University of South Bohemia in České Budějovice

Faculty of Science

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Ph.D. thesis

České Budějovice, 2010

Turnip ringspot virus: differentiation of new species in the *Comovirus* genus

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Koloniuk I., 2010: Turnip ringspot virus: differentiation of new species in the *Comovirus* genus. PhD thesis – 35 pages, University of South Bohemia, Institute of Plant Molecular Biology, České Budějovice, Czech Republic.

Annotation Complete sequences of TuRSV and RaMV isolates are presented. Obtained data support recognition of RaMV and TuRSV as distinct species of the *Comovirus* genus on the basis of molecular and biological criteria.

Prohlašuji, že v souladu s § 47 zákona č. 111/1998 Sb. V platném znění souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou, elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách.

V Českých Budějovicích, dne 18.01.2010

Igor Koloniuk

Financial support:

This work was supported by grant GAČR 522/07/0053 from the Grant Agency of the Czech Republic

List of publications

1. Petrzik, K., & **Koloniuk, I.** (2010). Emerging viruses in the genus Comovirus. *Virus Genes*. doi: 10.1007/s11262-009-0443-1.

2. **Koloniuk, I.,** & Petrzik, K. (2009). Complete genome sequence of turnip ringspot virus. *Archives of Virology*. doi: 10.1007/s00705-009-0511-9.

3. **Koloniuk, I.,** Špak, J., & Petrzik, K. (2008). Turnip ringspot virus recognised on Chinese cabbage in Russia. *European Journal of Plant Pathology*, *122*(3), 447-450. doi: 10.1007/s10658-008-9283-4.

Vyjádření spoluautorů k podílu Igora Koloniuka na přiložených publikacích

1. Petrzik, K., & **Koloniuk, I.** (2010). Emerging viruses in the genus Comovirus. *Virus Genes*. doi: 10.1007/s11262-009-0443-1.
2. **Koloniuk, I.,** & Petrzik, K. (2009). Complete genome sequence of turnip ringspot virus. *Archives of Virology*. doi: 10.1007/s00705-009-0511-9.
3. **Koloniuk, I.,** Špak, J., & Petrzik, K. (2008). Turnip ringspot virus recognised on Chinese cabbage in Russia. *European Journal of Plant Pathology, 122*(3), 447-450. doi: 10.1007/s10658-008-9283-4.

Prohlašujeme, že I.Koloniuk se zásadním způsobem podílel na získání experimentálních dat a na přípravě výše uvedených publikací.

Doc.RNDr. Karel Petrzik, CSc.

Prof. Ing. Josef Špak, DrSc.

Acknowledgments

I am very grateful to my supervisor Karel Petrzik for his support and guidance. I think I was very fortunate to have such nice colleagues who contributed for the pleasant and relaxed atmosphere in the lab. I would also like to thank to many people who entered to my life in České Budějovice.

Thank you very much!

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CONTENTS

Abbreviations

aa	–	aminoacid
APMoV	–	Andean Potato Mottle Virus
BPMV	–	Bean Pod Mottle Virus
CP–L	–	The large capsid protein
CPMV	–	Cowpea Mosaic Virus
CPs	–	The capsid proteins
CP–S	–	The small capsid protein
CPSMV	–	Cowpea Severe Mosaic Virus
nt	–	nucleotide
Pol	–	polymerase
Pro	–	protease
ProPol	–	protease–polymerase region
RaMV	–	Radish Mosaic Virus
RCMV	–	Red Clover Mottle Virus
RdRp	–	RNA dependent RNA polymerase
SqMV	–	Squash Mosaic Virus
TuRSV	–	Turnip Ringspot Virus
UTR	–	untranslated region

INTRODUCTION

The current work deals with the taxonomic status of two groups of virus isolates. One of the groups received the provisionally cumulative name Turnip ringspot virus (TuRSV) from the first published sequence DQ665367.1 in the GenBank database. Initiation of RaMV sequencing found that the RaMV collection at the department of plant virology was comprised of a mix of RaMV– and TuRSV–like isolates. Further analysis confirmed the results of early works on RaMV, which showed a great serological heterogeneity of its isolates (Plakolli & Stefanac, 1976). Later, a serological survey of European RaMV isolates was published in 2000 (Špak & Kubelkova, 2000).

At the beginning of the study, there was not any complete sequence of RaMV in the GenBank database. The work involved complete sequencing of two TuRSV and finishing the RaMV genomic sequence. In addition, the sequences of taxonomically important regions were obtained.

The obtained data allowed for a comparison of RaMV– and TuRSV–like isolates at the nucleotide and aminoacid levels. Unfortunately, the existing criteria for the demarcation of viruses at the species level did not allow for precisely establishing the taxonomic relationship between TuRSV and RaMV.

To investigate that, a cross–protection assay was conducted. The presence of RNAs of both viruses supported the idea that they represent two different species.

Comoviruses

Viruses of the family *Comoviridae* infect plants. The family is composed of three genera *Comovirus*, *Fabavirus*, and *Nepovirus*. The current (2009) taxonomic classification is entirely based on the VIIIth report of the International Committee on Taxonomy of Viruses (ICTV) (Fauquet, Mayo, Desselberger, & Ball, 2005). Cowpea mosaic virus (CPMV), the typical member of the *Comovirus* genus, is one of the most intensively studied plant viruses. A number of works are dedicated to the development of CPMV–based vectors for the expression of foreign peptides and polypeptides, including antigens and antibodies (Portney, Destito, Manchester, & Ozkan, 2009; Sainsbury, Liu, & Lomonossoff, 2009; Steinmetz, Lin, Lomonossoff, & Johnson, 2009). Additionally, studies on the structure and replication of comoviruses were done using CPMV as a model object (Goldbach et al., 1994; Lin & Johnson, 2003; Lomonossoff & Johnson, 1991).

The comoviruses have small icosahedral particles about 30 nm in diameter built from two species of proteins with M_r of about 37 and 23 kDa. Their virions sediment as three components, which are identical in their protein composition but vary in RNA content.

The infectious genome is divided between two molecules of positive–sense ssRNA, the 5′ end of each having a VPg and the 3′ end being polyadenylated (Goldbach & Wellink, 1996). The VPg is not essential for infectivity (Stanley, Rottier, Davies, Zabel, & Van Kammen, 1978). The viral RNAs encode long polyproteins. ORF of RNA1 contains proteins that are involved at replication. ORF of RNA2 encodes structural proteins and proteins required for cell–to–cell movement. Additionally, RNA2 has a second in–frame ORF that encodes replication and the same two structural proteins as the first

ORF. Functional viral proteins are produced by cleavages of the polyproteins by a viral–encoded protease at conserved processing sites (Pouwels, Van Der Krogt, Van Lent, Bisseling, & Wellink, 2002).

The host range of comoviruses is narrow. Eleven of the 15 members of the genus infect plant species of the family *Leguminosae*. APMoV, RaMV, SqMV, and Ullucus virus C (UVC) infect non–leguminous plants (Le Gall et al., 2005). Comoviruses have been shown to be vectored by leaf–feeding beetles in a semi– or non–persistent manner. Also they can be readily transmitted by sap inoculation (Daubert, Bruening, & Najarian, 1978). The infection induces characteristic cytopathological effects in the cytoplasm of infected plants (Francki, Milne, & Hatta, 1985). No tentative species were mentioned in the VIIIth report of ICTV.

