoviruses is shown at the nucleotide level in the Table 4, and at the protein level in Table 5. Taken together these data clearly demonstrated a close relationship between OMV1c and other mitoviruses.

Alignment of the putative RdRp encoding region of the OMV1c nucleotide sequence with accordant regions of all other *Ophiostoma* mitoviruses (1a, 1b, 3a, 3b, 4, 5, and 6) revealed several high nucleotide identities intrinsic to mitoviruses in the region from ~1000 bp to ~1400 bp in the OMV1c genome (Figure 5a). There were no blocks with 100 % similarity. Meanwhile, when the same RdRp regions translated into amino acids were aligned, four well-conserved motifs with 100 % homology were found (labeled as Motifs 1 - 4 in Figure 5b). Motif 1 consisted of 3 and 4 identical amino acids separated by different amino acids. Motif 2 consisted of 6 and 2 amino acids, and each of motifs 3 and 4 had four identical amino acids in a row (Figure 5a). These four well conserved motifs in the RdRp region in mitoviruses were previously described by Park *et al.* (2006).

Table 4. List of mitoviruses with the highest nucleotide identity to mitovirus OMV1c.

Mitovirus	Query coverage	Max identity	Accession
Ophiostoma mitovirus 1b	66%	82%	AM087549.1
Tuber aestivum mitovirus	9%	67%	HQ992989.1
Ophiostoma mitovirus 1a	6%	80%	AM087550.1
Botrytis cinerea mitovirus 1	3%	75%	EF580100.3
Ophiostoma mitovirus 3a	3%	74%	AJ004930.1
Ophiostoma mitovirus 3b	3%	74%	AM087550.1
Sclerotinia homoeocarpa mitovirus	3%	74%	AY172454.1

Table 5. List of sequences having significant protein similarity to OMV1c (BLASTx).

Virus	Query coverage	Max identity	Accession
Ophiostoma mitovirus 1b	76%	68%	CAJ32467.1
Tuber aestivum mitovirus	50%	38%	AEG79311.1
Ophiostoma mitovirus 1a	50%	36%	CAJ32466.1
Ophiostoma mitovirus 3a	44%	35%	NP_660176.1
Thanatephorus cucumeris	38%	42%	AAD17381.1

OMV1b -----TAAA 4
 OMV1c TAACCATACCTGTTTAAATCAAACCTGCCTGCAGATTCCTCATTAGAGGGAAAACCTGAAGAA 360
 **

OMV1b GCCTTTAATACTAAAGAAAAGTCACATACAAT----AATTTGATAAAAATTACTGCAAGCA 60
 OMV1c GGAAATGGTATTAAATTAATAATACTTGCACCTAACCACTTTTACATAAAATTATGATCA 420
 *

OMV1b ACACTCTTTAGAATGAAGAATTTATATCTTGGCAAAGAGGTTGAAAGACCTCTTCCAT 120
 OMV1c TGTATCAACGTTGTTAGATGTACATTTCTTGGCACAAGG--TCTAAAATCCCTTGACTT 478
 ** *

OMV1b GTTAATA-AAGGACTATTTTTAGTTCTTAAATTAGTCTGGGGTGAACAA--CTATATCC 177
 OMV1c ATTAGTCTGACCACTTTTACAAGTATAAGACTAGTAAGGGGTTGTTTAAAGCCAGATTT 538
 *

OMV1b AAATTAAGTCGTTTTATGTGTACTAGTTCACCTTAAAACATTACAAGAAAATCAGGT 237
 OMV1c AA-TCAAAGCAGTATT-TGTTTTCGTGAAAAGATCAAGTTCTCTTCAGAAAACCTGGAGGT 596
 *

OMV1b CTGTCATATACTTGTCAGTATATGAAGCCTTGCCATCTATGTAATGAAATACGTGGCT 297
 OMV1c TTAAAGTTCGTGCTTTATATATATAAAGCTTGTCAATCTACACTATGCAATTTGTTGCT 656
 *

OMV1b A----AGGATCCCAATAAGTTAGCA----ATTAATAGTTTTTCTATTTTA-GTATCCTTA 348
 OMV1c AGTGGAGGTGTAGACAACTCCCTCATAACATCAACATGTTATGGAGTAAACGTATCTCTA 716
 *

OMV1b ACTGATTCGGGTATACCTAGAATTATTCCTAGTTATATCAGGTACCAATTAAGAAGAGGA 408
 OMV1c ACTTCCGGTGGTCTACCGAGAATTCCTCTATTTATTTAAGAAGATTGGTTACTTCCACAG 776
 *

OMV1b AATAAGAATCTATTAAGCTCTATTGACAGTCTTCAATCTGTATAGGGTCTGGAATAC 468
 OMV1c AACAAAGAAGGTATCAAATTTGTTTTAACTTTATTTAACCTATATAGGGTCTTACCTAT 836
 *

OMV1b CCGGGAGTATTAAACTCTCGACTATTACAGATTCTAGTAGTTTTACTC---TACCTTCG 525
 OMV1c CCAGGAAAAGTAAAATTATCAACCATAACTGATA--AATGATCCGGATCATACTCCCTTAG 894
 *

OMV1b GATTTTTCAGGTGATTGGCAGAAATTA-----TGAGATCACAGTCTATGACTGTAC 577
 OMV1c ATATGATCTCATTTCCTAAGTTTTGGTTGCTTCTAAGATCCCAAGGAAAGATAGCTC 954
 *

OMV1b CTTTTAAAATT-TCTGATCTTC-TTAATCCTTTCT-TGATTTATTCAAGTGGACCCGTGT 634
 OMV1c CTTTTACCTTTATTTCGATCACCATTCGCCATATCAGCGAGTGGCTCAATTACAG---GTT 1011
 *

OMV1b CACCCAAAGGTATGAATAACACTGCAGGTTGGATTCTAGCCTTTAGAAGATTGA-AAGCC 693
 OMV1c TTGGGAAACATCTTCTCCATGTCTGGTTTCTTTAAAGCTCTTCTATTCCTAAGAAGAG 1071
 *

OMV1b TTGTTTCT--TGACAAGGAATTTCTTCTTACTTTGCT-AGATCCCTATAAAGAGAGAAG 749
 OMV1c AGGATTCTCTGTGACAATCACCTCAATGATTTTTTACAGAGGCCCTTTAAGAAGGGGAT 1131
 *

OMV1b ACAATGGTGAGAATCTTCTCCTTTTAT----TAAGTACTTTAATGCGA---TCCTCCCTG 802
 OMV1c GAGCTTCTAGAATCTACGATGTGACAATCTATGGATTTTATTTCCAGATGTTTACTTG 1191
 *

OMV1b ACTTTGACGACAATGT-----TTCCTTAGGGAAACTAGCTTTTAAAGGAGGAACCGG 853
 OMV1c TTGCAGGTCAAAGAGTGTATCTTCTCCTTTAGGAAAGTTAGCCTTTAAGGAGGAACCGG 1251
 *

OMV1b GAAAGGTAAGAGTGTGCTATGGCAGACTGTATAACTCAATGAGTGTACATCCTCTTC 913
 OMV1c GAAAAGTTAGGTATTTCGCTATGGCGGACTGTATAACTCAATGAGTATTACATCCTCTTC 1311

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**** * * * * * *****
OMV1b ATCAGTACTTATTTCTATCTTGAACAAATTAGCATCGTTGATGCTACCTTGATCAAG 973
OMV1c ACCAGTATTTATTTCAATCTTGAACAAATAAGCATCGTTGATGCAACATTTGATCAAG 1371
* * * * * *****

OMV1b AAGAAGGGGTAAAACCTTTCTCGGAAAATAAAGCTGGAAAGAGAGTAGTATTCTCTC 1033
OMV1c AAGAAGGGGNAAGAACCCTTTCTCAGAAATAAAATCTGGTAAGAAGATTGTATTCTCCT 1431
***** * ***** ** ***** * * * * *

OMV1b TTGATTTATCAGCAGCCACAGACAGAYTACCTCTAATCAAGCTCAGATTCTGAACC 1093
OMV1c TGGATTTATCAGCCGCTACAGACAGATTACCACTAACAATCAAGCAGAGATCCTGAATC 1491
* * * * * ***** ** ***** * * * * *

OMV1b ATATAGTTCCTCAAATTAGGTGACCATTGAGCTAATCTTCTGGTGAACAGAGATTACTCAG 1153
OMV1c ATATAGTTCCTCAAAGTTAGGGGACCCTGGGCCAATCTTCTGGTGAACAGAGATTACTCAG 1551
***** * * * * * *****

OMV1b TGCCGAACCATACTACTCTACCAGTTAATCCTGGTACTGTTAGGTATGGAGCGGGCAAC 1213
OMV1c TGCCAAACCATATTACTCTACCAGTTAATCCTGGTACTGTTAGATATGGAGCGGGCAAC 1611
**** ***** *****

OMV1b CTATGGGGCTTATAGTTCATGAGCTATGCTTGCCCTAACACATCACTTCATTGTTGAGT 1273
OMV1c CTATGGGAGCTTATAGTTCATGAGCTATGCTTGCCCTAACACATCACTTCATAGTACAGT 1671
***** *****

OMV1b ATTGCGCTTCAAAGTCTACCACACTAATCTTCAAAGATTATTTAATCTTGGGAG 1333
OMV1c ATTGTGCTTCAAAGTCTATCATTCTAATCTTCAAAGATTATCTAATCTTAGGAG 1731
**** ***** ** *****

OMV1b ATGACTTATTGTTGTTAGATGCGAAAGTGCAGAAACAGTACTTGCAAGTGATGAACCAAC 1393
OMV1c ATGATTATTATTGTTAGACGCGAGAGTTGCAAAACAGTACTTAAAAGTCATGAGCCAGC 1791
**** ***** ***** ** *****

OMV1b TGGATGTCGGAGTTAATCTAGCAAAAATCCCTAATCTCAGTTAGAGGTTATGGTGAATTTG 1453
OMV1c TGGATGTAGGAGTGAACTAGCAAAAATCCCTAATCTCTGTTAGAGGTTATGAGAAATTTG 1851
***** *****

OMV1b CTAACAATTTCTATCTCCAGATGGTCTCTTCAAGGAGTTAGTTTAAAGAGAATTTCTCGT 1513
OMV1c CTAACAATTTCTATCTCCAGAGGACCTTTACAAGGGTCAAGTTTAAAAGAAATTTCTTT 1911
***** ***** ** * * *****

OMV1b CGTTAAAAGATGGAATGTCTAACATTCTATCCTTATCGACAAAAGATGAATCTTAAACCCG 1573
OMV1c CGTTAAAAGATGGAATGTCTAACATTCTATCCTTATCGGTTAAAATGTCTCTTAAACCTT 1971
***** ***** ** * * *****

OMV1b CTCTCTGTTGAGATTGTTGGGTTGCGTTCTAAATCAATTGGTCATTCTACTTTACCTT 1633
OMV1c CCTTATGTTAAGGCTGTTGGGTTGCGTTCTAAATCAAGTTGGTCATTCTCACTTTACCTT 2031
* * * * * ***** *****

OMV1b TCACAAGGTATTCAGTACTGAGATCTTCTCTGATCACTGTTTCATCAGTCCGCTTGCGAAT 1693
OMV1c TTTCTAGATACTCATTGAGATCCAGTCTTGATCATTCTTCTGAGTCCGCTTGCGAAT 2091
* * * * * ***** * * * * *

OMV1b CTTCGAATAAGTGATTAGATTATTTCTCATGTATAAGACATTTAAAATCTCTCACTCTAA 1753
OMV1c CTCTGAATAGTTGACTAGATTATTTCTCGTGATTAGACATTCAAAATCTTATTGATTA 2151
** ***** ** ***** * * * * *

OMV1b ATAACTCATTATTGAGATATTTCTTGTGGGGATTTGAATTTCTAAAATACAAGTGAGTCT 1813
OMV1c TTAAGTCTTTATTGGCTTACTTTCTATGAGGTTTGAATTTATCAAATACAATGAGTTT 2211
*** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

OMV1b ATATGAAAGTGAGGGAGAATGAACAACCTTATGTTGAAACATGGAACGCATCACTACCATC 1873
OMV1c TCAAGAAGGTTAGGGAGAATGAGCAACTGATGTTGCGACATGGAACACATCGCTACCATC 2271
* * * * * ***** ***** ***** ***** ***** *****

OMV1b AGATTGTAAGTGAAACCTTACTGAATTACTGATGTCCTTCTCCTACGGATGAGGTGTTGA 1933
OMV1c AATTGGTAAGTGAGACTTTACTGAGTTACTGATGTCCTTCTCCATCAGAAGGATTGTA 2331
* * * * * ***** ***** ***** ***** * * * * *

OMV1b AATCACCGAATATTGCTGATTGTGTCAGCAGGATTCTTCTTTCAACTCCTCCC--ATAG 1990

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OMV1c      AATTACCAGAAGTTGCTGATTATCAGCTAACTTCTTCTTTTCAACTCCTCCTCCGGTGA 2391
          *** ** * ***** ***** *****
OMV1b      ATAAGGCATCTTTTAAGGTGTTGATGATTAATCACATCAACATCTTGTTTTTCATGTGGGT 2050
OMV1c      ATAAGGCATCATCAAGTTATTGATGATTAATCATATCAATATCTTGTTCTCATGTGGGT 2451
          ***** ** ** * ***** ***** *****
OMV1b      CTCTCTTTACAGAGCTGATGAATGGTGGTGAATGGATCTAGTCCCTAAATTCCTAACAT 2110
OMV1c      CTCTCTTTTCAGAATTGATGAATGGAGGTGAATGAATCTAGTCCCTAAATTCCTAACAT 2511
          ***** ***** ***** ***** *****
OMV1b      TTGAGTTGTCCTTTGAAGAGGGCTACTCAGATTTTGAGAATGTTGATAACGATCATCTAT 2170
OMV1c      TTGAGTTGTCCTTTGAAGAGGGTTACTCAGATTTTGAGAAAGTGGAGAATGACTTAT 2571
          ***** ***** ***** ** * ** ***** **
OMV1b      GATCCGTTATCACAAGGGATCCGAAATCTTGGGTTAAACCCAATAATTTGGAGGAAACA 2230
OMV1c      GATCAGTCATCACAAGGGATCCAAAAGTTGGGTTAAACCCAAT-----A 2616
          **** * ***** ** *****
OMV1b      ATTTTGATATTGTTGAATTTATGAGCCAGCAACCATCGGTAGAGTACCACCAACCTAGAA 2290
OMV1c      ATTTTGATATTGTTAAATTTATGAGCCAGCAACATCTGTAGAGTTTCACAAACCAAGGA 2676
          ***** ***** ***** ***** ** *
OMV1b      CTCTTAAAGAGATTTCTTTAGCTTTACAACCTGAAGAAAGATCTCTTTGATAGTGGTTGG 2350
OMV1c      CTCTTAAAGAAATTTCTTTAGCTTTACAACCTAAAGAAGGATTTCTTTAATAGTAGTTGG 2736
          ***** ** ***** ***** ***** *****
OMV1b      TAGTGGATCTCAAACATACTCGATGTACCTCTCTCAATAAGAAAGGGTAGACTGGAC 2410
OMV1c      TAAATGGATCTCAAACCATACTAGATGTGCCCTCTGGTAAACCAAAGGCTAGGTGACTC 2796
          * * ***** ***** ***** ** ***** **
OMV1b      TCTGACAAGAGGTCTTGCTCTGGTTGAGAATATACAGTTGGAGGTGACC---ATTATA 2466
OMV1c      ATTAGCAATCATAGTGATCTCTGGTTGAGAATACTCAGTTAAAGGTGATCGAGAATTATG 2856
          * ** * ***** ***** * *****
OMV1b      ATTACTTCGTAATTATAATAGGAAGACACTCCCGAGTTATATTTATAACAGTAGACTAG 2526
OMV1c      AA-ATTATTCATTTCTTTTCGAAAGACCCTTTCGTGTAGTATTTATAACAGTAGATCAG 2915
          * * * * ** * * ***** ** * ***** **
OMV1b      AGATGAGACACCTTCGTCATTGAACATTTTCAGTCCCTATAAAGGA----- 2572
OMV1c      AGATC---TATTATTGTTGCTAAATGTTT-CATTCCTCATAAAGGAGATGTTCCAAAATA 2971
          **** * ** * ** * *****