Radish mosaic virus group

RaMV is a member of the genus *Comovirus* in the family *Comoviridae*. It is a beetle–transmitted virus that infects cruciferous plants, causing mosaic, ringspots, and leaf crinkling. RaMV was first reported in California by Tompkins (Tompkins, 1939) and was rediscovered later in the same region by Campbell (Campbell, 1964; Campbell & Tochihara, 1969). This crucifer–infecting virus has also been detected in Europe, Iran, and Japan (Farzadfar, Pourrahim, Golnaraghi, Jalali, & Ahoonmanesh, 2004; Koenig & Fischer, 1981; Petrzik, Špak, & Holá, 2005; Tochihara, 1968).

A partial sequence of the RaMV genome was published in 2005 (Petrzik, Špak, & Holá, 2005). After that, the complete genomic sequences of Japanese (in 2007), Californian (in 2008), and Czech (in 2009) isolates were reported (Koloniuk & Petrzik, 2009; Komatsu et al., 2007; Komatsu et al., 2008).

The complete genomic sequence of RaMV–J RNA1 is 6064 nucleotides long, excluding the 3′–terminal poly(A) tail (Fig.1). The sequence begins with UAUUAAAAU, which is the consensus sequence found at the 5′ termini of many comoviruses. Analysis of the nucleotide sequence revealed a single ORF that begins at an AUG codon (nt 340–342) and terminates with a UAA codon (nt 5899–5901). The 5′ untranslated region consists of 339 nt, and the 3′ UTR is 163 nt long. The predicted translation product of the ORF is 1853 amino acids in length with a calculated M_r of 210,469 Da (a 210 kDa protein). The 210 kDa protein encoded by RaMV–J RNA1 showed 42 to 44% amino acid identity to RNA1 polyproteins of other comoviruses (Chen & Bruening, 1992; Chen & Bruening, 1992; Di, Hu, & Ghabrial, 1999; Komatsu et al., 2007).

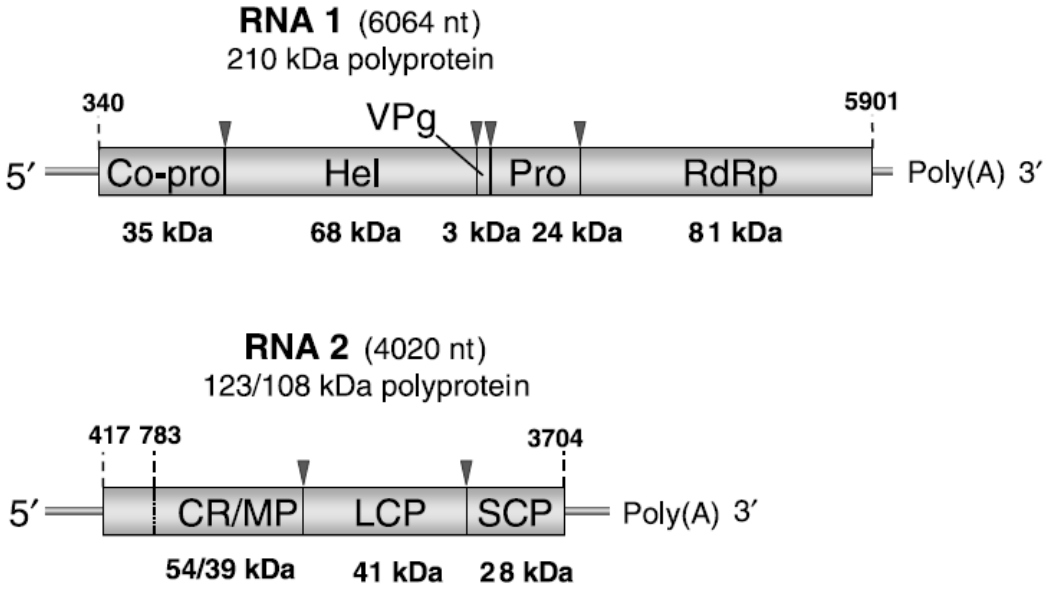

Figure 1. Genome organization of *Radish mosaic virus*. The positions of the first nucleotide of the start codons (including the second start codon of RNA2, which is translated by leaky scanning) and the last nucleotide of the stop codons of each long ORF are indicated above the rectangles. The putative viral proteinase processing sites are indicated by triangles. Deduced M_r values of the corresponding gene products are indicated below RNA1 and RNA2 (from Komatsu et al., 2007).

Turnip ringspot virus group

In 2006, a second comovirus after RaMV in the genus that infects plants of the family *Brassicaceae* appeared in the GenBank with the name *Turnip ringspot virus* (TuRSV, accession numbers: DQ665367, EF191015). The virus was found in Toledo (OH, USA) and did not hybridise with some RaMV–specific primers. The infection resulted in systemic symptoms. They included chlorotic ringspots, line patterns, lesions, and mild stunting (Rajakaruna, Khandekar, Meulia, & Leisner, 2007).

Another isolate M12 was obtained from Chinese cabbage (*Brassica pekinensis*) that was collected in the botanical garden of Moscow State University, Moscow, Russia (Špak & Kubelkova, 2000). The partial sequences of the M12 and Toledo isolates were found to be highly similar (Koloniuk, Špak, & Petrzik, 2008). In 2009, the complete genomic sequence of the Toledo TuRSV isolate was published in the GenBank database (accession numbers NC_013218, NC_013219) (Khandekar, He, & Leisner, 2009).

Virus species concept

Debates have continued for many years about virus species — whether or not they exist and, if they do, how they might be defined. The outcome was the adoption by the ICTV of the following definition which was first proposed by Regenmortel: ‘a virus species is a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche’.

The key feature of the definition is the recognition that the species is a polythetic class; that is a class whose members always have several properties in common, although no single property need be common to all members. No single criterion can be used to assign a virus to a species. Of course, a sufficient similarity in certain criteria might very well indicate membership, but when a

virus has similarity closer to the criterion, other criteria would also be used to assess its classification.

Thus, a list of criteria exists for every genus that would indicate whether or not two viruses belong to the same or different species. List of demarcation criteria in the genus *Comovirus* looks like (Le Gall et al., 2005):

- Large CP amino acid sequence less than 75% homologous;
- Polymerase amino acid sequence less than 75% homologous;
- No pseudo–recombination between components possible;
- Differences in antigenic reactions.

For potyviruses there is a good visual representation of the value of homology and species/isolates differentiation (Fig.2).