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Figure 3. Nucleotide similarity between RdRp regions of OMV1c and of OMV1b (ClustalW2).

```

OMV1b      MKNLYSWQRGSKDLFHVNKGLFLVLKLVWGGTTISKLKSFYVLLVHLKTLQEKSGLAYTC 60
OMV1c      MYIFLAQGTKSTDLLVWPT--LQVMRTVVRGCLSPDLIKAVFVFKRSSSTQKTGGLKQFVA 58
*   :   :   *.*.*   .   :   *:: * *   :   .:*::*::*::*   :   .:   *:.**   :..

OMV1b      QYMKACAIYVMKYVAKD---PNKLAINSFSLVSLTDSGIPRIIPSYIRYQLRRGNKNSI 117
OMV1c      LYKACAIYTMQFVASGGVQRQSFMTSTCYGVNVSTTSGGTPRITPIYLRLVTSQNKEGI 118
*   ***   **.*::**..   .   ::   ..:::   **   *..*   ***   *   *.*   :   **:.**

OMV1b      KALLTVFNLYRVLEYPGVKLKSLTITDSSSFTLPSDFSGWLAEF--MRSQSMVTPFKISD 174
OMV1c      KIVLTLFNTYRVLPYPGVKVLSTMTDKWGSYPLDMISFIPKFWLTRSQGMAPPFTF-- 176
*   :.*.*   **   ***   :****:*.*   *   :   *   *   :   :..:*   *   **..   .**..

OMV1b      LLNPFLIYSSGPCS--PKGMNNTAGWILA---FRRLKALFLDKEFFLTLDDPYK----ER 225
OMV1c      IRSPFAMSASGSITGFGKHTSMSGGFKATFTTREDSTWQSTQWFFTEAPLRRGWASRT 236
:   .*.*   :   :.*.   :   *   ..:*:: *   **   ..   :   :..:*.*   :   .

OMV1b      RQWWESEFFIKYFNAILPDFDDNVSLGKLAFFKEEPGKVRVFAMADCITQWVLHPLHQYLF 285
OMV1c      TRCWQSMDFISRLTLVAGQKVVSPLGKLAFFKEEPGKVRVFAMADCMTQWVLHPTHQYLF 296
:   :.*   **..   :   ::   :   .   *****:*****   *****   *****

OMV1b      SILKQISIVDATFDQEEGVKTLSAKIKAGKRVVFSLDLSAATDRLPLTIQAQILNHIVPK 345
OMV1c      SILKQMSIVDATFDQEEGXRSTSQKMKSGKKIVFSLDLSAATDRLPLTIQAQITNHMVPK 356
*****:*****   :.*   *   *.*:*.*:*****   *****   **:*.*

OMV1b      LGDHWANLLVNRDYSVFNHTTLPVNPVTVRYGAGQPMGAYSSWAMLALTHHFIVQYCAFK 405
OMV1c      LGDHWANTTVNRDYSVFNHITTPVNPVTVRYGAGQPMGAYSSWAMTATTHHFVQYCAFK 416
*****   *****   *   *****   *****   *   *****:*****

OMV1b      VYHTNSFFKDYILGDDLLLLDAKVAQYLVQVMQLDVGVNLAKSLSVIRGYGEFAKQFL 465
OMV1c      VYHSNSFFKDYIILGDDLLLLDARVAQYLVQVMQSDVGVNTAKSLSVIRGYGEFAKQFT 476
**:*.*   *****   *****:*****:*. *   *****   *****

OMV1b      SPDGPLQGVSLREFSSLKDGMSNILSLSTKMNLPALLLRLFGFGSKSIGHSTLPFTRYS 525
OMV1c      SPEGPLQGVSLKEFSSLKDGMSNITLSVKMSTKPSLLLRTFGFGSKSVGHSTLPFSRYS 536
**:*.*   *****:*****   ***.*.   **:*.*   *****:*****:***

OMV1b      LRSSLDHLFISPLANSSNKWLDYFSCIRHLKSLTLNNSLLRYFLWGFELFKYKWVYMKVR 585
OMV1c      LRSSTDHTFLSPTANSTNSWTDYFSCIRHSLKSLLSLLAYFTWGFELFKYKWVFKVR 596
****   **   *:*.*   ***:*. *   *****   *   *   :.*.*   **   *****:*****:   **

OMV1b      ENEQMLKHGTHHYHQIVSETLLNYWCPSPTDEVLSKSPNIADLSAGFFFSTPP-IDKASF 644
OMV1c      ENEQTMLRHGTHRYHQLVSETLTSYWCPSPEEVLKLEPADSSANFFFSTPPPVKASF 656
****   **:*.*:***:***:*****   .*****:*****   *:*.*   **   *****   ::*.*

OMV1b      KVLMINHINILFSCGSLFTELMNGGAMDLVPKFLTFELSFEEGYSDFENVNDHLWSVIT 704
OMV1c      KLLMINHINILFSCGSTFSELMNGGAMNTVPKFLTFELSFEEGYSDFEKVENDHLWSVIT 716
*.*.*   *****   *:*.*   *****:*****:*****:*****:*****

OMV1b      RDPKSWKPNNFGGNNFDIVEFMSQQPSVEYHQPRTLKEISLALQLKDLFDSGLVSGSQ 764
OMV1c      RDPKSWKPN----NFDIVKMSQQTSVEFHKPRTLKEISLALQTKKDFNSSLVNGSQ 771
*****   *****:*****   *.*.*   *:*.*   *****   *****:*. *.*   **

OMV1b      TILDVPLFNKKG 776
OMV1c      TMTDVPTGKPKG 783
*   :   ***   :   **

```

Figure 4. Amino acid alignment between the OnuMV1c and OMV 1b (ClustalW2): *- three nucleotides are the same, :-two nucleotides are the same, .- one nucleotide is the same in a codon

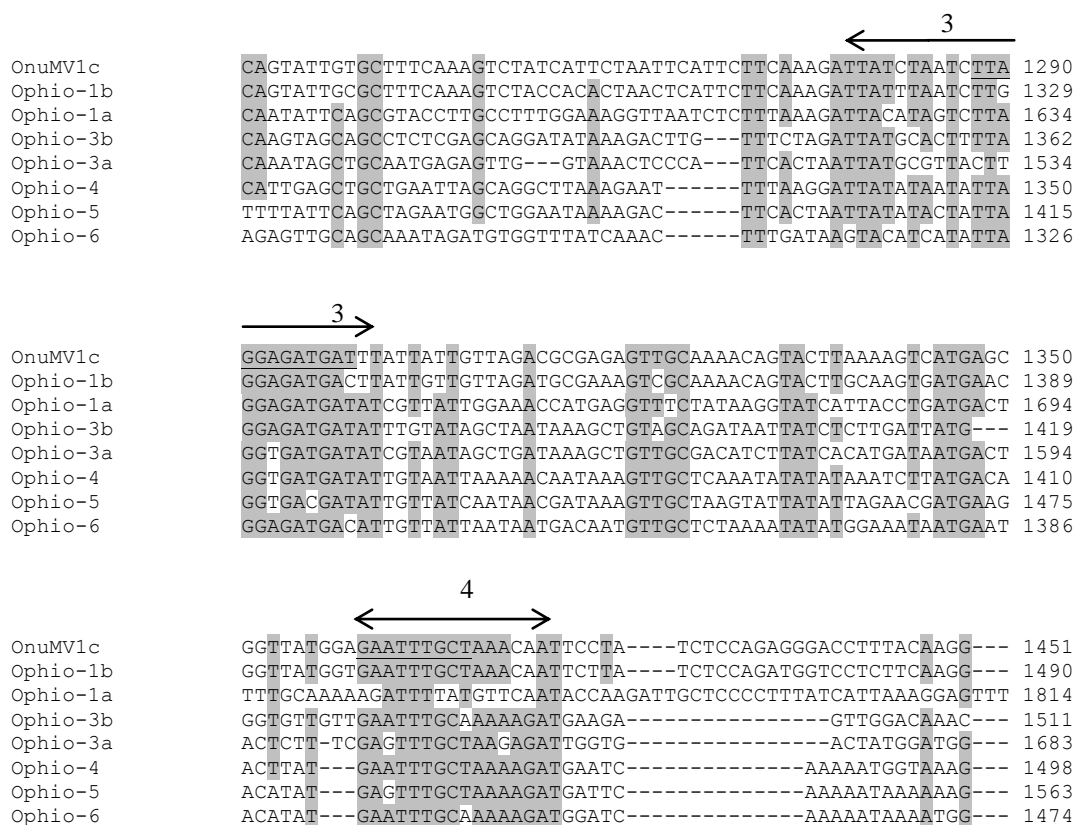


Figure 5. Alignment of the amino acid (a) and corresponding nucleotide (b) sequences of the putative RNA-dependant RNA polymerases of OMV1c and *Ophiostoma novo-ulmi* mitoviruses 1a, 1b, 4, 5, 3a, 3b, 4, 5, and 6. In amino acid alignment (a) well conserved regions are highlighted grey. Four conserved motifs were found (marked with arrows and numbers from 1 to 4); in nucleotide alignment (b) well conserved regions are highlighted grey, arrows and numbers above show the parts of conserved motifs that correspond to appropriate conserved regions in the protein sequence.

Part of conserved motif 3 (GDD) also corresponded to the conserved region VI in an alignment of viruses of the family Partitividae (Shamoun *et al.*, 2008). The disagreement in similarity level between nucleotides, which showed lower similarity, and amino acids, with higher similarity, alignments is explained by the fact that the same amino acid can be encoded by several different nucleotide codons (Figure 6). We suggest that this new dsRNA virus is a member of the Genus *Mitovirus*, Family Narnaviridae. This is supported by its utilization of the mitochondrial codon pattern, the presence of an ORF with a potential to encode RNA-dependent RNA polymerase on the positive strand, the sequence of a double stranded stage in its replicative cycle. This new dsRNA had high similarity to OMV1b with less, but still obvious, homology to other mitoviruses. Hence we named this new mitovirus OMV1c.

2.3.5 Potential Secondary Structures of OMV1c from *O.novo-ulmi* Isolate 93-1224

The 5'- and 3'- untranslated regions (UTR) were examined for potential secondary structures using the RNAfold software which predicts the structure summarizing free positive or negative energy change associated with all possible pairing. An examination of the positive strand of RNA sequence showed that the 44 bp of 5'- terminal sequence of the positive strand

(CCGTATGGGGTCGCTGACTTTCGCGAGTCAGAAACCTCCGTACG) could

potentially be folded into a double-stranded structure (free energy -24.80 kcal/mol) with some mismatches and 1 unpaired nucleotide on the very end (Figure 7-a). The 18 bp of 3'- terminal sequence (TTTCCCCGTTAAGGGAAA) also had a potential to be folded into a perfect double-stranded stem-loop structure with free energy -6.70 kcal/mol

New virus	GAT	TTA	TCA	GCC	GCT	ACA	GAC	AGA	1020
OnuMV1b	GAT	TTA	TCA	GCA	GCC	ACA	GAC	AGA	1059
OnuMV1a	GAC	CTA	TCG	GCA	GCC	ACA	GAC	AGA	1373
OnuMV3b	GAT	TTA	TCT	GCT	GCA	ACT	GAT	CGA	1103
OnuMV3a	GAT	CTA	TCT	GCT	GCT	ACC	GAT	AGA	1290
OnuMV4	GAC	TTA	TCA	GCT	GCA	ACA	GAT	AGA	1104
OnuMV5	GAC	CTA	TCT	GCT	GCA	ACT	GAT	CGT	1169
OnuMV6	GAT	CTG	TCT	TCT	GCA	ACT	GAT	AGA	1080
Base number	123	123	123	123	123	123	123	123	
Amino acid	D	L	S	A	A	T	D	R	

Figure 6. Variety of bases in the third and the first codon positions of amino acids in conserved motif 1 (highlighted grey).

(Figure 7-b). The whole RNA could potentially be folded into a complex secondary structure with a thermodynamic free energy -1088.68 kcal/mol (Figure 8). Other fully sequenced *Ophiostoma* mitoviruses (OMV3a, OMV4, OMV5, and OMV6) as well as the *Tuber aestivum* mitovirus that had relatively high similarity with OMV1c, were assessed for potential secondary structure. All of them could potentially be folded into complex “snowflake”- like shapes similar to OMV1c (Figure 9), but there were no obvious similarities or conserved motifs in the secondary structures.

2.3.6 Phylogenetic Analysis of OMV1c from *O. novo-ulmi* Isolate 93-1224

Sequence comparisons of the OMV1c RdRp gene indicated that this virus grouped most closely with a mitovirus from the truffle *Tuber aestivum*, as well as *Ophiostoma* mitovirus1b and *Ophiostoma novo-ulmi* mitovirus 4 Ld segment 10. Also, it grouped within a larger clade with *Cryphonectria cubensis* mitoviruses 2a, 2b, and 2c (Figure 10). Phylogenetic analysis of the whole viral genomes (OMV1c, other eight mitoviruses, and narnavirus *Saccharomyces* 20S as an out-group) was conducted. The OMV1c was seen to group more closely with the *Tuber aestivum* virus than with any mitoviruses described in *Ophiostoma*. *O. novo-ulmi* mitovirus 4 Ld segment 10 again comes together within the larger clade (Figure 11).

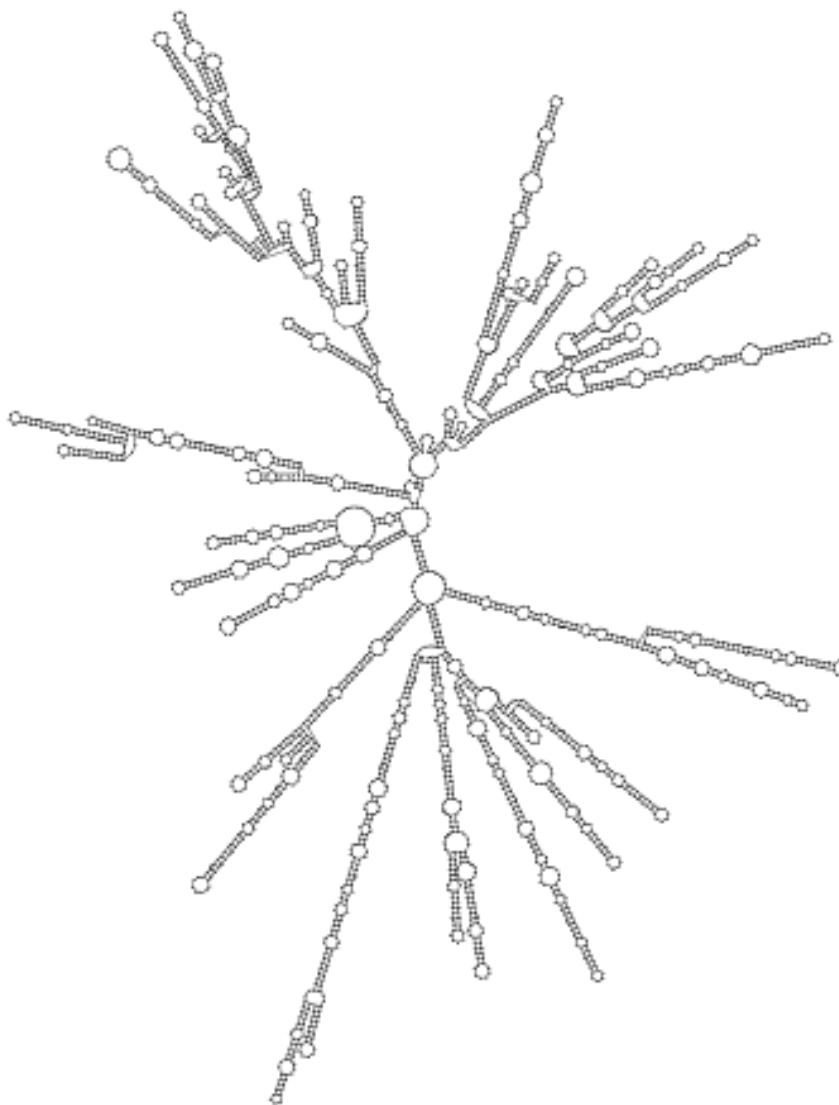
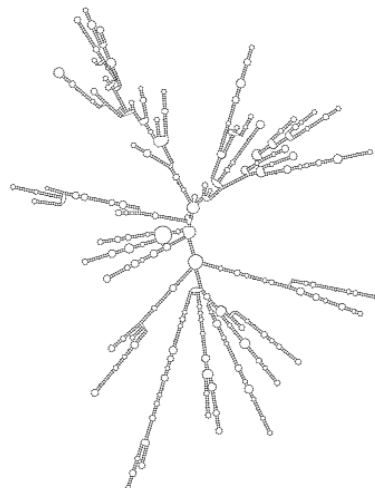
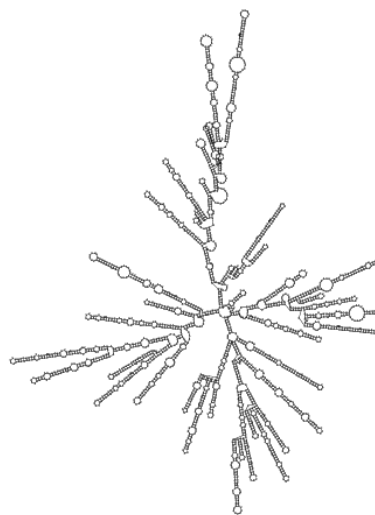


Figure 8. RNA secondary structure prediction of OMV1c.

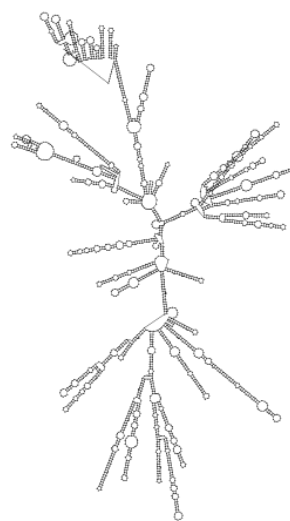
a) OMV1c



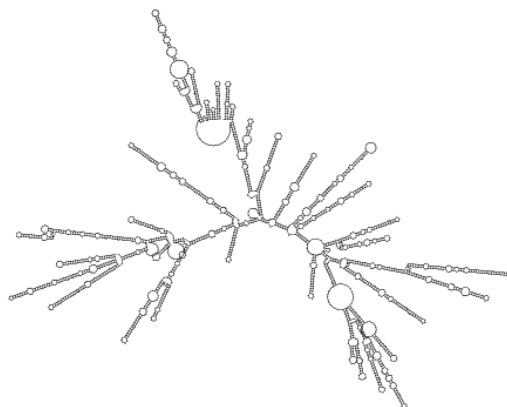
b) OMV3a



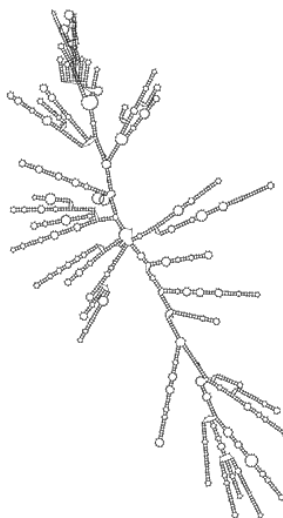
c) OMV4



d) OMV5



e) OMV6



f) *Tuber aestivum* mitovirus

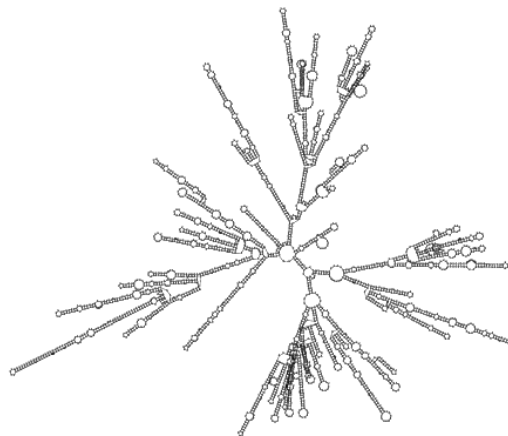


Figure 9. Potential secondary structures of *Ophiostoma* mitovirus RNAs.

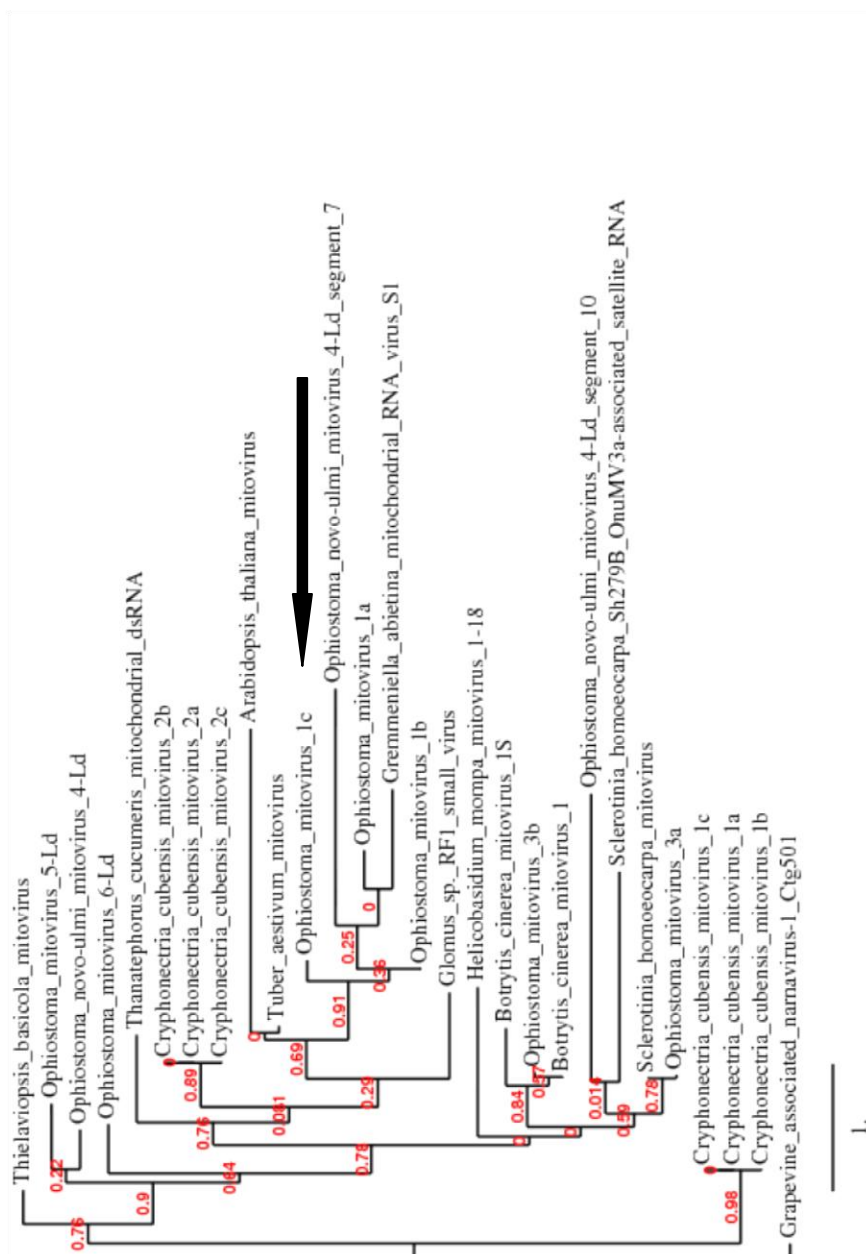


Figure 10. Phylogenetic analysis based on Maximum likelihood of the putative RdRp regions of a new virus OMV1c (indicated by an arrow) and 23 selected fungal viruses from genus *Mitovirus*, with branch lengths measured in expected substitutions per site calculated using PhyML. Out-grope sequence – Grapevine associated narnavirus-1 Ctg 501. Scale bar indicates substitutions per site.

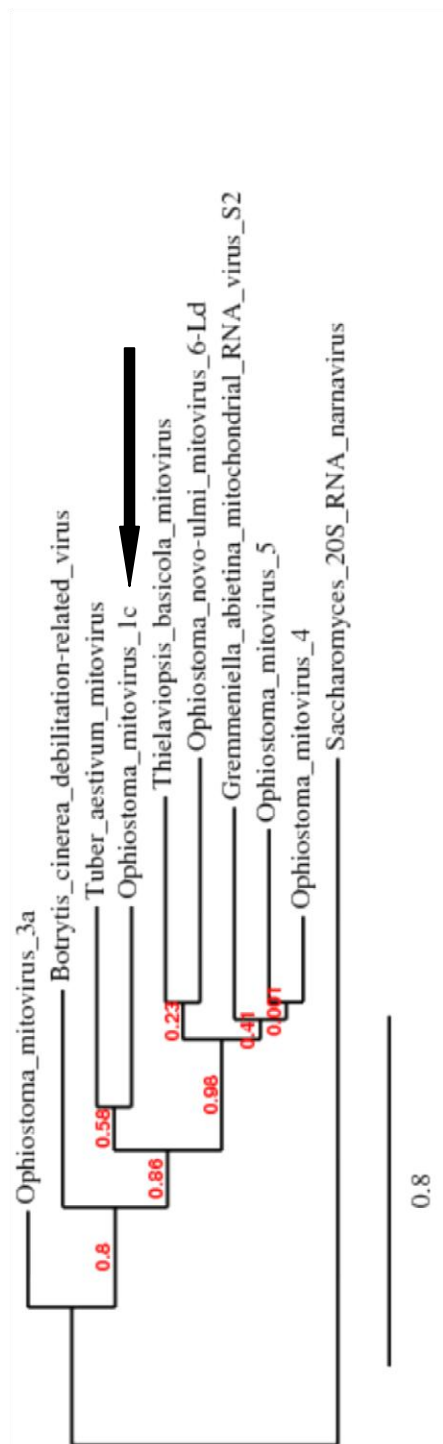


Figure 11. Phylogenetic analysis based on Maximum likelihood of a new - OMV1 (indicated by an arrow) and eight previously published mitovirus complete genomes with branch lengths measured in expected substitutions per site calculated using PhyML. Out-group sequence – *Saccharomyces 20S RNA narnavirus*. Scale bar indicates substitutions per site.

2.3.7 Screening *O. novo-ulmi* 93-1224 for the Presence of *Ophiostoma* Mitoviruses: 1a, 1b, 3a, 3v, 4LD, 5LD, and 6LD

As there was a possibility of there being multiple mitoviruses in *O. novo-ulmi* 93-1224 beyond the virus OMV1c, we assessed whether isolate *O. novo-ulmi* 93-1224 harbored any of the viruses found in European isolates of *O. novo-ulmi*. Mitovirus specific primers were designed according to the genome sequences of *Ophiostoma* mitoviruses: 1a, 1b, 3a, 3v, 4LD, 5LD, and 6LD. These were used to screen for the presence of similar elements from cDNA constructed from total RNA from 93-1224. For each screen, a separate target cDNA was made using a specific primer designed according to the genome sequences of *Ophiostoma* mitoviruses: 1a, 1b, 3a, 3v, 4LD, 5LD, and 6LD. The only combination which resulted in a visible amplification product was for primers designed according to mitovirus OMV1b (Figure 12). All other combinations yielded no amplification products. The amplification product using the mitovirus 1b-specific primers was purified and its sequence determined. The amplicon corresponded to OMV1c, not mitovirus 1b, hence it was concluded that there was no mitovirus OMV1b present in *O. novo-ulmi* 93-1224. It appeared that *O. novo-ulmi* isolate 93-1224 did not harbor any previously characterized mitoviruses: 1a, 1b, 3a, 3v, 4LD, 5LD, or 6LD.

2.3.8 Attempts to Cure *O. novo-ulmi* Isolate 93-1224 from Viruses

A putatively virus-free strain of *O. novo-ulmi* 93-1224-PVC was developed by serially re-culturing the hyphal tips onto new medium. Control RNA extraction was performed for both 93-1224 and 93-1224-PVC. The double band (~2,100 bp and ~2,300 bp) was

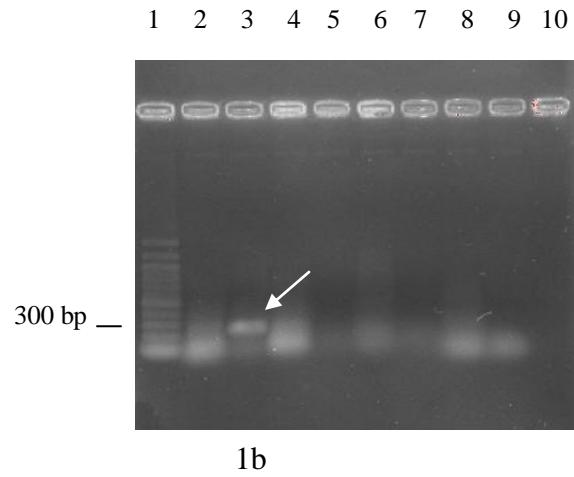


Figure 12. Agarose gel of PCR amplification for screening *O. novo-ulmi* 93-1224 for the presence of *Ophiostoma* mitoviruses: 1a (lane 2), 1b (lane 2), 3a (lane 3), 3b (lane 4), 4LD (lane 6), 5LD (lane 7), and 6LD (lane 8). An arrow indicates PCR amplification was obtained in isolate *Ophiostoma* mitovirus 1b.

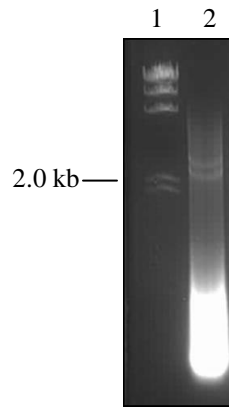
seen on gel electrophoresis typical for a dsRNA infected isolate 93-1224 (Figure 13-a), while gel electrophoresis for 93-1224-PVC apparently showed the absence of the dsRNA double band originally observed by Temple *et al.* (2006) in isolate 93-1224 (Figure 13-b).

As it was not known whether the hyphae had indeed been cured of virus or whether the titre had simply been reduced to a level that could not be visualized by gel electrophoresis, a set of primers with an expected amplification product size of 826 bp was designed according to the OMV1c genome sequence and used to test *O. novo-ulmi* 93-1224-PVC for the presence of OMV1c. Amplification products for the OMV1c were observed in amplifications of cDNA from both *O. novo-ulmi* 93-1224 (positive control) and *O. novo-ulmi* 93-1224- PVC (Figure 14). VA30 and H327 did not show amplification products, indicating the lack of this mitovirus. Isolate *O. novo-ulmi* 93-1224-PVC retained the mitovirus even after repeated hyphal transfer.

2.3.9 Screening of 12 New *O. novo-ulmi* Isolates from Winnipeg for the Presence of OMV1c

Having determined the nucleotide sequence of OMV1c permitted us to develop a more rigorous screen for the presence of this virus in other *Ophiostoma* isolates in our culture collection including strains collected at the Western Canada disease front. Because the virus could be present despite not being visible on agarose gels it was of interest to see how prevalent OMV1c might be amongst *O. novo-ulmi* isolates from the Winnipeg front disease. To determine the prevalence of OMV1c at the disease front we screened for the presence of this mitovirus using a set of primers designed according to OMV1c (Fwd: CCTCCGTACGATGAGGAAGA; Rev:

a)



b)

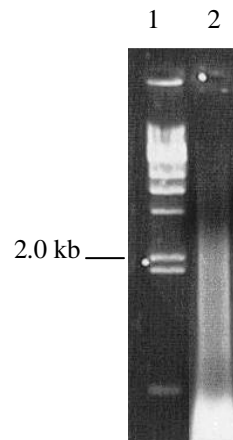


Figure 13. dsRNA extraction from *O. novo-ulmi* 93-1224-PVC:
a) Lane 1 - DNA Lambda ladder Hind III. Lane 2 - dsRNA extraction from *O. novo-ulmi* 93-1224; b) Lane 1- DNA Lambda ladder Hind III. Lane 2 – dsRNA extraction from *O. novo-ulmi* 93-1224-PVC. The lack of a band in b) lane 2 potentially indicates the absence of a mitovirus.



Figure 14. Screening *O. novo-ulmi* isolates: 93-1224, 93-1224-PVC, H327, and VA30 for the presence of OMV1c. Agarose gel: lane 1 - 100-bp DNA ladder; lane 2 - *O. novo-ulmi* 93-1224 (positive control); lane 3 - *O. novo-ulmi* VA30 (negative control); lane 4 - *O. novo-ulmi* H327; lane 5 - *O. novo-ulmi* 93-1224-PVC. An arrow shows an amplification product of size 826 bp in lanes 2 and 5.

TTGAGCCACTCGCTGATATG) (Carneiro, unpublished) and used the Rev primer for first-strand cDNA synthesis followed by PCR screening using both primers to survey for the presence of OMV1c. All 12 samples of *O. novo-ulmi* isolates from Winnipeg screened gave negative results (Figure 15, Carneiro, J – unpublished) indicating the absence of OMV1c in this portion of the population. Also, OMV1c also was not found in isolate H327 (from Bratislava, Slovakia) nor in VA30 (from Virginia, USA).