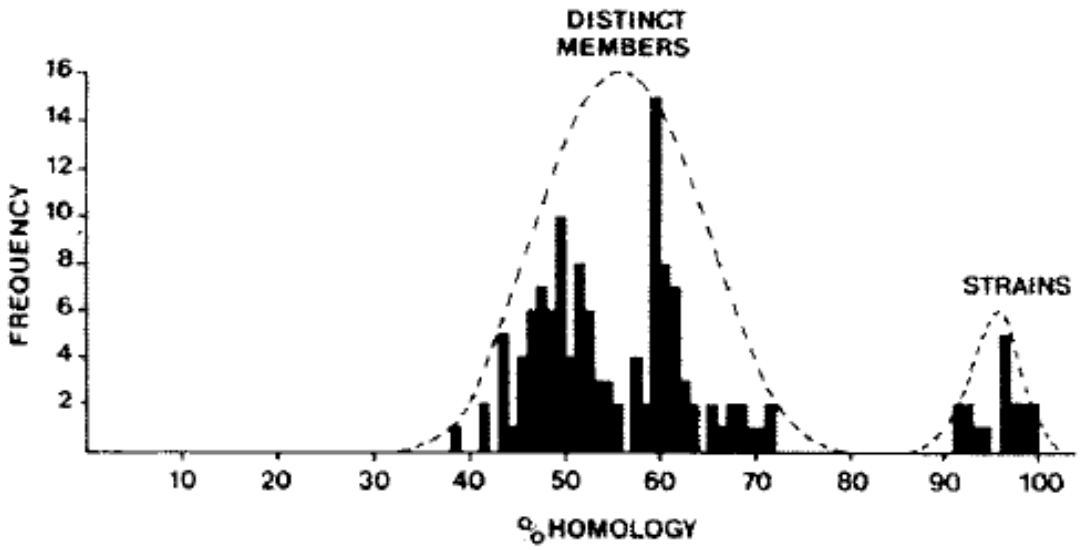

Figure 2. Demarcation between the extents of amino acid sequence homologies in coat proteins amongst distinct individual potyviruses (left–hand distribution) and between strains of the same virus (right–hand peak) (Shukla, Thomas, McKern, Tracy, & Ward, 1988).

In biology, no taxonomy is perfect as it is always an attempt to impose a discontinuous structure onto a naturally dynamic and often continuously varying natural world (Fauquet, Mayo, Desselberger, & Ball, 2005). As a result a classification system is dynamic and is modified and refined to take account of new research findings.

A common problem is to determine whether a new virus is truly a new species or a strain of an existing species. Conversely, what was considered to be a strain may, on further investigation, turn out to be a distinct species. This is because of the population structure of viruses, which due to continuous production of errors in replication, can be considered a collection of quasispecies (Hull, 2009).

Taxonomic confusions in the *Comovirus* genus

There have occurred similar cases in the taxonomy of viruses due to their particular properties. Demarcating species from strains is quite a puzzling task. It is sometimes problematic to determine how different should be two viruses to be recognized as separate species. The more data which are available, then the more precise could be the answer.

In Japan, Tochihara described a virus from radish with many characteristics similar to RaMV but initially referred to it as Radish Enation Mosaic Virus (REMV) (Tochihara, 1968). After a subsequent study showing that REMV and RaMV were serologically indistinguishable, REMV was considered an isolate of RaMV (Campbell & Tochihara, 1969).

The virus, which is now known as *Cowpea severe mosaic virus* (CPSMV), was originally referred to as the severe strain of CPMV (Agrawal, 1964).

Future perspectives of the taxonomy of picorna–like viruses

Recently, two proposals were published which deal with the taxonomy of comoviruses.

The first proposition is about the creation of a new order *Picornavirales* (Le Gall et al., 2008). The order would include viruses infecting plants (*Comoviridae, Sequiviridae, Cheravirus, Sadwavirus*), vertebrates (*Picornaviridae, Caliciviridae*), insects (*Iflavirus*), arthropods (*Dicistroviridae*), and algae (*Marnaviridae*). All of these families and unassigned genera were known and referred to as the picorna–like group. Other distantly related 'picorna–like' viruses (*Potyviridae*, *Caliciviridae*, and *Hypoviridae*) were not proposed as members of the order. However, the authors neither discuss any revisions within the *Comoviridae* family nor changes of demarcation criteria at the level lower than family level.

The other proposal is for merging the *Sequiviridae* and *Comoviridae* families together with the unassigned *Cheravirus* and *Sadwavirus* genera into a new family *Secoviridae* (Sanfaçon et al., 2009). Additionally, a new genus *Torradovirus* within the mentioned family was proposed to be established. The family *Comoviridae* would be converted into subfamily *Comovirinae*, preserving the existing genera. The criteria defining species within the *Torradovirus* genus were considered by analogy with criteria established for other known genera within the proposed family *Secoviridae*:

- Less than 75% aa sequence identity in the CPs
- Less than 80% aa sequence identity in the proteinase–polymerase region
- Type of biological vector
- Host range
- Absence of serological cross–reaction
- Absence of cross–protection

There were several changes and additions in comparison with criteria established in the VIIIth report of the ICTV (Le Gall et al., 2005). First, all CPs are subjected to sequence analysis. Capsid proteins are organized in a module containing three related jelly–roll domains. It is quite obvious, as most taxa have three CPs, that *Comovirus*, *Fabavirus*, and *Sadwavirus* have two CP and some members of the *Nepovirus* genus have a single CP. Second, instead of the polymerase sequence, the proteinase–polymerase region was proposed for comparison (however, it does not include the complete protease and polymerase sequences, but parts of them). Third, absence of pseudo–recombination is replaced by absence of cross–protection between distinct species.

Sequential infection by related viruses can lead to the second virus being suppressed. Thus, the infected plant should be immune against the challenging strain or isolate. This has been termed cross–protection (Hull, 2009). Cross–protection has been utilized to protect crops against highly virulent strains of viruses (Prins, 2003; Sudarshana, Roy, & Falk, 2007). The most rational explanation of the phenomenon is the RNA silencing mechanism (Soosaar, Burch-Smith, & Dinesh-Kumar, 2005).

Materials

The isolates used in this study (Tab.1) were obtained as fresh or freeze–dried infected leaves from relevant researchers, or isolated earlier from symptomatic plants. The virus isolates were maintained on *Sinapis alba* and *Nicotiana glutinosa* plants using mechanical inoculation. Samples were stored at –20˚C until RNA extraction.

Table 1. Virus isolates used in the study.

Group	Abbreviation	Geographic origin
RaMV	RaMV1	Czech Republic
	CB1	Czech Republic
	CB9	Czech Republic
	PV0306	USA
TuRSV	M12	Russia
	M92	Russia
	CH210 (B)	Croatia
	CH246	Germany
	CH247	UK
	CH250	Italy
	CH594	Croatia
	HZ117	Croatia
	Toledo	USA
	Vl98	Russia

RNA extraction and cDNA synthesis

Total RNA was extracted from infected leaves using the RNeasy® Plant Mini kit (Qiagen, Germany) following the manufacturer's protocol and stored at –20˚C.

cDNA was synthesized by reverse transcription (RT) using the iScript cDNA Synthesis kit (Bio–Rad, USA) or using a gene–specific primer and MonsterScript Reverse Transcriptase (Epicenter, Technologies, USA) following manufacturers' instructions. The RT was performed in a total volume of 20 µl. 4

µl total RNA was used in each RT reaction. In the first step of the gene–specific RT, 4 µl of the isolated RNA, 4 µl of 5× first strand buffer, 1 µl (5 pM) of a gene–specific primer, and 9 µl of nuclease–free water were mixed and denatured at 70˚C for 5 min in a thermocycler with heated lid and then chilled on ice for 5 min. To this mixture, 0.5 µl (40 U/µl) of RiboLock™ RNase Inhibitor (Fermentas, Canada), 1 µl (50 U/µl) of MonsterScript Reverse Transcriptase, and 0.5 µl of 10mM dNTPs were added. The reaction was incubated at 42˚C for 5 min and then at 45–60˚C (depending on the T_m of the used primer) for 40 min. The synthesized cDNA was stored at –20˚C.