2.4 Discussion

The study of the Western Canada *O. novo-ulmi* disease front done by Temple *et al.* in 2006 in Winnipeg revealed that a single *Ophiostoma* isolate (93-1224) was infected by dsRNA virus. Reexamining isolate 93-1224 we found and characterized a novel mitovirus OMV1c which we propose belongs to the genus *Mitovirus* in the family *Narnaviridae*. The most compelling evidence for this is the very high nucleotide similarity of OMV1c to that of the previously described RdRp regions of mitovirus OMV1b (85% identity) and less, but still significant similarities to several other complete genomes of mitoviruses, OMV1c had a genome size of 3003 bp which is in the range of other sequenced mitoviruses including *Ophiostoma novo-ulmi* mitovirus 6-Ld (2,343) bp and *Tuber aestivum* mitovirus (3,480 bp). The genome had a low GC content (37%), consistent with the low GC of other mitoviruses. The GC content of *Gremmeniella abietina* mitovirus is 30.6 % (Tuomivirta and Hantula, 2003) while *Botrytis cinerea* mitovirus-1 has a GC content 33.2 % (Wu *et al.*, 2010). The predicted molecular mass

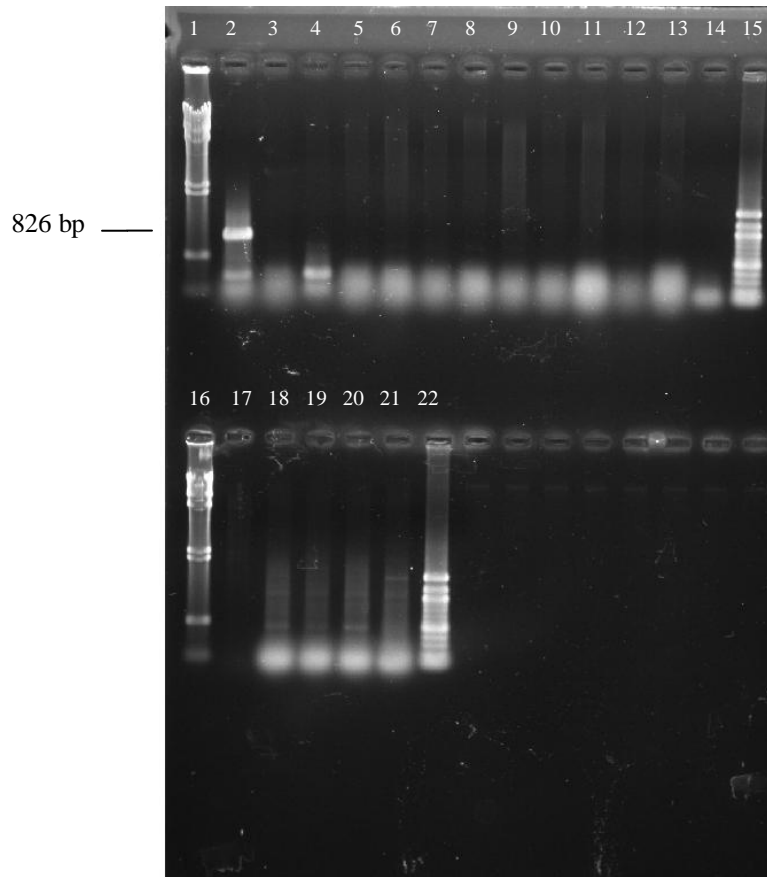


Figure 15. Screening *O. novo-ulmi* populations from Winnipeg for the presence of OMV1c. Agarose gel electrophoresis showing the presence of the OMV1c in lane 2 (isolate 93-1224, positive control) with amplification product size 826 bp. Total RNA was reversed transcribed with specific primers from the OMV1c sequence. Two negative controls – isolates VA30 (lane 3) and H327 (lane 4) were used and no OMV1c were found in either one. All 14 new isolates sampled in Winnipeg in 2010 (lanes 5-14, 18-21) show no amplification product of the expected size.

(77.23 kDa) of the putative RdRp is also within the range of RdRp proteins of other mitoviruses: 86.1 kDa in OnuMV5-Ld, and 80.0 kDa in OnuMV6-Ld of *O. novo-ulmi* (Hong *et al.*, 1999); and 60.29 kDa in *Botrytis cinerea* mitovirus 1 S (Wu *et al.*, 2010). Like other mitoviruses, such as *Chalara elegans* mitovirus (CeMV), the OMV1c genome has a single ORF with the potential to encode an RdRp protein and utilizes the mitochondrial genetic code (Park *et al.*, 2006). There are four significant well conserved motifs in RdRp gene of OMV1c that are common between *Ophiostoma novo-ulmi* mitoviruses 1a, 1b, 4, 5, 3a, 3b, 4, 5, and 6, that were earlier found by Park *et al.* (2006) between *Chalara elegans* mitovirus, *Cryphonectria parasitica* mitovirus, *Gremmeniella abietina* mitochondria RNA virus, *Sclerotinia homoeocarpa* mitovirus; *Ophiostoma novo-ulmi* mitovirus 3a-Ld; *Ophiostoma novo-ulmi* mitovirus 4; *Ophiostoma novo-ulmi* mitovirus 5; *Ophiostoma novo-ulmi* mitovirus 6; and *Rhizoctonia solani* M2-1A1 dsRNA (2006). These well-conserved regions also partially correspond to well conserved regions of mitoviruses as determined by Bruenn (1993) and Poch *et al.* (1989).

A common feature characteristic of all mitoviruses is the presence of 5' and 3'-terminal untranslated regions of variable length. It has been suggested that these terminal residues act as stem-loop structures for RdRp recognition and initiation of replication (Deng and Boland, 2004). It also was suggested that the potential of RNA to be folded into a secondary stem-loop structures at the ends may be a characteristic of mitoviruses (Hong *et al.*, 1998) and that these structures may act as promoters for RNA replication (Buck, 1996). Such structures were predicted in *Chalara elegans* mitovirus (CeMV) (Park *et al.*, 2006), OMV3a (Hong *et al.*, 1998), and *Sclerotinia homoeocarpa* mitovirus (Deng *et al.*, 2003). Similarly a potential secondary structure was also predicted for both

the 5' and 3'- ends of OMV1c. Predicted secondary structures at the ends in all these mitoviruses share many similarities. They all are “stem-loop” structures of 20 - 47 residues with four to six residues making an internal loop. OMV1c and *C. elegans* both have (one and two, respectively) mismatches in the “stem” while OMV1c and the *S. homoeocarpa* mitovirus has unpaired residues on the 3' and 5' ends (4 and 1, respectively).

It has been reported earlier that mitoviruses can be co-purified with mitochondria, suggesting a mitochondrial location in at least some stage of their life cycle (Deng *et al.*, 2003; Polashock and Hillman, 1994). It has been suggested that mitovirus RNA is translated in the mitochondria (Cole *et al.*, 2000) due to their mitochondrial codon usage. OMV1c's low GC content (37%) is not only consistent with other mitoviruses, but also with the low GC contents of fungal mitochondrial genomes (Cummings *et al.*, 1990; Paquin *et al.*, 1997; Unseld *et al.*, 1997). Our results showed that the ORF of OMV1c has fifteen AUA codons which in mitochondrial codon usage encode methionine. The RNA genome of OMV1c has a high (~ 78%) preference of either adenine or uracil in the third, or wobble, position of codons, which is a characteristic of mitochondrial codons (Deng *et al.*, 2003). The codon usage of ORF in *Sclerotinia homoeocarpa*, as shown by Deng *et al.*, (2003) has a preference of A or U over G or C at the third or wobble position, and 33.3, 33.6, 17.2, and 15.8% of the total codons had A, U, G, and C at this position, respectively.

Following the sequencing of the OMV1c virus, we hypothesized that the newly characterized virus might correspond to the two dsRNA bands observed by Temple *et al.* (2006). Because single-stranded mitoviruses have a dsRNA stage during their replicative

cycle it was possible that the two bands observed by electrophoresis corresponded to the ssRNA and dsRNA versions of OMV1c. We attempted to cure isolate 93-1224 of the dsRNA viruses and examine whether the disappearance of the dsRNA band would correspond to a disappearance of the OMV1c. One method for potentially curing a fungal isolate of a cytoplasmically carried dsRNA is repeated hyphal tip culture (Rivera-Varas *et al.*, 2007). A putatively virus-free strain of *O. novo-ulmi* 93-1224 was developed by multiple re-culturing of hyphal tips followed by screening for the presence of the dsRNA by gel electrophoresis. In this way a potentially cured strain, designated 93-1224-PVC, was developed which no longer showed the presence of the dsRNA doublet. However, screening this cured isolate for the presence of OMV1c by making cDNA and performing PCR using genome specific primers showed the retention of OMV1c. If the virus was harboured in the mitochondria it would be expected to persist despite repeated hyphal tip culturing as those tips lacking mitochondria would not be able to survive. The observation that OMV1c was not curable confirmed the mitochondrial nature of OMV1c and is consistent with results obtained by Park *et al.* in 2006. So far there has been no evidence of curing mitoviruses by the hyphal tip reculturing since mitochondria, which have their own genomes, propagate by division and are distributed into daughter cells of growing hyphae through pores and cannot appear *de-novo*. It is not without precedent that dsRNA viruses are not visible by gel electrophoresis but can be detected by more sensitive methods such as RT-PCR. For example, mitovirus OnuMV3a dsRNA in *S. homoeocarpa* was detected by RT-PCR but not by agarose gel electrophoresis or northern blot analysis (Deng *et al.*, 2003). A similar observation was made for one of the dsRNAs present in *R. solani* (Lakshman and Tavantzis, 1994).

Screening of 12 *O. novo-ulmi* isolates from the Winnipeg disease front showed that neither a double band of the specific sizes on a gel, nor RT-PCR amplification product of the specific size in any of 12 samples. Therefore, only one isolate, 93-1224, showed this specific dsRNA.

The observation that the band consists of two closely located bands might be explained by the fact that single stranded RNA viruses have a double stranded stage in their replication and that since positive and negative strands have different (complementary) sequence of nucleotides, they may have different secondary structures leading to differential migration in the agarose gel.

An alternate explanation for the presence of double band is that *O. novo-ulmi* 93-1224 might carry more than one type of virus. It is not uncommon that a fungus may harbor more than one virus. Two genetically distinct types of dsRNA were detected in *S. homoeocarpa* (Deng and Boland, 2004), two dsRNA elements were reported to co infect *C. elegans* (Park *et al.*, 2005), and a diseased isolate Log1/3-8d2 of *O. novo-ulmi* was reported to contain 12 mitoviruses (Hong *et al.*, 1999).

The greatest interest in studying mitoviruses lies in the potential to use them as a biological control of pathogenic fungi. The presence of dsRNA in fungi can effect physiological and biochemical processes and even change the morphology of fungi (Brasier, 1983; Buck, 1986; Nuss and Banerjee, 2005; Dave and Nuss, 2001; Jian *et al.*, 1997). Some mitovirus infections can be latent as is the case for OnuMV3a in *Sclerotinia homoeocarpa* (Deng *et al.*, 2003). Other examples of dsRNA latency have been observed in *Rhizoctonia solani* (Lakshman and Tavantzis, 1994) and CeMV in *C. elegans* (Park *et al.*, 2006). As suggested by Ghabrial (1998), having a latent infection,

defined by a virus being present but not causing disease symptoms, might be beneficial to fungi as it may confer protection against infection by other viruses.

The rarity of OMV1c at the western Canadian disease front raises the question of the origin of this virus. Because there were two waves of infection spread through Europe and North America with the less aggressive *O. ulmi* being replaced by the more aggressive *O. novo-ulmi* it may be that the virus was harbored in the older and ancestral infection wave. Usually, when *O. novo-ulmi* arrives at a “new” area it rapidly replaces resident *O. ulmi* (Brasier, 1986a). During this replacement process, the close proximity of *O. ulmi* and *O. novo-ulmi* in the bark beetle galleries provides the physical opportunity for interspecific genetic exchange. Rare *O. ulmi*–*O. novo-ulmi* hybrids do occur in nature but are transient, quickly disappearing in competition with the parent species (Brasier *et al.*, 1998). There exists a possibility: that the deleterious viruses that spread in the *O. novo-ulmi* clones were also acquired from *O. ulmi*. Sexual hybridization between these two *Ophiostoma* species is likely quite rare but there may opportunity virus transfer through transient hyphal anastomosis (Brasier, 1977; Kile and Brasier, 1990). A preliminary comparison of viruses in *O. ulmi* and *O. novo-ulmi* isolates obtained from the same epidemic front site in Europe indicated a very close similarity in their RNA sequences. *Ophiostoma novo-ulmi* may have become infected with debilitating virus infections from *O. ulmi* (Abdelali *et al.*, 1999).

The mitovirus OMV1c was not found in any of twelve screened *O. novo-ulmi* isolates from Winnipeg. These data are insufficient to fully support the idea that OMV1c is absent in Winnipeg *Ophiostoma* population, but it did show that OMV1c may be rare in this population. Since mitoviruses are transmissible only through hyphal fusion and

not through sexual spores, the presence of a mitovirus might serve as a marker to better understand the origin and spread of the fungus. To address whether OMV1c was present in *Ophiostoma* isolates transported to North America at the beginning of the last century we will have to screen many more isolates for the presence of OMV1c. Neither the isolate VA30 from Virginia (USA) nor H327 from Bratislava (Slovakia) showed the presence of OMV1c. There is also a possibility of horizontal transfer from other yet unidentified species. Further screening of *O. novo-ulmi* isolates for OMV1c from across North America could better address the question of the origin of OMV1c.

Chapter 3: The Potential for Additional Viruses Infecting the Plant Pathogen *Ophiostoma novo-ulmi*, Isolate 93-1224

3.1 Introduction

3.1.1 General Description of Viruses

Viruses are genetic elements that replicate independently of a cell's chromosomes but are dependent on the host cells for their ability to replicate. Many viruses have an extracellular form that enables them to exist outside the host for some time and provides for the transmission from one host to another (Madigan and Martinko, 2006). During replication in the intracellular stage, viruses redirect host metabolic functions to support their own replication and the assembly of new virions. Viruses contain DNA or RNA genomes and come in many different shapes, ranging in size from 20-300 nm. A typical virion (virus particle) consists of nucleic acids located within a protein shell (capsid), assembled in a specific array of structural subunits (capsomers), which are composed of protein. Many viruses have an envelope which consists of a lipid bilayer derived from the host cell membrane in which are embedded virus encoded glycoproteins (Madigan and Martinko, 2006).

3.1.2 Mycoviruses

Viruses that infect lower fungi, such as *Saccharomyces*, were first discovered in 1936. The presence of viruses that infect higher fungi were first suspected when Sinden and Hauser (1950) reported on two degenerative disease of mushrooms. This led to the

widespread discovery of viruses in fungi and spawned the field of mycovirology (Ghabrial and Suzuki, 2009). Almost all mycoviruses have RNA genomes (ssRNA or dsRNA) and fungal viruses typically consist of isometric particles 25-50 nm in diameter. Their RNA genome can be undivided or segmented, with the coat protein and RdRp genes often appearing on separate dsRNA segments. The number of segments can vary from 2 to 12 (Ghabrial and Suzuki, 2009). Screening of many phytopathogenic fungi for the presence of dsRNA shows a prevalence of fungal viruses. Almost 80% of screened fungi were found to be potentially infected (Ghabrial and Suzuki, 2009). Over eighty mycovirus species have been officially recognized and have been classified into ten virus families (Pearson *et al.*, 2009). Mycoviruses are usually located in the cytoplasm of the fungal host (Polashock and Hillman, 1994), although certain viruses have been found to be specific to the mitochondria (mitoviruses). Mitoviruses have no capsid and consist of a single strand of naked RNA, which encodes a single protein for RNA-dependent RNA polymerase (RdRp) (Doherty *et al.*, 2006; Ghabrial, 1998). Replication is catalyzed by the RdRP encoded by the viral genome (Cole *et al.*, 2000), thus viruses ensure their persistence within fungal cells though little is known about the transmission of mitoviruses.

Mycovirus infection can result in a wide range of phenotypes of their hosts. Although a large number of the fungal viruses have been reported to be virulent, there is some evidence that phenotypic consequences of harbouring some viruses or RNA molecules can range from no symptoms to severe debilitation and from hypovirulence to hypervirulence (Buck, 1986; Nuss and Koltin, 1990; Pearson *et al.*, 2009).

Mycoviruses are believed to be of ancient origin and dsRNA viruses are thought to have evolved in close association with their hosts. In some cases there were mutual benefits. For example, the viruses linked with killer phenotypes in yeast confer a strong advantage to their hosts by encoding toxins which are lethal to sensitive cells (non-killer strains of yeast and some bacteria), helping the yeast to compete for resources. Together with the toxins, these viruses encode proteins that provide immunity to these toxins (van Diepeningen *et al.*, 2006; Wickner, 1996). The host cells are only able to tolerate a defined viral titre, beyond which, increased viral load becomes pathogenic. Host death may lead to virus elimination (Ghabrial, 1998). Mycoviruses do not have an extracellular phase. In nature these viruses are transmitted only intracellularly: vertically during host cell division and sporogenesis (by sexual and asexual spores) and horizontally during cell fusion as a result of hyphal anastomosis (Buck, 1998; Ghabrial, 2009; Pearson *et al.*, 2009). Mixed infections with two or more related or unrelated viruses and accumulation of defective and/or satellite dsRNAs are common features of mycovirus infections. Examples of mixed infections include the *Sphaeropsis sapinea* victoriviruses SsRV1 and SsRV2, *Sclerotinia sclerotiorum* viruses SsDRV and SsRV-L and *Diplodia destructiva* partitiviruses DdV1 and DdV2 (Jacob-Wilk *et al.*, 2009; Hong *et al.*, 1999; Deng and Boland, 2004).

3.1.3 Taxonomy and Morphology of Mycoviruses

Mycoviruses with double-stranded (ds) RNA genomes are classified into the families: *Chrysoviridae*, *Endornaviridae*, *Hypoviridae*, *Partitiviridae*, *Reoviridae* and *Totiviridae* (Attoui *et al.*, 2000; Mayo, 2002; Pearson *et al.*, 2009; Ghabrial and Suzuki, 2009).

Approximately one-third of all the characterized mycoviruses are ssRNA viruses and mycoviruses with single-stranded (ss) RNA genomes belong to the families *Barnaviridae*, and *Metaviridae* (Pearson *et al.*, 2009). To date only one virus, from the genus *Rhizidiovirus* (60 nm), has isometric virions with a monopartite dsDNA genome (Pearson *et al.*, 2009). SsDNA and negative-strand RNA viruses have not been found (Ghabrial and Suzuki, 2009). Even if most mycoviruses are isometric, a variety of other particle morphologies have been reported including rigid rods (McDonald and Heath, 1978), flexuous rods (Howitt *et al.*, 2001; Howitt *et al.*, 2005), club-shapes (Dodds, 1980), bacilliform (Revill *et al.*, 1998) and even a herpes-like virus (Kazama and Schornstein, 1972).

3.1.4 Replication and Gene Expression Strategy

Little information is available on the replication cycles of dsRNA mycoviruses. What is known has mostly been derived from *in vitro* studies of virion-associated RNA polymerase activity and the isolation of particles representing various stages in the replication cycle. Genome transcription is a very important stage in the life cycle of a virus. This is the process by which the viral genetic information is presented to the host cell protein synthesis machinery for the production of the viral proteins needed for genome replication and progeny virion assembly. DsRNA faces a particular challenge because host cells cannot transcribe from a dsRNA template. Therefore, dsRNA viruses must encode all of the necessary enzymatic machinery to synthesize complete messenger RNA (mRNA) transcripts without the need for disassembly (Lawton *et al.*, 2000).

The RdRp activity associated with certain mycoviruses in the families *Totiviridae* and *Partitiviridae* was shown, *in vitro*, to catalyze end-to-end transcription of dsRNA to

produce mRNA for the coat protein, which is released from the particles. In the case of the yeast L-A virus, purified ScV-L-A virions, isolated from log-phase yeast cells, contained a less dense class of particles, which packaged only plus-strand RNA. During *in vitro* reactions, these particles exhibited a replicase activity that catalyzed the synthesis of minus-strand RNA to form dsRNA. The resultant mature particles were capable of synthesizing and releasing plus-strand RNA (Wickner, 1996; Ghabrial and Suzuki, 2009).

Hypoviruses have linear RNA genomes 9–13 kb in size. The dsRNA forms of *Endornaviruses*' genomes comprise single large dsRNAs 14 to 17 kbp long with a site-specific nick within 3 kbp of the 5' end of the coding strand. They encode a single long ORF, which contains conserved motifs for an RdRp and an RNA helicase. In members of the families *Hypoviridae* and *Endornaviridae*, replication of naked RNA mycoviruses may occur in infection-specific, lipid-membranous vesicles presumed to contain viral RdRp and RNA helicase. These vesicles are able to synthesize *in vitro* both plus and minus RNA at a ratio of 1:8 (Ghabrial and Suzuki, 2009).

3.1.5 Mycoviruses in Genus *Ophiostoma*

A total of ten mitochondrial viruses have now been found in *Ophiostoma novo-ulmi*. They all consist of naked positive ssRNA, and have one ORF encoding RdRp and range in size between 2.3 to 3.5 kbp (Cole *et al.*, 1998, Kassatenko Chapter 2).

Recently, a virus was isolated and characterized from *Ophiostoma himal-ulmi* that was designated *Ophiostoma partitivirus 1* and represented a new member of the genus *Partitivirus* (Crawford *et al.*, 2006). The new virus has two dsRNA molecules: S1 and S2, sized 1,744 bp and 1,567 bp respectively. RNA S1 had a potential to encode a

protein of 539 amino acids and was found to be closely related to RdRps of members of the genus *Partitivirus* in the family *Partitiviridae*. RNA S2 had the potential to encode a protein of 430 amino acids, which was related to capsid proteins of members of the genus *Partitivirus*. Isolated virus particles were shown to be isometric with a diameter of 30 nm. A phylogenetic analysis of RdRp's of members of the family Partitiviridae showed two lineages of viruses from genus *Partitivirus*. One of them contained viruses that infect both fungi and plants, raising the possibility of the horizontal transmission of partitiviruses between plants and fungi (Crawford *et al.*, 2006).

Partial sequences of two other dsRNA elements from *Ophiostoma minus* (a pathogen of *Pinus contorta*) and *Ophiostoma quercus* (a pathogen of *Ulmus carpinifolia*) revealed a single ORF with the potential to encode a portion of RdRp. The *O. minus* dsRNA was most closely related to an RdRp gene in a virus classified in the genus *Totivirus* (family Totiviridae) with an overall sequence identity of 45%. For the *O. quercus* dsRNA the partial sequence had the most similarity with the *Rhapanus sativus* (family Partitiviridae) with an overall sequence identity of 37% (Doherty *et al.*, 2007). Deng and Boland (2007) reported the natural occurrence of a partitivirus in *Ceratocystis resinifera*. The two dsRNA molecules of this virus were 2305 and 2207 bp in length. A comparative analysis of these two sequences with sequences in the databases showed similarities with CP and RdRp sequences from earlier characterized fungal partitiviruses. This virus was highly related (87.3 % and 95.6 % amino acid similarity for CPs and RdRps, respectively) to the partitivirus in *Ceratocystis polonica*. These results support the hypothesis that interspecific transmission of these mycoviruses is possible between close species (Deng and Boland, 2007).

3.1.6 Current Study and Research Objectives

While we did characterize a single mitovirus from *O. novo-ulmi* 93-1224, it is possible that this isolate harbours more than one type of viral element. The main objective of this study was to determine whether the isolate 93-1224 of *O. novo-ulmi* harboured novel cytoplasmic viruses in addition to the mitovirus OMV1c. Electron microscope analysis of isolate 93-1224 revealed the presence of two flexuous rod-shaped structures, of different diameters, which we hypothesized to represent filamentous viruses.

3.2 Materials and Methods

3.2.1 Fungal Growth and Culture Maintenance

Ophiostoma novo-ulmi (strains 93-1224, H327, VA30) were maintained and grown as described in Chapter 2. A putatively virus-cured culture of *O. novo-ulmi* 93-1224-PVC was obtained by multiple re-culturing of hyphal tips derived from infected isolate *O. novo-ulmi* 93-1224 (Rivera-Varas *et al.*, 2007).

3.2.2 Morphology and Colony Growth Comparison of *O. novo-ulmi* Wild and a Putatively Cured Strains: 93-1224 and 93-1224-PVC

To determine whether there might be phenotypic effects associated with the presence or absence of virus, the growth characteristics of the putatively cured isolate were compared to the wild-type for isolate 93-1224. The growth rate and colony morphologies of the putatively cured isolate 93-1224-PVC and a wild-type virus-containing strain 93-1224 from Winnipeg (Temple *et al.*, 2006) were compared when grown on OCM agar. An 8 mm² agar plug from the actively growing mycelium of each strain was placed on a OCM agar plate and incubated at 25° C for 10 days until the fastest growing colonies spread to the edge of the Petri dishes. The diameter of each colony was measured daily after inoculation until the mycelium had covered the entire plate and the colony morphology was visually assessed. Four replicate plates were used for each strain and data were averaged.

3.2.3 Application of a Virus Purification Procedure to *O. novo-ulmi* 93-1224 and *O. novo-ulmi* VA30

Mycelia were crushed in liquid nitrogen into fine powder using a mortar and pestle. Three volumes of 0.05 M sodium phosphate buffer were added to the powder and samples were shaken at 4°C for 15 min. The mix was centrifuged at 11,000 rpm in a GST rotor at 4°C for 30 min to pellet cell debris. The supernatant was collected and adjusted to 8% polyethylene glycol-6000 and 1% NaCl. This mixture was stirred on ice for 2 hours then centrifuged at 11,000 rpm at 4°C for 30 min. Supernatant was collected and centrifuged at 55,000 rpm in a 70.1 Ti rotor at 4°C for 80 min. The pellets were re-suspended in 100 µl of 0.1 M sodium phosphate buffer, placed in a micro centrifuge tube and centrifuged at 11,000 rpm for 15 min (Shamoun *et al*, 2008). Supernatants were kept at -80°C.

3.2.4 Density Gradient Centrifugation of *O. novo-ulmi* 93-1224 and *O. novo-ulmi* VA30

Continuous sucrose gradients consisting of 20, 30, 40, and 50% sucrose in 0.05 M phosphate buffer were prepared by layering progressively less dense sucrose solutions upon one another and storing the centrifuge tubes at 4° C overnight to equilibrate. The supernatant was thawed on ice and layered onto the gradients and centrifuged at 15,000 rpm for 3.5 hours in a SW 25.1 rotor at 4°C. Fractions were collected using bottom illumination. Volumes were adjusted to 25 ml with 0.05 M phosphate buffer, and pelleted by centrifugation at 45,000 rpm for 3 hours in a 70 Ti rotor. The pellet was re-suspended in 100 µl of 0.01 M phosphate buffer and centrifuged at 11,000 rpm for 15 min to remove debris. Supernatant was collected and examined by EM.

3.2.5 Electron Microscopy of Fractionated *O. novo-ulmi* 93-1224 and *O. novo-ulmi* VA30

3.2.5.1 Grid Preparation

Grids (300 square meshes, copper) were cleaned in acetone by sonication for 1-2 minutes and air-dried. A glass slide was lightly wiped with a Kimwipe and placed in the upper part of the filmforming device previously cleaned with 1% Formvar. A 1% Formvar solution was slowly poured in (~10 cm deep) and quickly poured out. The slide was taken out and air-dried in a dust-free space. The edges of the slide were scored to release the formvar film. The glass slide with the scraped film was lowered at the angle of 45° into the water surface. The floating film was a silver-gold color indicating a thickness 60-100nm. The cleaned and dried copper grids were placed up-side down onto the floating film. The surface of the film grids was gently touched with a section of Parafilm and lifted. The film adhered to the Parafilm, sandwiching the grids in between. Dry, plastic-coated grids were arranged on a clean glass slide (with coated side up) and then the slide was placed in a vacuum evaporator, where the grids were allowed to be coated with ~ 5 nm-thick carbon films and then stored in covered Petri dishes (Hayat, 1989).

3.2.5.2 Negative Staining

Two different negative stains were used: 1% uranyl acetate (UA) and 2% phosphotungstic acid (PTA). 3 µl of specimen were placed directly on the grid and air dried. A drop of negative stain was placed on the piece of Parafilm. The grid with specimen was touched to the surface of the drop of negative stain (coated surface down). Excess fluid was removed with torn filter paper, and the grid air dried (Doane and

Anderson, 1987). Negatively stained specimens were examined on a transmission electron microscope (TEM) and photographed.

3.2.5.3 Electron Microscopy

Resulting supernatants from initial purification and sucrose gradient centrifugation were stained with uranyl acetate or phosphotungstic acid and viewed and photographed using a Hitachi H-7000 transmission electron microscopy at 30000 – 150000x magnifications (Electron Microscopy Resource Center, Biology Department, University of Victoria).

3.2.6 Mass Spectrometry

3.2.6.1 Protein Isolation from O. novo-ulmi 93-1224 and O. novo-ulmi VA30

Homogenates from the initial purification and sucrose gradient fractions were treated by 1 M dl-dithiothreitol (DTT) and 4 x sodium dodecyl sulfate (SDS) loading buffer, mixed briefly by vortexing, then heated for 5 min at 98°C. Pre-cast two-dimensional gel NuPAGE Novex Bis-Tris Gel and NuPAGE MES SDS running buffer from Invitrogen (Grand Island, USA) were used to separate proteins by size. SDS-polyacrylamide gel electrophoresis was performed for 1 hour at 150 V. The gel was fixed for 10 min in 40 % ethanol, 10% acetic acid, and stained overnight in 0.01 % Coomassie Brilliant Blue, and then the gel background was destained overnight to visualize the proteins. Protein bands from the SDS-PAGE gel of sucrose gradient fractions were individually excised and in-gel digested with trypsin. Upon excision of the gel pieces they were shrunk by dehydration in acetonitrile, which was then removed, and were lyophilized until dry. The peptides were reconstituted with 0.1 % TFA (trifluoroacetic acid) and 50% ACN (acetonitrile) solution in preparation for Mass Spectrometry analysis.

3.2.6.2 Mass Spectrometry (MS) and Tandem Mass Spectrometry (MS/MS)

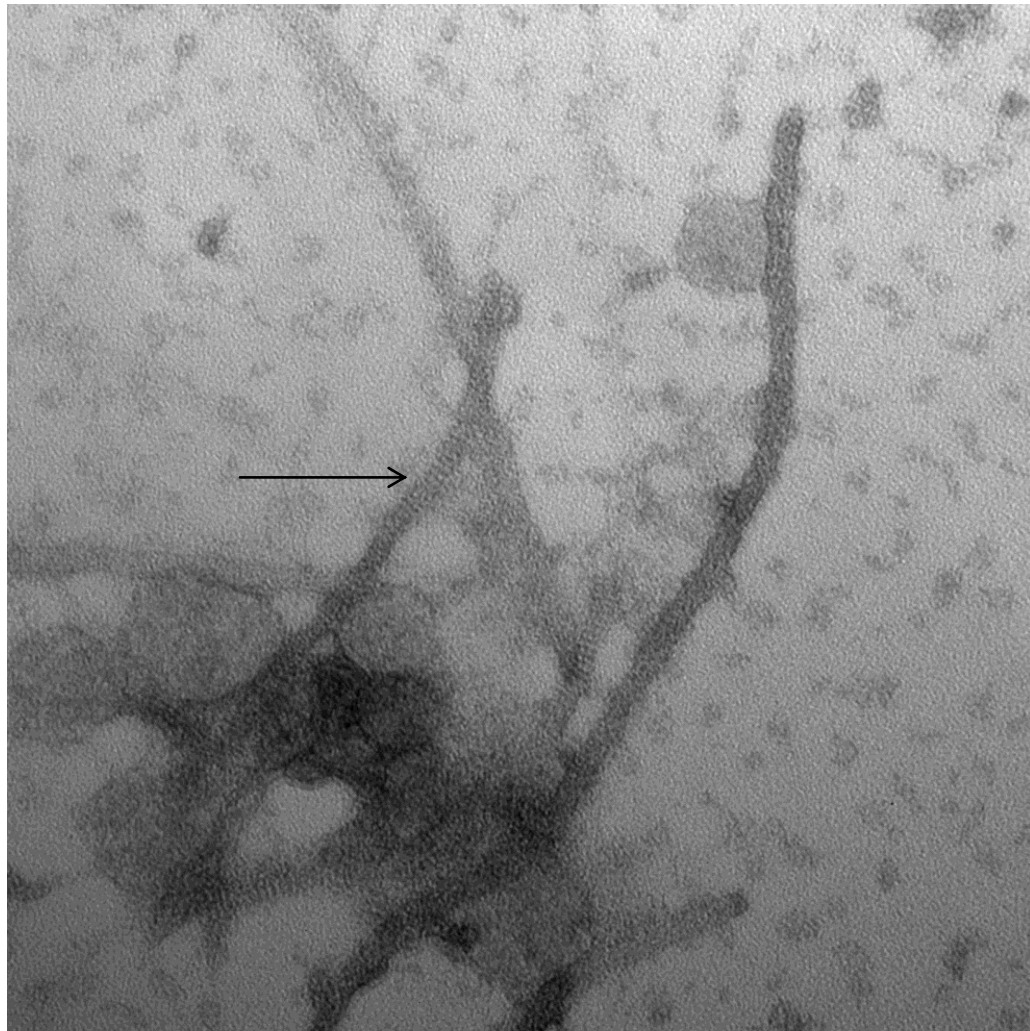
One μ l samples of the reconstituted tryptic digests were spotted on a MALDI plate, and overlaid with 1 μ l of matrix (R-cyano-4-hydroxy-*trans*-cinnamic acid and nitrocellulose were dissolved in acetone/2-propanol (1:1 v/v) to a concentration of 20 and 5 g/L, respectively). All mass spectra were obtained on a 4800 ABI MALDI TOF/TOF-MS (University of Victoria Genome British Columbia Proteomics Centre). Data were visualized using the program DataExplorer AB SCIEX (available on-line at www.matrixscience.com). The mass fragments with the highest m/z in the MS/MS spectra of the tryptic digests were assembled into short peptide sequences using the molecular weight information.

3.3 Results

3.3.1 Screening for the Possible Presence of Encapsidated Viruses in *O. novo-ulmi*

In order to screen for the possible presence of encapsidated viruses, homogenates of *O. novo-ulmi* 93-1224 and VA30 were subjected to sucrose density gradient centrifugation and various fractions were examined by electron microscopy. Originally we had expected that isolate 93-1224 might show the presence of a novel virus, because we had previously observed the presence of two dsRNA bands typical of this type of particle, and we expected that the VA30 would serve as a negative control as there was no evidence for the presence of a virus in this second isolate. Following density gradient centrifugation, we were surprised to find a type of non-enveloped flexuous rod-shaped particle in the purified preparation of both *O. novo-ulmi* isolate VA30 and 93-1224 upon negative staining with 1% uranyl acetate (UA) (Figure 16, 17). The particles had a clearly visible helical capsid structure typical of viral coat proteins. Many of the particles were damaged during the extraction or ultracentrifugation hence the complete length of intact particles could not be determined. The longest measured between 2,800 and 2,900 nm long and had a thickness of 15 nm. These will henceforth be referred to as the “thick” rod-like structures.