PCR

PCR assays were carried out using PPP Master Mix (Top–Bio, Czech Republic), Long PCR Enzyme Mix (Fermentas, Canada), and the Advantage® 2 PCR Enzyme System (Clontech, USA). Amplification of fragments with expected size up to 1.5 kbp was performed using PPP Master Mix, while longer fragments were amplified with Long PCR Enzyme Mix. Advantage® 2 PCR Enzyme System was successful in the 5′ RACE reaction. Usually, 1/10 (2 µl) of the cDNA reaction was taken to the PCR. The final concentration of $MgCl_2$ was 1.5 mM, while for each primer it was 0.5–1 µM. The cycling parameters were set up according to the T_m of the primers and the polymerase type used in the reaction. The first denaturation time was 95˚C for 1 min. The denaturation time in each cycle was 30 sec at 95˚C. The annealing time was 30 sec. The extension time was equal to the expected target size — 1 min for every 1 kb. Not more than 35 cycles were set up.

Molecular cloning

The obtained DNA fragments were cloned using the CloneJET™ PCR Cloning Kit (Fermentas, Canada) or the TOPO TA Cloning® Kit for Sequencing

with pCR®4 vector (Invitrogen, USA). Mach1™T1R, TOP10, TOP10F′ (Invitrogen, Carlsbad, USA), and NEB 10–beta and NEB Turbo (New England Biolabs, USA) competent cells were used in the cloning procedures following the protocols of the manufacturers.

Real–time reverse transcription PCR assay

Real–time reverse transcription PCR (RT RT–PCR) assays were carried out in iCycler thermal cycler (Bio–Rad, USA). Two-step approach was chosen.

Universal pair of primers, which flank the 176 bp DNA region coding part of the CP–L, and two fluorescent, group–specific to TuRSV and RaMV probes were designed (Tab. 2). For the best output, the probes were labeled at their 5′ end by FAM or HEX dye, which were different in excitation and detection wave lengths. BHQ–1™ was used as the 3′ end quencher. The reaction mixture contained 1 µl of cDNA, 2 µl 10× 10X DreamTaq™ Buffer (Fermentas, Canada), 0.5 µl dNTP mixture (10mM), 0.5 µl (5U/µl) DreamTaq™ DNA Polymerase (Fermentas, Canada), 4 µl of primers (2 µM each), and 0.5 µl of each specific hydrolysis probe (5 µM). Sterile deionized water was added to a final volume of 20 µl. The cycling parameters were: 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. For each cycle, fluorescence readings were performed at the 60°C step. To ensure the absence of contaminants, each run included a negative (water) control. If required, additional positive controls were also included.

Table 2 Fluorescent probes and universal primers

The content of the table is removed in this version of the thesis.

For a comparative quantification RT-PCR with SYBR Green was carried out. Plant ribosomal 18S RNA was used as a reference gene. The TuRSV– and RaMV–specific primer sets were described earlier (Kasalova, 2008). The reaction volumes and concentrations were not changed. The comparative C_t method ($2^{-\Delta\Delta Ct}$ method) was used to calculated relative concentrations of TuRSV and RaMV in the cross–protection assay.

Analysis of amplified products

Aliquots (2–30 μl) of the products were electrophoresed in 1.5–2% agarose gels in 0.5× TBE buffer for 15–25 min at 100 V and stained with ethidium bromide. The gels were visualized by ultraviolet light illumination at 312 nm. Sizes of fragments were determined by comparison with Low, Middle, and High Range FastRuler™ DNA Ladder molecular weight marker sets (Fermentas, Canada). The marker set was chosen according to the expected product size.

Amplified fragments were purified from the agarose gels with the NucleoSpin® Extract II kit (Macherey–Nagel, Germany).

Virus specific primers

Highly conserved regions were determined from sequence alignments of all available sequences from the GenBank of the National Center for Biotechnology. The alignments were carried out with the program ClustalW and Muscle (Edgar, 2004; Larkin et al., 2007). Primers were designed following general recommendations (avoid primer–dimers, hairpins, and self–complementary sequences).

Cross–protection assay

The scheme of the assay is shown in Fig.3. Altogether, 56 plants of *Sinapis alba* were used. The Czech isolate RaMV1 and the Croatian isolate of TuRSV–CH210 were used. One of the limitations to cross–protection in studying taxonomic relationships includes the fact that in some pairs of related virus isolates, cross–protection operates in one order of inoculation, but not the reverse (Fulton, 1978). To overcome this, the inoculations were done in two orders simultaneously: TuRSV→RaMV and RaMV→TuRSV. Homogenized in 0.1 M phosphate buffer tissues of positively tested plants were used for mechanical inoculation. To increase the efficacy, abrasive addition was used. The second inoculation was done 14 days after the first one. Several samples from each plant were collected at 30 days after the first inoculation. To avoid false–positive results, non–inoculated leaves were taken for RNA isolation.

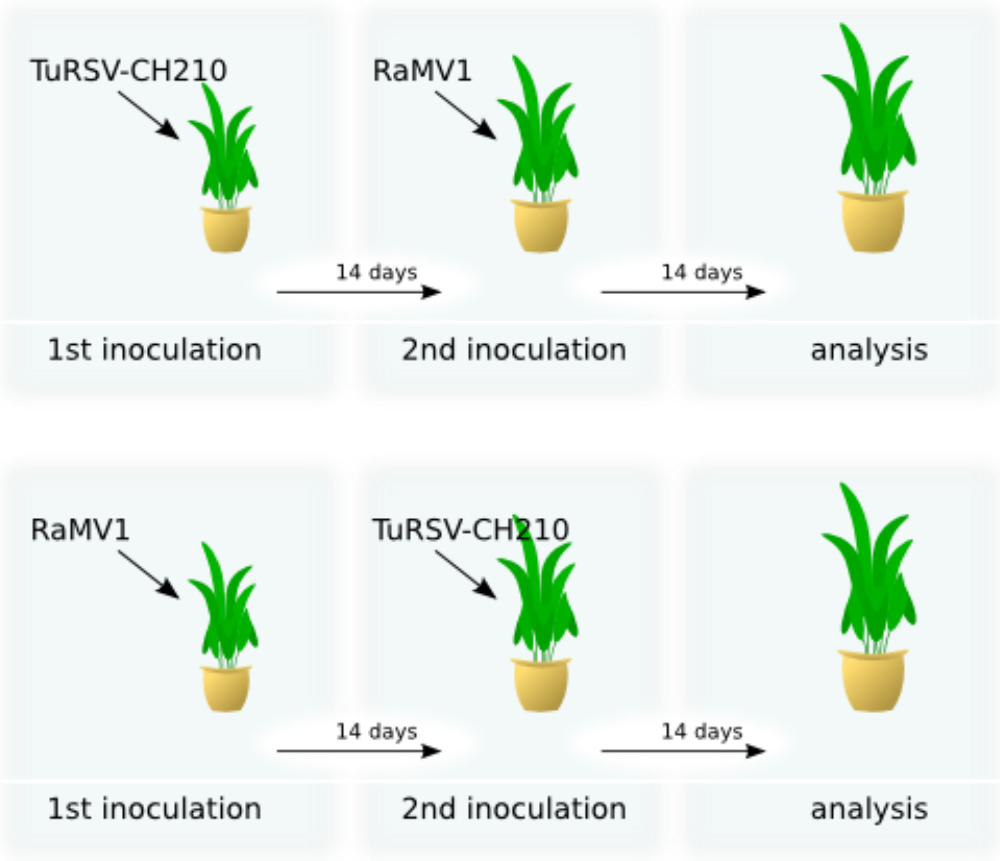

Figure 3. The scheme of cross–protection assay.