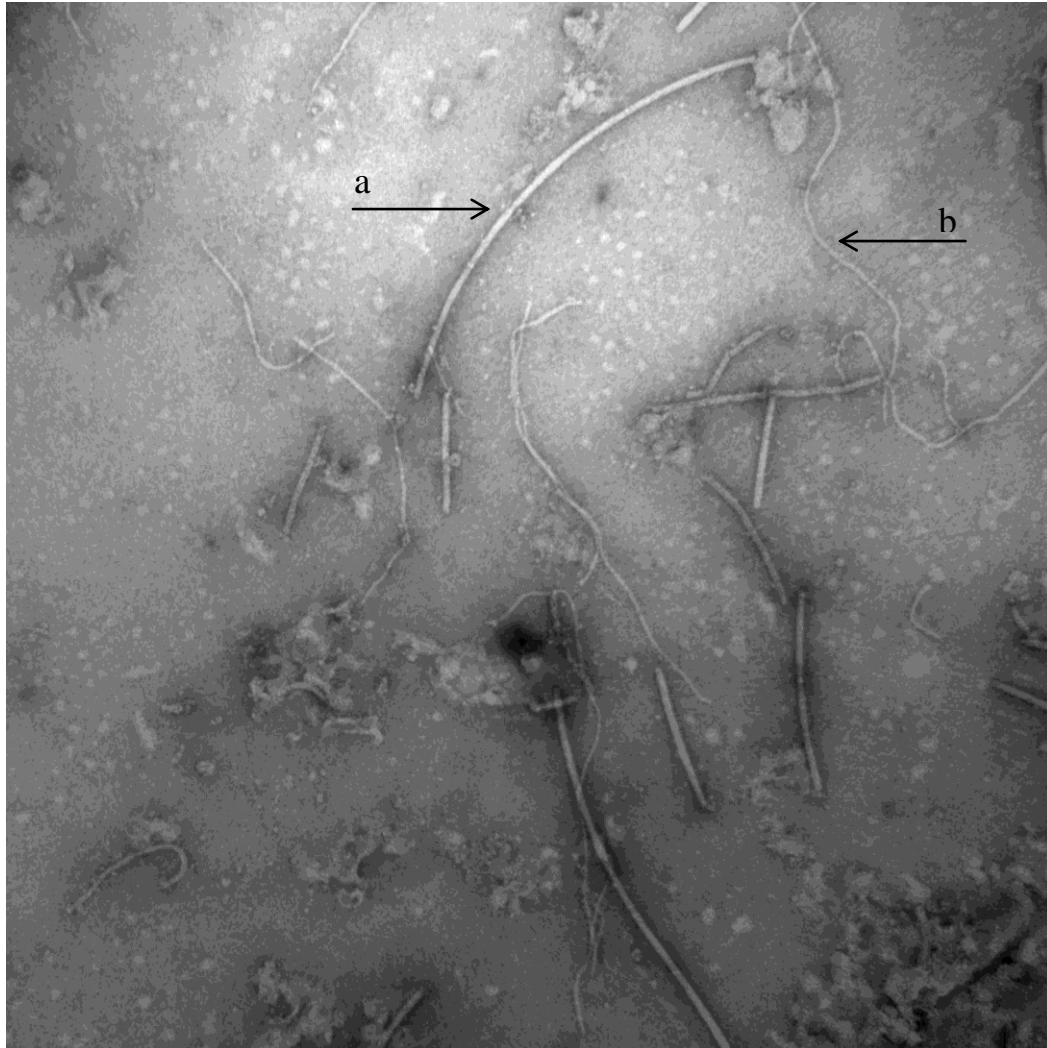
When we examined the sucrose density gradient fractions for isolate 93-1224 we observed two different types of rod-like structures (Figure 17). The second type was long, thin, flexible rod-like particles that ranged in length from 2,300 to 2,500 nm in length and measured 10 nm in thickness (Figures 17, 18). These will henceforth be referred to as the “thin” rod-like structures. There also were very few isometric



VA30-UA-1to10-UA-200000-20.tif
onu.VA30-UA.1to10
26 June 2009
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1:30 06/27/09
TEM Mode: Imaging

100 nm
HV=75kV
Direct Mag: 200000x
AMT Camera System

Figure 16. Electron micrograph of rigid rod-shaped structures with a length $\sim 2,900$ nm in a purified preparation of *O. novo-ulmi* isolate VA30 negatively stained with 1% uranyl acetate, magnification 200,000 x. An arrow indicates possible helical structure of coat protein.



onu10-UA-1to10.tif

onu_virus UA 1to10 image10

4 June 2009

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1 TEM Mode: Imaging

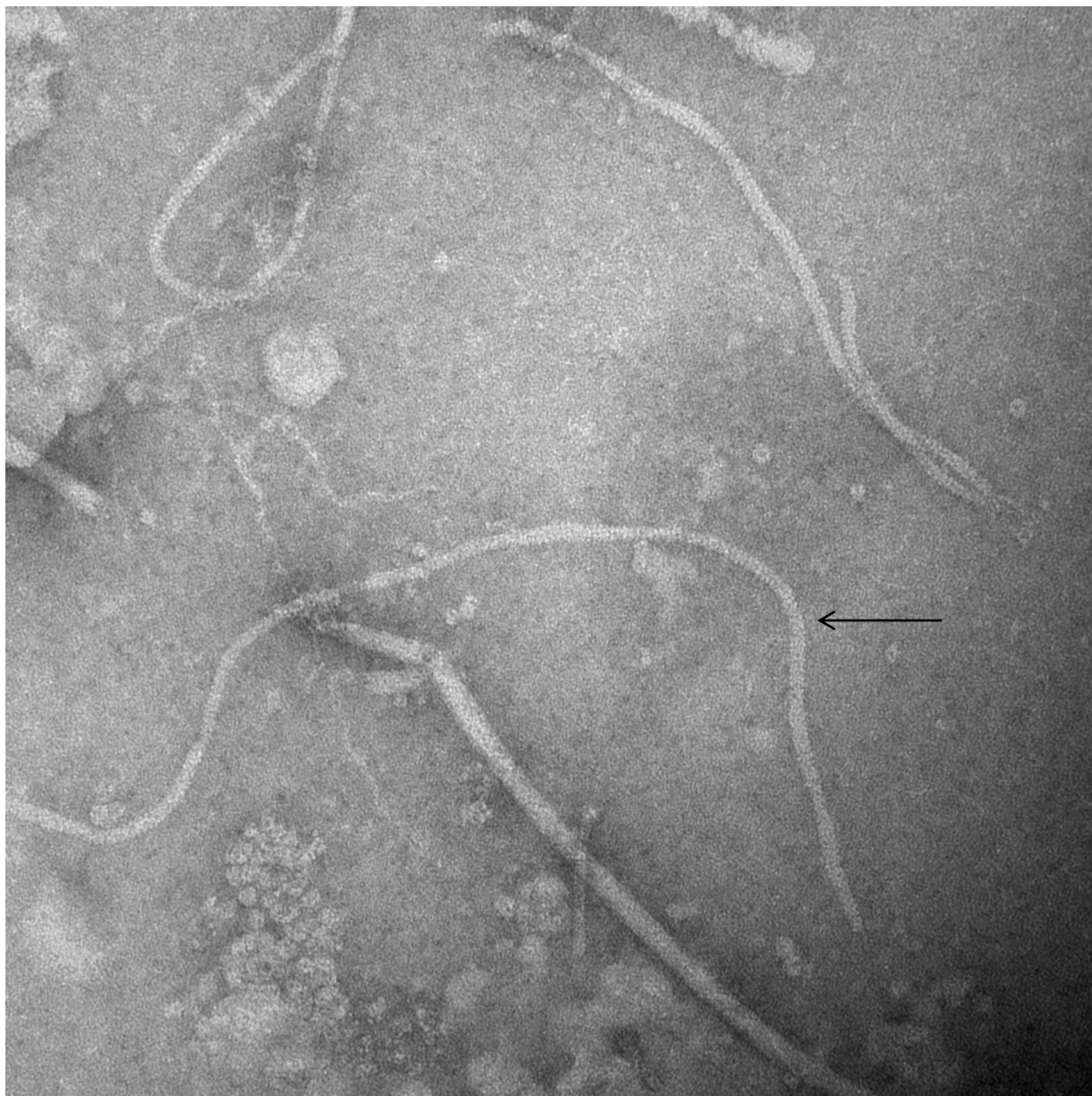
500 nm

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Figure 17. Electron micrograph of two types of rod-like structures in a purified preparation of *O. novo-ulmi* isolate 93-1224, negatively stained with 1% uranyl acetate, magnification 50,000 x. Arrows indicate a) “thick” and b) “thin” morphology of the rod-like structures.



onu11-UA.1to10.tif

onu_virus UA 1to10 image11

4 June 2009

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TEM Mode: Imaging

100 nm

HV=75kV

Direct Mag: 150000x

AMT Camera System

Figure 18. Higher magnification of the two types of rod-like structures in a purified preparation of *O. novo-ulmi* isolate 93-1224, negatively stained with 1% uranyl acetate, magnification 150,000x. Indicated are the thick and thin flexuous rod-like structures. An arrow indicates helical structure.

structures that ranged in diameter from 60 to 80 nm and displayed angled sides consistent with an icosahedral shape (Figures 19-a, b, c.). The “thick” rod-like structures from isolate VA30 had the same size and shape and appeared very similar to the “thick” rod-like structures from isolate 93-1224. The migration on the sucrose gradient was also similar for both the 93-1224 and VA30 extracts (Figure 20). SDS polyacrylamide electrophoresis demonstrated that the proteins corresponding to the thick rod-like structures from both isolates migrated at the same size. Both had an apparent molecular weight of ~ 40 kDa (Figure 21) hence the question arose as to whether these two rod-like structures were the same in these two fungal isolates. Protein extracted from fraction 1, corresponding to the thin rod-like structures, migrated at ~ 47 kDa. The highest migrating protein bands for each fraction were extracted and subjected to analysis by mass spectroscopy.

3.3.2 Mass Spectrometry, Tandem mass Spectrometry, Spectra Interpretation and de-novo Sequencing

Proteins from purified and separated rod-like structures were extracted, and separated according to their size by SDS-polyacrylamide gel electrophoresis. Discrete bands were excised and digested in-gel with trypsin and prepared for MALDI-TOF (Matrix-assisted laser desorption/ionization) time of flight mass spectrometry. Mass spectroscopy (MS) and mass spectroscopy tandem (MS/MS) were both performed. The resultant profiles were distinctly different for the thin and the thick rod-like structures indicating that these molecules were distinct. MS spectra of proteins from rod-like structures having the “thick” morphology from *O. novo-ulmi* isolates 93-1224 (lower) and VA30 both had the same profile suggesting similar sets of peptides (Figure 22). This was further confirmed by Mass spectroscopy tandem analysis which revealed that peptides with a

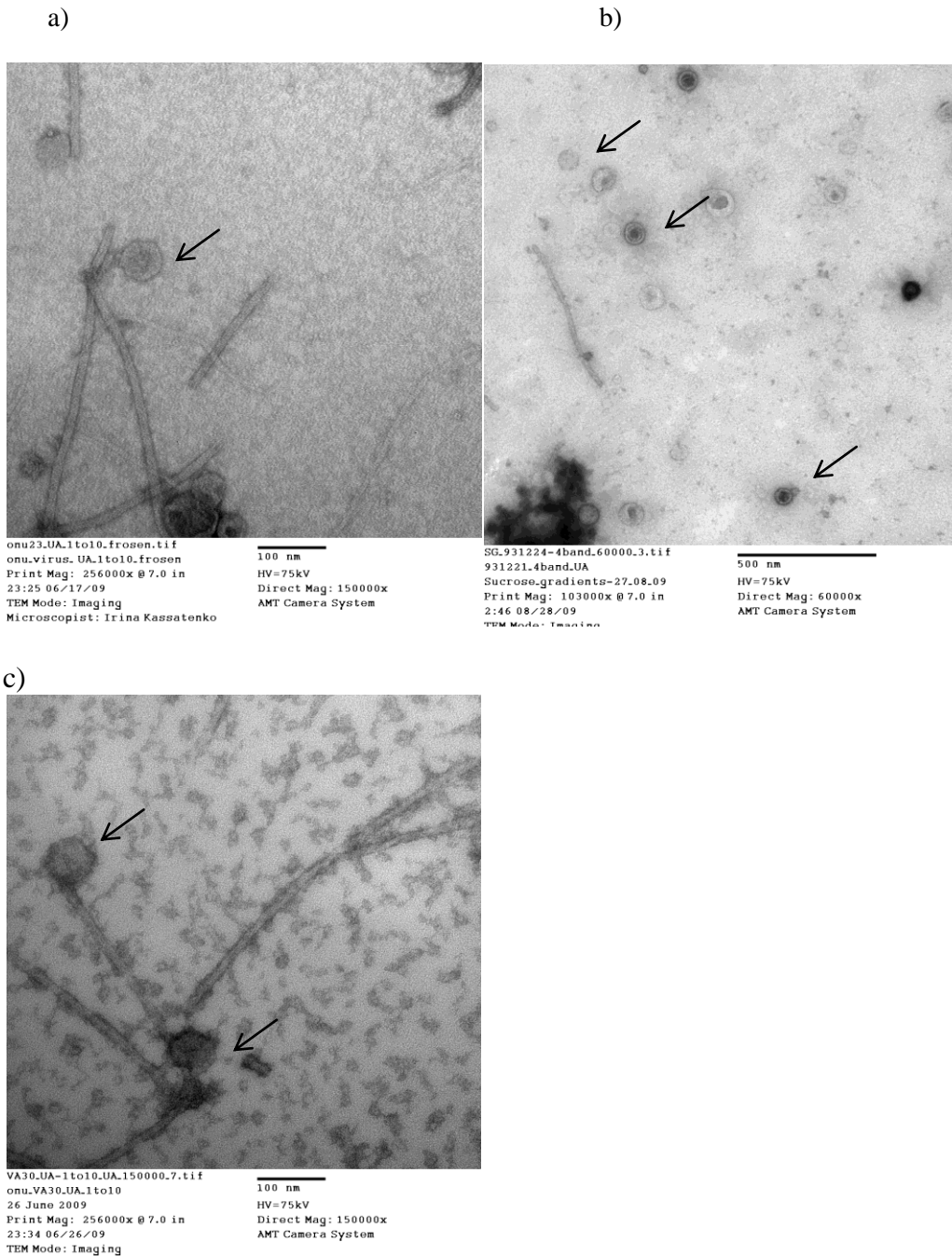


Figure 19. Electron micrograph of possible isometric virus particle (indicated by arrows) of size ~60-80 nm in relation to the “thick” and “thin” rod-like structures negatively stained with 1% uranyl acetate a) in a purified preparation *O. novo-ulmi* isolate 93-1224, magnification 150,000x; b) in sucrose gradient of *O. novo-ulmi* isolate 93-1224, magnification 60,000x; d) in a purified preparation of *O. novo-ulmi* isolate VA30 with clearly seen angles of icosahedral shape, magnification 150,000x.

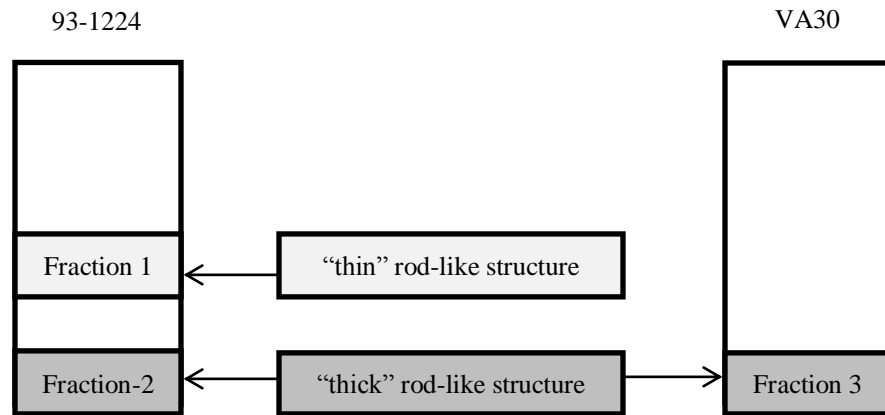


Figure 20. Separation of rod-like structures by sucrose density centrifugation from *O. novo-ulmi* isolations 93-1224 and VA30. There were predominantly thin rod-like structures observed in fraction 1 (93-1224) and thick rod-like structures were observed in fraction 2 in (93-1224) and fraction 3 (VA30).

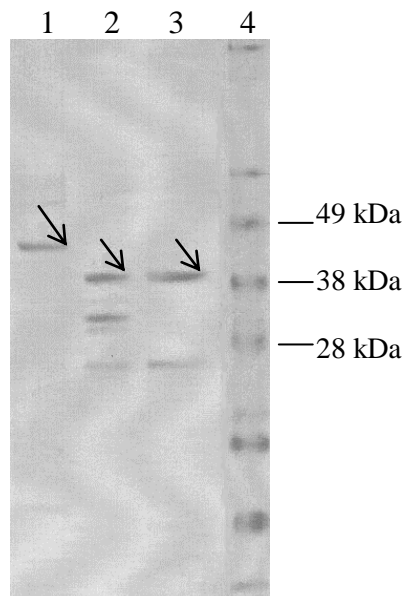


Figure 21. SDS-PAGE gel electrophoresis of proteins isolated from sucrose gradient from *O. novo-ulmi* 93-1224, fraction 1 (Figure 20) containing thin rod-like structures (lane 1), *O. novo-ulmi* 93-1224 fraction 2 (Figure 20) containing thick rod-like structures (lane 2) and VA30 fraction 3 (Figure 20) containing thick rod-like structures. Lane 4 – SeeBlue Plus2 Pre-Stained Standard ladder. Arrows indicate bands that were excised for MS analysis.