Data analysis

All sequences obtained from GenBank database and used in the present study are listed in Tab. 3. The similarity/identity matrix generator computer program MatGAT (Matrix Global Alignment Tool) v.2.02 was used for comparing pairwise nt and aa identities of discrete protein–coding regions or the whole genomes (Campanella, Bitincka, & Smalley, 2003). Standard deviation was calculated using the STDEV.P function in Microsoft Excel v14.0. Average identity for the genus was calculated using only one isolate/strain per species.

Multiple alignments were produced using MUSCLE (Edgar, 2004). Phylogenetic analyses were conducted in MEGA4 (Tamura, Dudley, Nei, & Kumar, 2007). The evolutionary history was inferred using the Minimum Evolution (ME) method (Rzhetsky & Nei, 1992). The optimal trees are shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckercandl & Pauling, 1965) and are in units of the number of amino acid substitutions per site. The ME tree was searched using the Close–Neighbor–Interchange algorithm (Nei & Kumar, 2000) at a search level of 3. The Neighbor–joining algorithm (Saitou & Nei, 1987) was used to generate the initial tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

Table 3. Sequence accession numbers from GenBank database, used in the work.

Sequence name, type	Accession number
BPMV RNA1	NC_003496.1
BPMV RNA2	NC_003495.1 AY744933.1
BPMV K–Hopkins1 RNA1	AF394608.1
BPMV K–Hopkins1 RNA2	AF394609.1
BPMV K–Hancock1 RNA1	AF394606.1
BPMV K–Hancock1 RNA2	AF394607.1
BPMV subgroup I RNA1	AY744931.1
BPMV subgroup II RNA1	AY744932.1
BPMV RNA2	AY744933.1
TuRSV Toledo RNA1	FJ712026.1
TuRSV Toledo RNA2	FJ712027.1
TuRSV B RNA1	GQ222381.1
TuRSV B RNA2	GQ222382.1
TuRSV M12 RNA1	FJ516745.1
TuRSV M12 RNA2	FJ516746.1
RaMV1 RNA1	EU450837.1
RaMV1 RNA2	EU450838.1
RaMV–J RNA1	NC_010709.1
RaMV–J RNA2	NC_010710.1
RaMV–Ca RNA1	AB456531.1
RaMV–Ca RNA2	AB456532.1
RCMV RNA1	NC_003741.1
RCMV RNA2	NC_003738.1
CPMV RNA1	NC_003549.1
CPMV RNA2	NC_003550.1
SqMV RNA1	NC_003799.1
SqMV RNA2	NC_003800.1
SqMV CH 99/211 RNA1	EU421059.1
SqMV CH 99/211 RNA2	EU421060.1
SqMV Kimble RNA2	AF059533.1
SqMV Arizona RNA2	AF059532.1
CPSMV RNA1	NC_003545.1
CPSMV RNA2	NC_003544.1
APMoV CDS RNA2	L16239.1
APMoV polymerase	M84483.1

Descriptions of the TuRSV–B&M12 genomes

Characteristics and comparisons of genomic sequences of TuRSV–M12 and CH210(B) against RaMV isolates were reported and discussed in the article "Complete genome sequence of turnip ringspot virus" (Koloniuk & Petrzik, 2009); see the supplements.

Comparison of intra– and interspecies variability

Whole RNAs and polyproteins comparison

Average interspecies nt identity values in the *Comovirus* genus were about 55% (RNA1) and 51% (RNA2). However, intragroup nt identities ranged from 84 to 98% (RNA1) and from 82 to 91% (RNA2) (assuming that TuRSV is a discrete species). TuRSV and RaMV are the closest in the genus. They share 67.7±0.2% of the overall nt identity in RNA1 and 64.2±0.4% in RNA2.

Intragroup TuRSV and RaMV aa identity levels were 95.6±2.3% and 93.3±1.5% in polyproteins encoded by RNA1 and RNA2, respectively. This is similar to the comparison with BPMV isolates. They shared more than 95% (RNA1) and 92% (RNA2) of aa identity. Lower values, ranging from 65±0.3% (RNA2) to 73.2±1.5% (RNA1), were estimated in the comparison of RaMV and TuRSV. However, these values were higher than the average identity between species of the genus. They shared only 46% (RNA1) and 37% (RNA2) of aa identity.

The distribution of the identity values over the whole genomes of RaMV–Ca and TuRSV–CH210 is shown in Figure 4. Changes are spread more or less evenly. The most variable are both N– and C–ends of the polyprotein coded by RNA2.

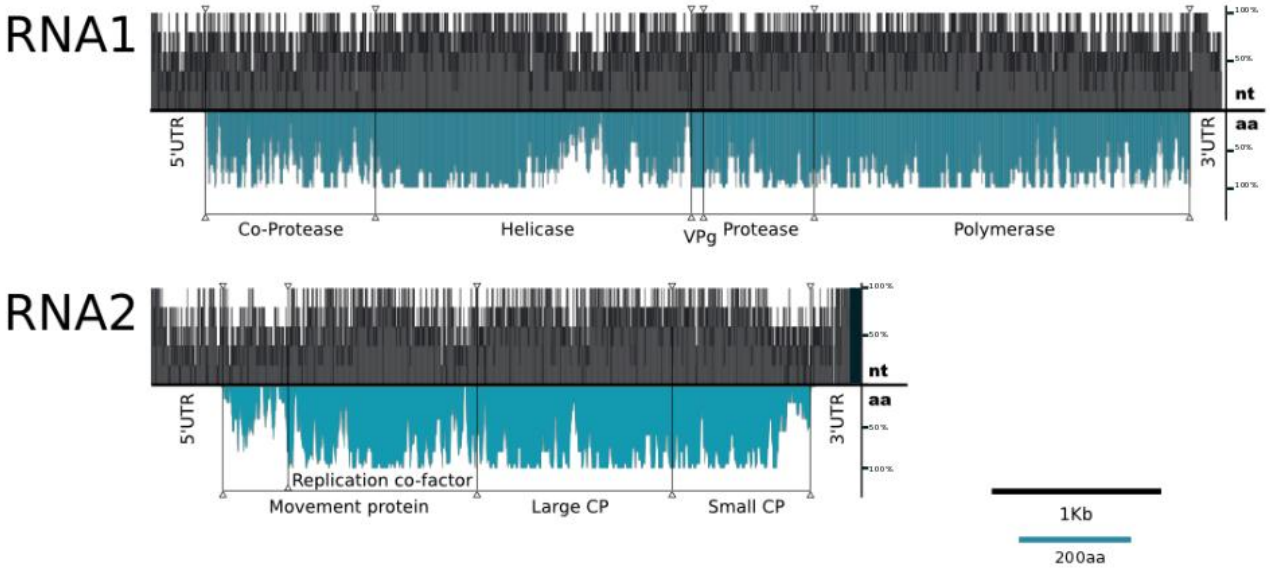

Figure 4. Schematic distribution of nt (dark grey) and aa (light blue) identities for TuRSV–CH210(B) and RaMV–Ca.

Table 4. Comoviruses CP–L aa identities (%). Conditional formatting was applied. The color bar is under the table.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1. APMoV		36.1	36.3	36.1	36.1	35.1	29.7	35.8	36.7	36	38.8	37.3	37	37	38.3	34.9	34.4	36.1	34.4	34.7	33.8	34.1	34.4
2. BPMVa			99.2	99.2	99.5	55.9	55.9	45.6	44.9	45.5	57.9	56.7	57	57	57	46.7	46.7	45.9	46.9	45.3	45.6	46.1	46.4
3. BPMVn				98.9	99.2	55.9	55.6	45.6	44.9	45.5	57.9	56.4	56.7	56.7	56.7	46.7	46.7	45.9	46.9	45.3	45.6	46.1	46.4
4. BPMV K–Hancock					99.7	55.9	55.6	45.6	44.9	45.5	57.9	56.1	56.4	56.4	56.4	46.7	46.7	45.6	46.9	45.3	45.6	46.1	46.4
5. BPMV K–Hopkins						55.9	55.9	45.6	44.9	45.5	57.9	56.4	56.7	56.7	56.7	46.9	46.9	45.9	47.2	45.6	45.9	46.4	46.7
6. CPMV							49.5	43.7	43.5	43.9	54.6	49.7	48.9	49.5	50	44.4	43.9	44	43.9	44.1	43.9	43.9	44.9
7. CPSMV								44.1	43.9	43.6	49.3	49.7	49.5	49.7	49.7	43.7	43.7	43.9	43.7	43.5	42.4	42.9	43.5
8. RaMV–Ca									98.4	97.6	44	47.6	47.1	47.1	47.1	80	79.5	79.8	79.7	79.5	77.9	78.9	79.5
9. RaMV–J										97.1	44	47.9	47.3	47.3	47.3	78.9	78.4	79.3	78.7	78.9	76.8	77.9	78.4
10. RaMV1											44.2	47.2	46.7	46.7	46.7	79.5	78.9	79.5	79.2	78.9	77.3	78.4	78.9
11. RCMV												53.6	53.8	54.1	54.4	46	46	44.2	46.3	45.8	46	45.8	46.6
12. SqMV Kimble													96.3	96.5	98.4	49.9	49.9	50.1	50.1	49.9	49.1	49.9	50.1
13. SqMV CH99/211														98.7	96.8	49.6	49.6	49.9	49.9	49.6	48.8	49.6	49.9
14. SqMV Arizona															97.1	49.6	49.6	49.9	49.9	49.6	48.8	49.6	49.9
15. SqMV																48.8	48.8	49.1	49.1	48.8	48	48.8	49.1
16. TuRSV–246																	98.4	93.9	98.7	96.3	96.5	97.3	98.1
17. TuRSV–247																		92.8	99.5	95.7	96	96.8	97.6
18. TuRSV–250																			93.1	93.1	91.5	92.3	93.1
19. TuRSV–594																				96	96.3	97.1	97.9
20. TuRSV–CH210																					94.4	95.2	96
21. TuRSV–M12																						97.6	97.9
22. TuRSV–Vl98																							98.7
23. TuRSV–Toledo																							

30 40 50 60 70 80 90 100

Table 5. Comoviruses polymerase aa identities (%). Conditional formatting was applied. The color bar is under the table.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1. APMoV		46.6	48.6	48.3	48.5	48.5	48.7	47.9	49.4	49.6	49.6	49.2	49.1	48.7	49.4	49.9	49.7	49.7	49.9	49.9	49.9	49.7
2. BPMV			94.7	96.2	96.2	94.5	56.4	54.7	54.1	54.1	53.9	56.3	51.7	51.1	53.8	54.2	54.6	54.2	53.8	53.8	54.1	54.3
3. BPMV K–Hancock				97.7	97.7	99.7	56.9	55.7	55.3	55.3	55.2	57.1	52.4	52.4	55	55.5	55.6	55.5	55	55	55.3	55.5
4. BPMV K–Hopkins					99.4	97.6	57.1	55.8	55.1	55.1	54.9	57.1	52.2	52.2	54.6	55	55.5	55	54.6	54.6	54.9	55
5. BPMVsbgI						97.6	57.1	55.8	55.1	55.1	54.9	56.9	52.2	52.2	54.5	55	55.5	55	54.6	54.6	54.9	54.9
6. BPMVsbgII							56.7	55.6	55.2	55.2	55.1	56.8	52.2	52.2	54.9	55.3	55.5	55.3	54.9	54.9	55.2	55.3
7. CPMV								53.9	55.6	55.7	55.1	61.4	56.4	56.1	55	56	55.8	56	55.7	55.8	56	55.1
8. CPSMV									55	55	55.4	51.2	53.7	53.3	53.7	54.6	54.6	54.6	54.5	54.8	54.8	54
9. RaMV–Ca										98.2	95.8	55.8	54	53.3	75	76	74.9	76	75.7	76	75.8	75.4
10. RaMV–J											95	55.8	54.3	53.6	74.9	76.4	75.3	76.4	76.1	76.4	76.3	75.3
11. RaMV1												55.7	54.4	53.7	74.6	75.8	75	75.8	75.6	75.8	75.7	75.1
12. RCMV													54.5	54.5	54.9	55.3	55.7	55.3	55.3	55.3	55.3	55.2
13. SqMV														97.3	52.2	53.1	53.7	53.1	53.1	53.3	53.3	52.6
14. SqMV CH99/211															51.5	52.3	52.9	52.3	52.3	52.4	52.4	51.9
15. TuRSV–246																92.7	92.7	92.7	93.1	92.8	93	99
16. TuRSV–247																	95.5	99.7	99.3	99.4	99.4	93.1
17. TuRSV–250																		95.2	95.4	95.6	95.5	92.7
18. TuRSV–594																			99	99.2	99.2	93.1
19. TuRSV–Cr210																				99.3	99.6	93.5
20. TuRSV–M12																					99.4	93.3
21. TuRSV–Vl98																						93.4
22. TuRSV–Toledo																						
						30	40	50	60	70	80	90	100									

Individual proteins

CP–L

Average value of aa identity in TuRSV group was 96±2.2% and 97.7±0.5% for RaMV (Table 4). Intragroup identity was 78.9% and ranged from 77% to 80%. BPMV isolates shared 99.3±0.3% of aa identity. However, the values of interspecies identity were far lower between other members of the *Comovirus* genus. The most distant was APMoV, which shared only 29–38% of aa identity with other comoviruses, while they share from 44% to 59%. The CP–L aa sequence of SqMV–K and –Z isolates had similarity of 96.5% (Haudenshield & Palukaitis, 1998).

RdRp

RaMV and TuRSV shared 75.6±0.5% identity for the aa polymerase sequence. Intragroup values were 96.3±1.4% for RaMV and 95.8±2.8% for TuRSV. Similarly, BPMV and SqMV isolates showed 97.1±1.7% and 97.3% of identity, respectively. The polymerase of APMoV was 49% identical with those of other comoviruses and thus is the most distant in the genus. Average identity of the polymerase between the genus species is about 53% (Table 5).

Polymerases of TuRSV–246 and Toledo isolates differed from the rest of the TuRSV isolates. There were several changes in the shared I, VII, and VIII conserved motifs (Fig.5).

CP–S

The small capsid protein is more variable compared with the CP–L. Intergroup aa identity between RaMV and TuRSV was 65.1±0.9%. TuRSV,

RaMV, SqMV, and BPMV isolates showed 93.2±3.8%, 89.9±0.5%, 97.4±1.3% and 96.6±2.4% of aa identity, respectively.