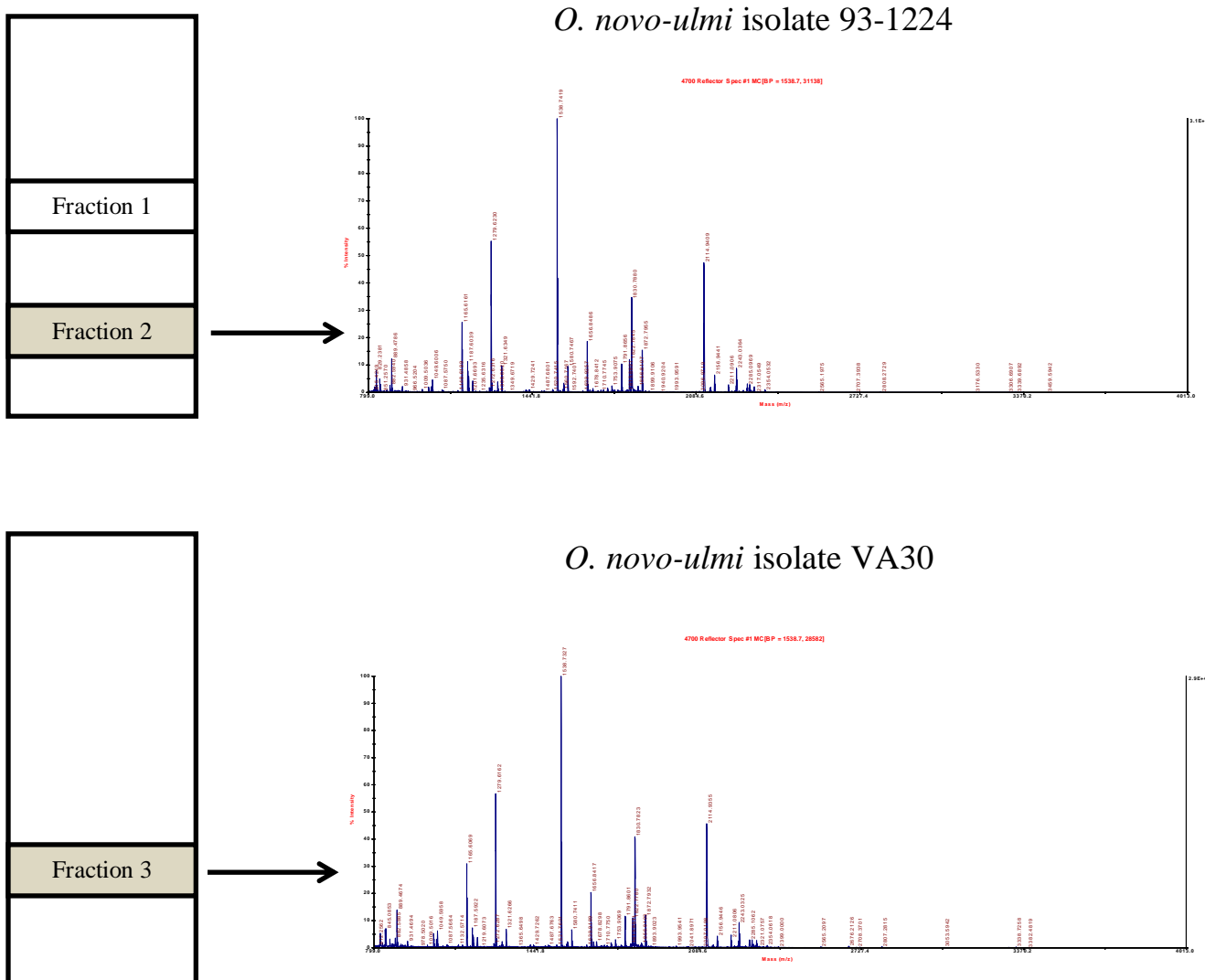


Figure 22. Mass spectrometry of proteins isolated from the “thick” rod-like structures from *O. novo-ulmi* isolates 93-1224 (upper) and VA30 (lower) demonstrate identical profiles.

mass of 1538.74 Da from MS from both isolates (VA30 and 93-1224) had exactly the same set of amino acids (Figure 23). These results demonstrated that the “thick” rod-like structures of both 93-1224 and VA30 shared a highly similar, if not identical, conserved protein sequence and represented the same structure in both of these fungal isolates. These data also showed that the particles having a thin morphology were unrelated chemically to those having the thick morphology. Mass fragments with the highest m/z in the MS/MS spectra of tryptic peptides were joined into short peptide sequence stretches. Together with the amino acid molecular weight information, they were assembled into five peptides (Table 6). A BLASTp search was done for each of the tryptic fragments. The ten best alignments are shown in Table 7. There were no amino acid sequences alignments with peptides in the non-redundant protein sequence (nr) databases detected for peptide fragment F-S-F-S-D-T-H-N-R. Peptide fragment N-E-T-F-T-L/I-E aligned with several proteins but none of them were viral derived. Fragment D-S-D-W-V-Y-P-H-N-N-V showed 100% maximum identity with 63% query coverage with chain X, atomic cryoem structure of a nonenveloped virus.

3.3.3 Curing of *O. novo-ulmi* Isolate 93-1224

As described earlier two flexuous rod-like structures having a ‘thick’ and ‘thin’ morphology and a helical structure were observed using EM in 93-1224 (Figures 17, 18). No rod-like particles, either of the thick or thin variety, were observed in the putatively cured isolate following electron microscopy. Three separate trials were done. Each experiment included a positive control of 93-1224. In each case no rod-like structures were visible

Table 6. Short peptide sequences were derived by joining mass fragments with the highest m/z in the MS/MS spectra of tryptic peptides using the amino acid molecular weight information.

Peptide sequence	Sucrose-gradient fraction of isolate 93-1224
F-S-F-S-D-T-H-N-R	Fraction 1
N-E-T-F-T-L/I-E	Fraction 2
D-S-D-W-V-Y-P-H-N-N-V	Fraction 2

Table 7. Protein BLAST search for peptide sequences: a) N-E-T-F-T-L/I-E;
c) D-S-D-W-V-Y-P-H-N-N-V, which showed some similarity to Aquareovirus virion.

a) N-E-T-F-T-L/I-E:

Protein	Maximum score	Query coverage	Maximum identity	Accession
chain A, Upf1-Rna complex	20.2	85%	83%	2XZL.A
chain A, crystal structure of susd superfamily protein (Bt_2365) from <i>Bacteroides Thetaiotaomicron</i> Vpi-5482	20.2	85%	83%	3MCX.A
chain A, alcohol dehydrogenase Adh-'a' from <i>Rhodococcus Ruber</i>	20.2	85%	83%	2XAA.A
chain A, structure of the Fanci-Fancd2	19.7	100%	86%	3S4W.A
chain A, dye-decolorizing peroxidase (Dyp) D171n	19.7	71%	100%	3MM1.A
chain B, the high resolution X-Ray structure of Papain complexed with fragments of the trypanosoma Brucei cysteine protease inhibitor Icp	23.1	85%	100%	2CIO.B
chain O, crystal structure of the photosynthetic A2b2-glyceraldehyde-3-phosphate dehydrogenase	21.0	100%	71%	2PKQ.O
chain A, crystal structure of Cppa protein from <i>Streptococcus Pneumoniae</i> Tigr4	21.0	100%	86%	3E0R.A
chain A, dye-decolorizing peroxidase (Dyp) D171n	20.6	85%	83%	3MM1.A
chain A, crystal structure of a decolorizing peroxidase	20.6	85%	83%	2D3Q.A

b) D-S-D-W-V-Y-P-H-N-N-V:

Protein	Maximum score	Query coverage	Maximum identity	Accession
chain T, crystal structure of the eukaryotic 40s ribosomal subunit in complex with initiation factor 1	25.2	54%	100%	2XZM.T
chain X, atomic cryoem structure of a nonenveloped virus suggests how penetration protein is primed for cell entry	22.7	63%	100%	3IYL.X
chain C, backbone model of an Aquareovirus virion by cryo-electron microscopy and bioinformatics	22.7	63%	100%	3K1Q.C
chain B, backbone model of an Aquareovirus virion by cryo-electron microscopy and bioinformatics	22.7	45%	100%	3K1Q.B
chain A, H553a mutant of phosphoketolase from <i>Bifidobacterium Breve</i>	22.7	45%	100%	3AHJ.A
chain A, H320a mutant of phosphoketolase from <i>Bifidobacterium Breve</i> complexed with acetyl thiamine diphosphate	22.7	45%	100%	3AHI.A
chain A, H142a mutant of phosphoketolase from <i>Bifidobacterium Breve</i> complexed with acetyl thiamine diphosphate	22.7	45%	100%	3AHH.A
chain A, H64a mutant of phosphoketolase from <i>Bifidobacterium Breve</i> complexed with a tricyclic ring form of thiamine diphosphate	22.7	45%	100%	3AHG.A
chain A, resting form of phosphoketolase from <i>Bifidobacterium Breve</i>	22.7	45%	100%	3AHC.A
chain A, structure of a family 4 carbohydrate esterase from <i>Bacillus anthracis</i>	22.7	72%	75%	2J13.A

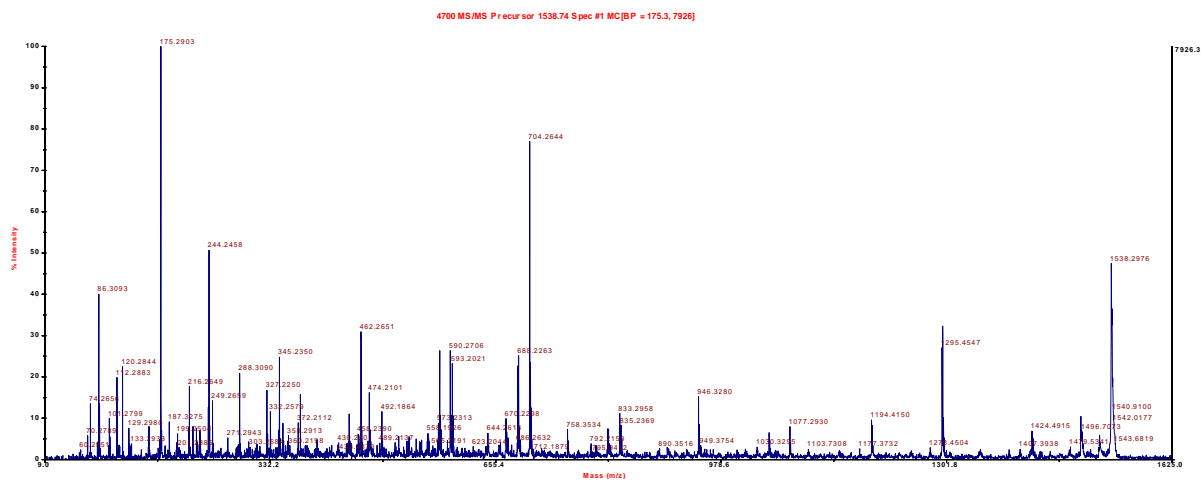
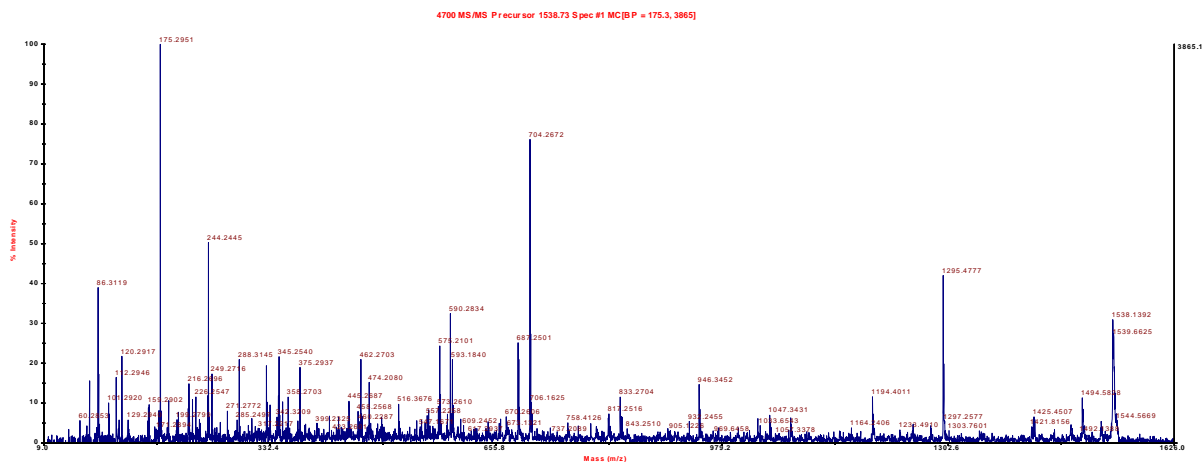
O. novo-ulmi isolate 93-1224*O. novo-ulmi* isolate VA30

Figure 23. Mass spectrometry tandem analysis of peptides with mass 1538.74 from rod-like structures having the “thick” morphology from *O. novo-ulmi* isolates 93-1224 (upper) and VA30 (lower) demonstrate exactly the same set of amino acids.

after curing but were consistently present in 93-1224. Repeated hyphal tip culture had apparently resulted in a loss of these structures (Figure 24 a, b).

3.3.4 Morphology and Colony Growth Comparison of *O. novo-ulmi* 93-1224 (Wild) and a Putatively Cured Strain *O. novo-ulmi* 93-1224-PVC

To determine whether there might be phenotypic effects associated with the presence of these virus-like particles, the growth rate and colony morphology of strain 93-1224-PVC was compared with its wild-type virus-containing strain 93-1224 from Winnipeg. Both strains were grown on Ophiostoma Complete Medium (OCM) and examined at 24 hour intervals for growth rate and morphological differences.

The strain 93-1224 colony had a white to cream color with a smooth and distinct roughly circular edge. The mycelial surface was dense, flat and not fluffy, had ridges that spread out radially from the centre (Figure 25-left; Figure 26-left). The 93-1224-PVC colony appeared less dense with a diffuse mycelium and had sectors that grew faster, which made the colony asymmetrical (Figure 25-right; Figure 26-right). The colour was the same for both 93-1224 and 93-1224-PVC (Figures 25, 26). Radial growth rate was 9.54 ± 0.32 mm/day for colonies of isolate *O. novo-ulmi* 93-1224, compared with 5.9 ± 0.5 mm/day for colonies of 93-1224-PVC, which indicated that virus containing isolate grew 1.6 times faster than putatively virus free isolate (Table 8, Figure 27).

Table 8. Comparison of colony diameters of *O. novo-ulmi* virus infected isolates 93-1224 and putatively virus-free 93-1224-PVC.