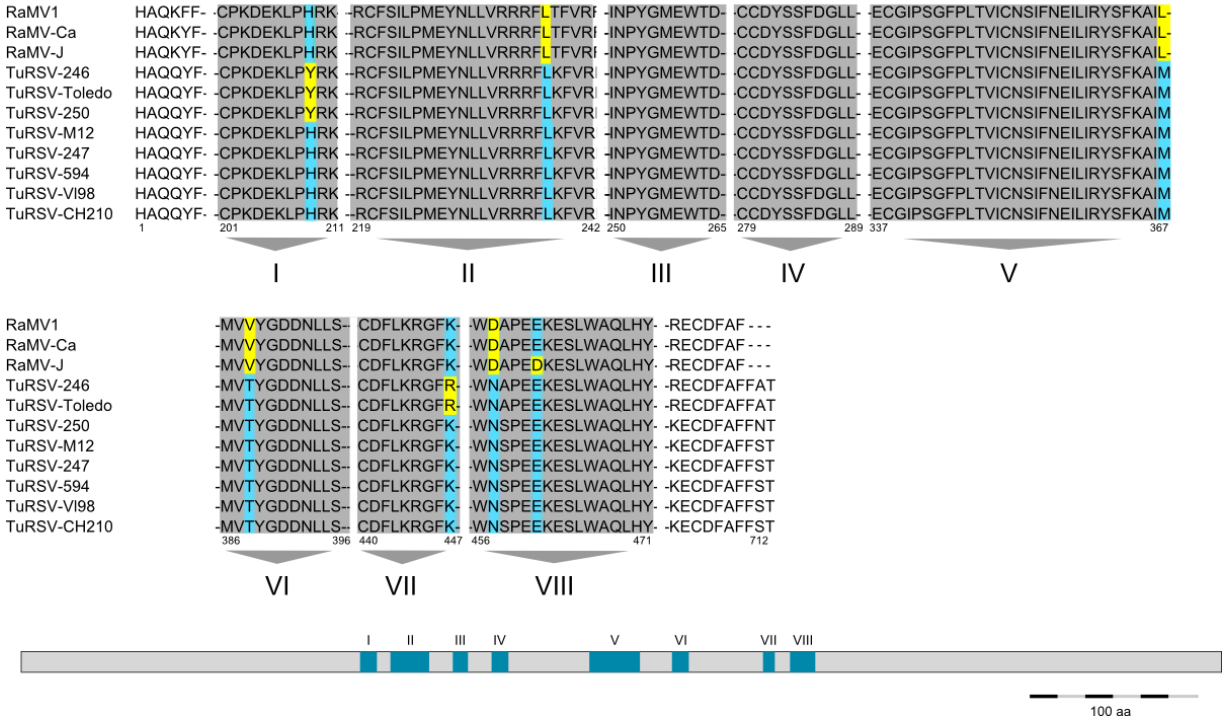

Figure 5. Multiple alignment of I–VIII conserved motifs of positive–strand RNA viral polymerases (Koonin, Choi, Nuss, Carrington, & Shapira, 1991). All other parts of the alignment except the start and the end were manually removed. Below is the scheme of motifs positions.

Design of RaMV– and TuRSV–specific fluorescent probes and using them in duplex RT–PCR

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Phylogenetic analysis

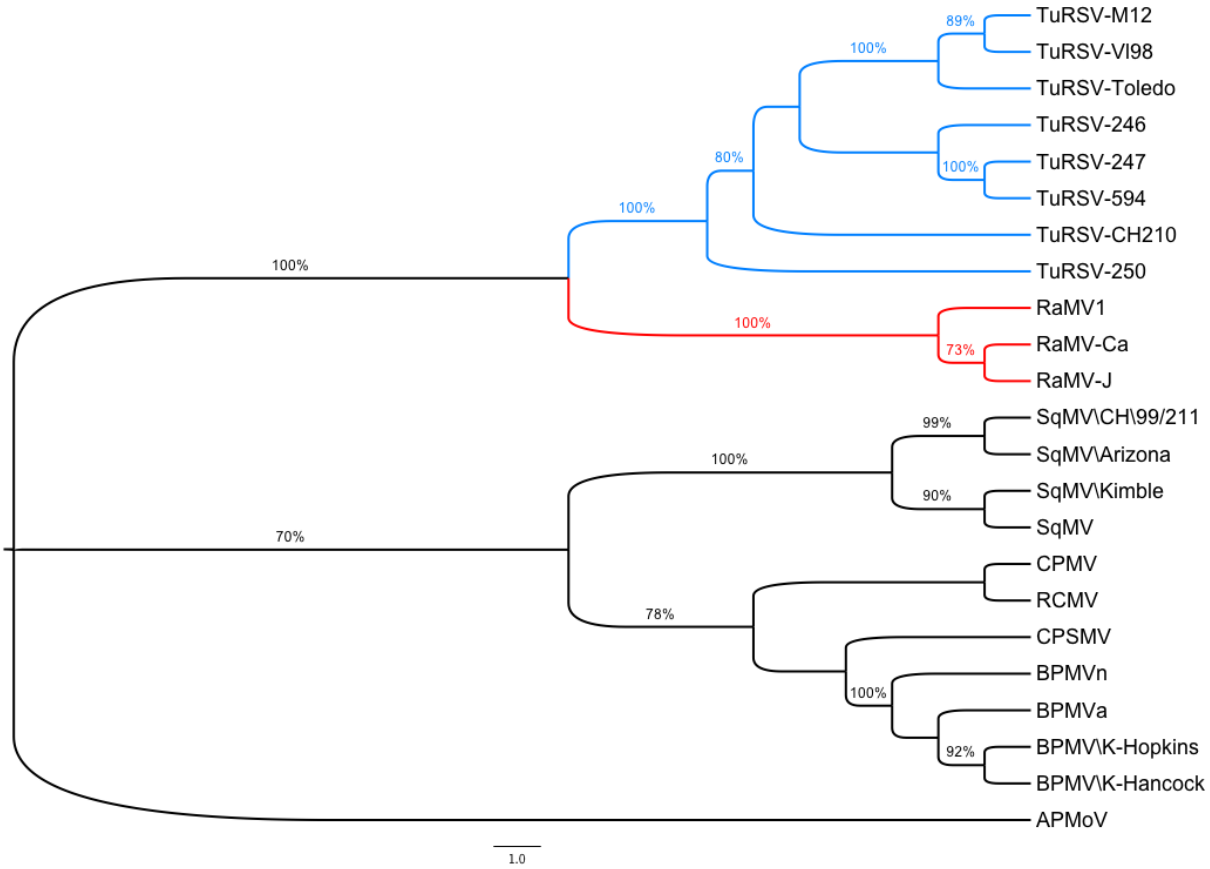

Figure 6. Evolutionary relationships of 19 taxa based on aa CP–L sequences. Description of the analysis is in section 'Materials and methods'. Abbreviations are given as in the text. Bootstrap values less than 50% are ommited.