<i>O. novo-ulmi</i> 93-1224										<i>O. novo-ulmi</i> 93-1224-PVC									
	Colony diameter 1 (vertical) (mm)				Colony diameter 2 (horizontal) (mm)				Avg. diam. 1 (mm)		Colony diameter 1 (vertical) (mm)				Colony diameter 2 (horizontal) (mm)				Avg. diam. 2 (mm)
Sample Day	1	2	3	4	1	2	3	4		6	7	8	9	6	7	8	9		
4	30	30	29	29	34	36	37	37	32.7	20	25	28	32	35	31	35	31	29.6	
5	38	39	39	38	43	45	45	45	41.5	25	25	29	33	36	34	32	33	30.9	
6	49	50	50	49	55	56	56	57	52.6	33	28	35	39	39	43	48	41	38.3	
7	57	59	59	58	62	64	64	66	61.1	41	38	40	44	46	48	52	47	44.5	
8	68	71	71	70	76	78	79	78	73.8	53	46	48	56	54	58	60	52	53.4	
9	75	78	78	82	81	83	84	82	80.4	55	52	56	68	60	58	62	62	59.1	
10	86 full	86 full	86 Full	86 full	86 full	86 full	86 full	86 full	86.0 full	59	65	63	76	68	65	71	72	67.4	

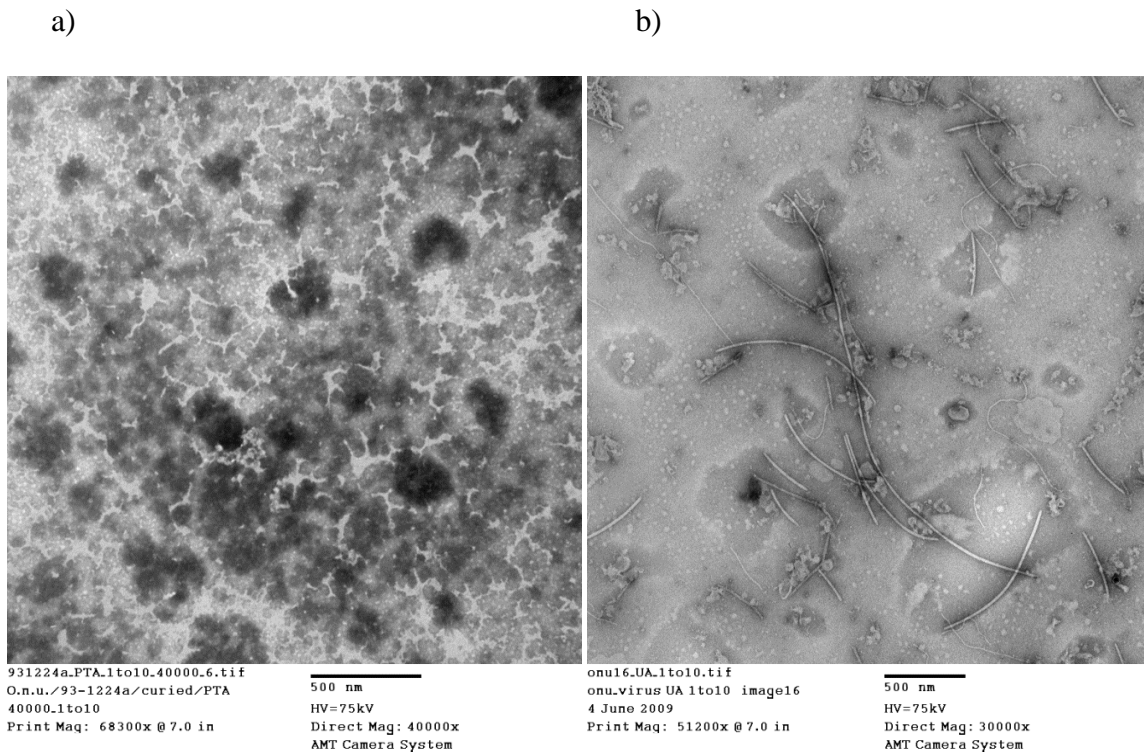


Figure 24. a) Electron micrograph of possible viral particles in a purified preparation of *O. novo-ulmi* isolate 93-1224-PVC, negatively stained with 1% uranyl acetate, magnification 40,000 x. No rod-like structures were observed. b) Positive control- electron microscopy of a purified preparation of *O. novo-ulmi* isolate 93-1224, negatively stained with 1% uranyl acetate, magnification 30,000 x.

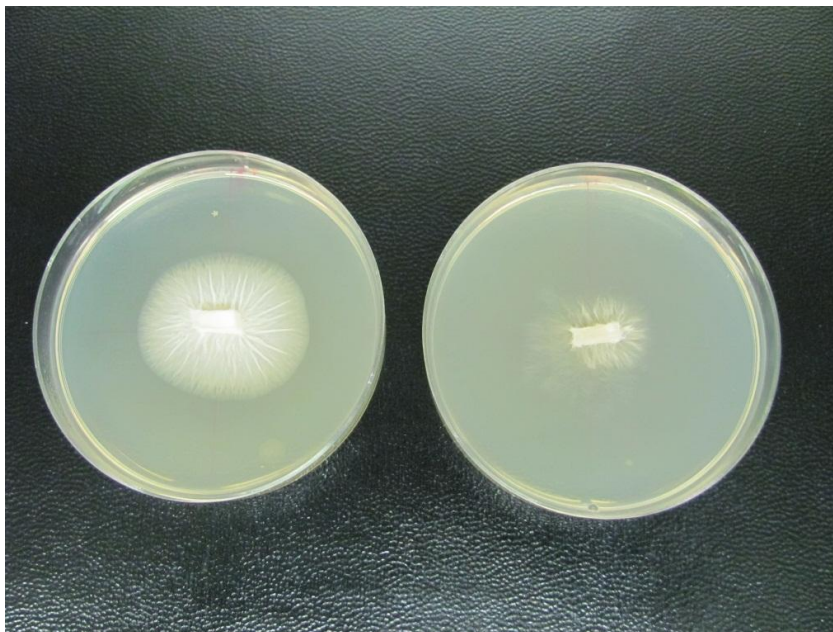


Figure 25. Morphology comparison of *O. novo-ulmi* 93-1224 (left) and *O. novo-ulmi* 93-1224-PVC (right) on day 5. Four replicates of each strain were done.



Figure 26. Morphology comparison of *O. novo-ulmi* 93-1224 (left) and *O. novo-ulmi* 93-1224- PVC (right) on day 10. Four replicates of each strain were done.

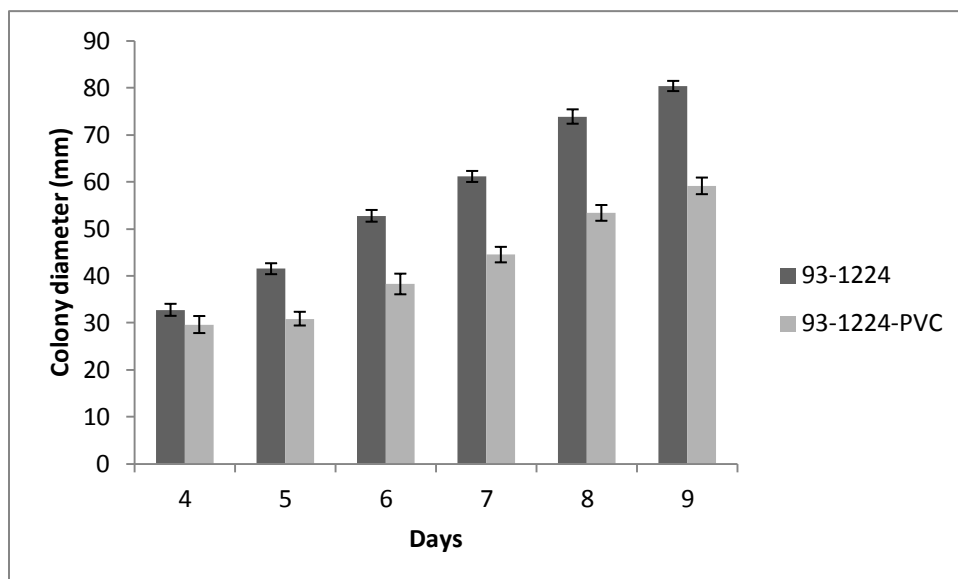


Figure 27. Comparison of radial growth of *O. novo-ulmi* isolates 93-1224 and 93-1224-PVC from day 4 to day 9: dark grey - isolates 93-1224, light grey – 93-1224-PVC. The bars indicate the standard deviation calculated from four replicates for each strain.

3.4 Discussion

Fungi are susceptible to infection by viruses from as many as ten virus families (Pearson *et al.*, 2009). There are eight well known mycoviruses that cause hypovirulence in fungal hosts providing their potential use as a biological control against pathogenic fungi (Buck, 1986). Studying mycoviruses, their properties and influence on their hosts is very important because it might provide a strategy to control fungal diseases in agriculture and forestry.

The majority of characterized mycoviruses either are encapsidated in isometric particles or occur as unencapsidated RNA elements (Buck, 1986). Mycoviruses of other morphological types have been observed in four fungal taxonomic divisions (Buck, 1986) however very few are characterized. Examples of non-isometric mycoviruses include bacilliform particles found in *Agaricus bisporus* (Hollings, 1962; Revill *et al.*, 1994), rigid rod-shaped particles in *Peziza ostracoderma* (Dielman-Van Zaayen, 1970), and a flexuous rod-shaped particle visualized in *Boletus edulis* (Huttinga *et al.*, 1975). Even though flexuous viruses are well known to infect plants, there are far fewer reports on rod-shaped viruses in fungi. There is only one example, the cowpea rust fungus, where more than one type of rod-shaped virus was found in the same isolate. Three types of rod-shaped viruses were found corresponding to a short rigid rod 260 x 12 nm, a long narrow-diameter flexuous rod 660 x 10 nm, and a long wide-diameter flexuous rod 740 x 16 nm (McDonald and Heath, 1978). The first genomic characterization of a flexuous fungal virus was reported by Howitt *et al.* in *Botrytis cinerea*. The *B. cinerea* flexuous rod-shaped mycovirus (BVF) resembled potexviruses and tymoviruses.

Assessing the western Canada disease front for the presence of virus we discovered one isolate 93-1224 that was infected with a dsRNA mitovirus. We were curious to determine whether this isolate might be infected with multiple viruses of different types. Based on our observations it appears that isolate 93-1224 in addition to mitovirus OMV1c may harbour virus-like particles of two types. Density gradient centrifugation followed by electron microscopy revealed the presence of two different types of abundant rod-like particles resembling encapsidated dsRNA viruses. Additionally, round particles were visualised by EM of sucrose-gradient fractions for both 93-1224 and VA30. These latter particles were not abundant and lacked the clearly staining dark outer line typical of the icosahedral coat protein of viruses hence are most likely fungal in origin. We hypothesized that the rod-like particles may represent viruses infecting the fungal host.

The fact that both types of rod-shaped particles with “thick” morphology (from isolates VA30 and 93-1224) showed exactly the same set of amino acids on mass spectroscopy tandem analysis could be explained by both isolates sharing the same virus or it could be that both isolate VA30 and 93-1224 were infected by viruses of the same type having a very conserved coat protein. Three short peptide sequence stretches were obtained from joined mass fragments with highest m/z in the MS/MS spectra of tryptic peptides using the amino acid molecular weight information. An alignment of these short sequences against protein Data Bank using BLAST revealed 63% similarity of the fragment D-S-D-W-V-Y-P-H-N-N-V with a nonenveloped *Aquareovirus* (family Reoviridae).

Putatively virus cured strain of *O. novo-ulmi* 93-1224-PVC was obtained by multiple re-culturing of hyphal tips derived from infected isolate *O. novo-ulmi* 93-1224. Electron microscopy of virus purification product from this cured strain confirmed the absence of rod-shaped particles. Though, 93-1224-PVC was shown to still carry the mitovirus (Chapter 2), the mitovirus consists of naked RNA and therefore would not be detected by electron microscopy. 93-1224-PVC was compared with its wild-type virus-containing isolate 93-1224 with regard to colony morphology and growth rate. The strain 93-1224 containing rod-like particles showed a healthy symmetric colony bigger in size with a smooth distinct circular edge. The putatively cured strain's colony was smaller (78.4% of the diameter of 93-1224 in 10 days of growth), with more translucent mycelium having sectors that grew faster and made the colony asymmetrical. One of our suggestions is that the observed changes in morphology and growth rate of 93-1224-PVC could mean that at least one of the rod-shaped structures might be beneficial to *O. novo-ulmi*, because when the fungus was cured from these structures, it grew more slowly and had abnormal asymmetrical mycelium. Typically, mycoviruses are symptomless but may also display hypovirulence or even provide beneficial effects towards the host (Pearson, 2009). As an example, a three-way symbiosis was discovered by Marques *et al.* (2006) whereby a plant-fungal-viral symbiosis between a tropical panic grass *Dichanthelium lanuginosum*, the fungus *Curvularia protuberata* and a virus infecting the fungus provided both organisms with the ability to grow at high soil temperatures. Fungal isolates cured of the virus were unable to confer heat tolerance, but heat tolerance was restored after the virus was reintroduced (Marques *et al.*, 2006). Alternately, it could be that the mitovirus provides some benefit to the 93-1224 and that a reduction in

titre, as demonstrated in the previous chapter, could result in the altered phenotype. Yet other processes, such as sectoring due to somatic mutations, could induce changes in colony morphology and growth. Sectoring of mycelia has been observed for many years in the mushroom industry and the biological mechanisms that are associated with this abnormal growth are not well understood. In some cases sectoring might indicate that the mycelium is actually composed of a mixture of fungi (Pitt and Hocking, 2009), but it is applicable only to newly isolated fungi. Our haploid culture of 93-1224 has been routinely cultured for a number of years. Never the less the phenotypic differences that we observed in colony growth of 93-1224-PVC could be due to sectoring and selection of somatic mutants by the process of isolating hyphal tips.

This study reports the first observation of rod-shaped structures with a potential to be viruses in *O. novo-ulmi*, and might indicate the first case of a co infection of *O. novo-ulmi* by viral particles from two distinct families: one of which was characterized as the mitovirus OMV1c from the family *Narnaviridae* and others are not yet defined. Evidences that these structures do represent viruses include: particles were isolated by a virus purification technique; they are of a size typical for viruses; they are visible by electron microscope and show helical structures typical for viral coat proteins. Mass spectrometry also proved the presence of proteins in their organization. The alignment of one of the short protein sequences from MS/MS with a nonenveloped *Aquareovirus* (family *Reoviridae*) showed some protein similarity however this must be interpreted with caution as there were as many other alignments with the same level of similarity that were not viral proteins. Both types (thick and thin) of these virus-like particles apparently disappeared following multiple rounds of hyphal tip transfer indicating that

they likely do not belong to regular fungal structures. We suggest what they might represent true viruses which do not belong to family *Narnaviridae*.

4 General Discussion

My general objective of this study was to explore potential diversity of viruses in a Canadian isolate of *O. novo-ulmi* 93-1224. The first evidence for the presence of a dsRNA virus in this isolate was the detection of two unique nucleic acid bands detected by agarose gel electrophoresis (Temple *et al.* 2006). A new virus, named *O. novo-ulmi* OMV1c, isolated from isolate *O. novo-ulmi* 93-1224, was determined to be 3,003 bp in length. The genome size, amino acid alignment, and phylogenetic analyses all indicated that OMV1c was closely related to members of the genus *Mitovirus* from family *Narnaviridae*. The putative RdRp gene of this virus was encoded by the single ORF located on a positive strand and demonstrated 85% identity to the RdRp gene of the mitovirus OMV1b. Viral RNA is relatively A and U rich (63%). The predicted molecular mass of the putative RdRp (77.23kDa) is also within the range of mitoviral RdRp proteins. Potential “stem-loop” secondary structures were predicted in OMV1c for both the 3' and 5'-ends. It also has been proposed that mitovirus RNA could be translated in the mitochondria (Cole *et al.*, 2000) and thus utilize mitochondrial codon usage. Our results also showed that the ORF of OMV1c has fifteen AUA codons which in mitochondrial codon usage encode methionine.

Because a dsRNA has previously been described in 93-1224 by Temple *et al.* (2006) we sought to determine whether these elements were related to the novel mitovirus. Results of the two tests (curing of the 93-1224 isolate by repeated tip re-culturing and screening additional isolates from the disease front) showed that Temple's double band visibly disappeared from the agarose gel, but that the mitovirus could still be detected by RT-PCR. The observation that strain 93-1224 could not be cured of OMV1c also

confirmed the mitochondrial nature of this virus. Screening twelve additional isolates for the presence of OMV1c showed that all tested isolates were negative for OMV1c; only 93-1224 contained the RNA doublet. This experiment showed OMV1c is relatively rare in the *O. novo-ulmi* population at the Winnipeg disease front.

We hypothesized that 93-1224 could potentially carry additional viruses. Further support for the potential presence of multiple viruses in *O. novo-ulmi* 93-1224 came from electron microscopy following a virus purification procedure. EM revealed two different types of rod-like structures in the dsRNA infected isolate 93-1224 and one type of rod-like structures in VA30. These appeared to have a helical capsid structure. Since the majority of characterized mycoviruses either are encapsidated isometric particles or occur as unencapsidated RNA elements (Buck, 1986), it was unusual to find a rod-shaped type of viral morphology. Even though flexuous rod-shaped viruses are well known to infect plants, there are few reports of rod-shaped viruses in fungi.

The putative virus-free 93-1224-PVC strain, developed through the serial transfer of hyphal tips, was checked by electron microscopy for the presence of both types of rod-like particles. Neither particle type was found in the 93-1224 PVC. When the colony morphology and growth rate of 93-1224 PVC was compared with its wild-type virus-containing strain 93-1224, the dsRNA containing wild type showed asymmetric colony shape and had a faster growth rate. The putatively virus-free isolate colonies were smaller, with a less dense and more transparent mycelium and appeared to be sectorial. The observed changes in morphology and growth rate of 93-1224-PVC could mean that at least one of the rod-shaped particles (or the presence of high titer of OMV1c) could be beneficial to *O. novo-ulmi*, because when the fungus was repeatedly sub-cultured it grew

more slowly and had an asymmetrically growing mycelium. The mass spectroscopy data together with electron microscopy suggested that the rod-shaped structures had coat protein. The absence of rod-shaped virus-like particles in putatively cured strain 93-1224-PVC strongly suggests that these are not naturally occurring fungal structures.

This study demonstrates the first observation of rod-shaped particles in *O. novo-ulmi*, and may represent the first case of a co-infection of *O. novo-ulmi* by viruses from two distinct families: one of which was characterized as a mitovirus OMV1c from family *Narnaviridae* and others that are not yet defined but could represent encapsidated rod-like viruses. In future experiments the newly characterized mitovirus OMV1c can be transferred to a naïve *O. novo-ulmi* strain to examine possible effects, such as hypovirulence or hypervirulence, without the complications of the hyphal tip transfer protocol. Similarly pairings of 93-1224 to other *O. novo-ulmi* can be conducted to determine the transmissibility of both the “thin” and “thick” rod-like structures. Such a transmission would definitively identify these structures as being viral in nature.

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