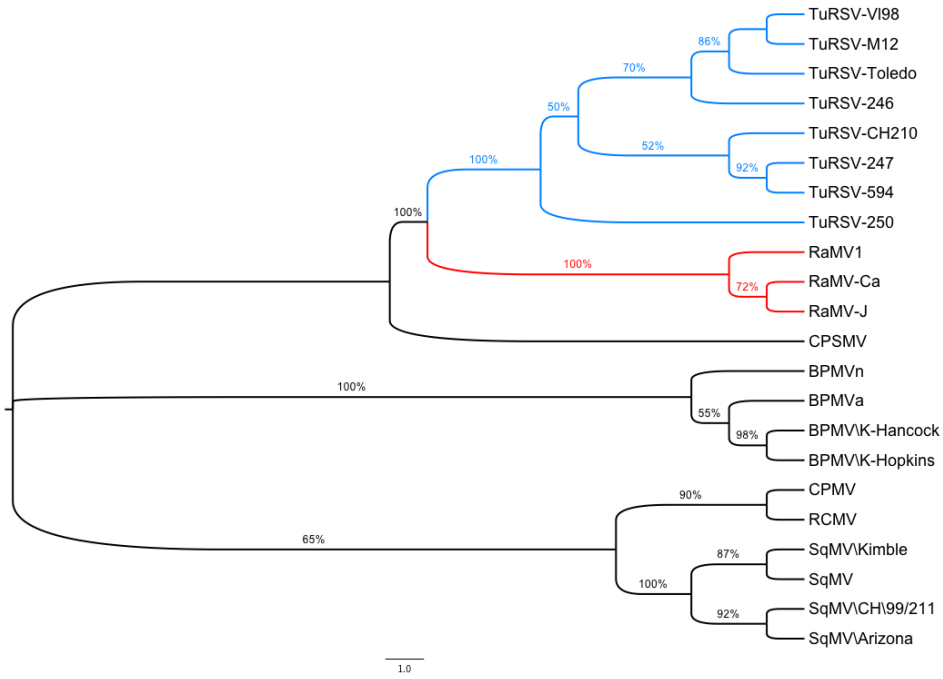

Figure 7. Evolutionary relationships of 18 taxa based on aa CP–S sequences. Description of the analysis is in section 'Materials and methods'. Abbreviations are given as in the text. Bootstrap values less than 50% are ommited.

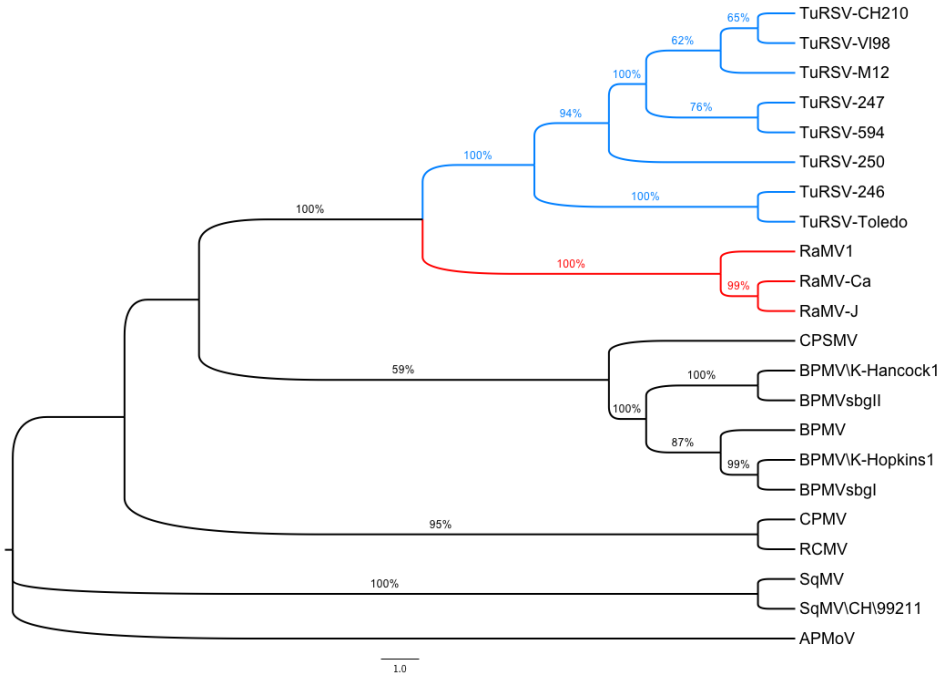

Figure 8. Evolutionary relationships of 22 taxa based on aa polymerase sequences. Description of the analysis is in section 'Materials and methods'. Abbreviations are given as in the text. Bootstrap values less than 50% are ommited.

Reconstruction of the phylogeny, using CP–L, CP–S, and polymerase aa sequences, divides TuRSV and RaMV isolates to different branches (Figs.6,7,8). As might be expected, they form a group that is distinct from other comoviruses. TuRSV–M12 and Vl98 isolates are clustered together in all three trees. Trees, based on both CPs aa sequences, have the same topology for all TuRSV isolates. A different grouping is observed in the tree based on polymerase aa sequences. TuRSV–246 and Toledo isolates are clustered together and are located as the most distant isolates in the whole TuRSV group.

Cross–protection

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Conventional PCR with specific primers was also used. The amplified fragments were purified, sequenced, and identified by pairwise alignment against TuRSV and RaMV sequences. Using this approach, specific regions of RaMV1 and TuRSV–CH210 were detected.

Possibility of inclusion of other criteria to species demarcation in the Comovirus genus

Much useful information might be also obtained by comparing nt sequence homologies between the various comoviruses and their strains and isolates.

The criteria, which were proposed for species demarcation in the *Secoviridae* family, have several significant differences from those, used in the VIIIth report of the ICTV. The older criteria do not allow precise distinguishing TuRSV and RaMV as different species using only sequences comparison. However, changing comparison targets, i.e. protease–polymerase (ProPol) region instead of the polymerase sequence and both CPs instead of the CP–L, allows for a clear discrimination of the studied viruses.

The ProPol sequence, 488aa long, between the Pro main motif and Pol motif VI is used for sequence identity estimation (Le Gall et al., 2008). The results of the analysis of the region for RaMV and TuRSV isolates are presented in Table 6.

Table 6. Comoviruses ProPol region aa identities (%). Conditional formatting was applied. The color bar is under the table.

	1	2	3	4	5	6	7	8	9	10	11
1. RaMV–CA		98.6	96.3	79.2	80	80	78.6	79.6	80	79.6	80
2. RaMV–J			96.1	78.8	80.4	80.4	78.2	80	80.4	80	80.4
3. RaMV1				78.8	80	80	78.2	79.6	80	79.6	80
4. TuRSV–Toledo					95.1	95.1	99	95.3	94.9	95.5	95.1
5. TuRSV–M12						100	94.5	98	99.8	99.6	100
6. TuRSV–247							94.5	98	99.8	99.6	100
7. TuRSV–246								95.1	94.3	94.9	94.5
8. TuRSV–250									97.7	97.5	98
9. TuRSV–594										99.4	99.8
10. TuRSV–CH210											99.6
11. TuRSV–Vl98											
		70	80	90	100						

RaMV and TuRSV shared 79.6 ±0.62% of aa sequence in the ProPol section. Some isolates showed 80.4% of intergroup (RaMV–J vs. TuRSV–M12,–247,–594,–Vl98) aa identity in the ProPol region. However, that is exactly on the edge, as the proposed criterion is 80% (Sanfaçon et al., 2009). TuRSV–M12, –247, and –Vl98 shared 100% of the identity in the ProPol aa sequence.

Different results were obtained after analysis of both CPs of TuRSV and RaMV (Table 7). As was shown, CP–S is more variable than CP–L. The average aa identity value of both CPs was 73.3±0.71%. This is below the proposed demarcation value of 75%. For more information, see "Emerging viruses in the genus *Comovirus"* (Petrzik & Koloniuk, 2010) (supplement 1).

Table 7. Comoviruses CPs region aa identities (%). Conditional formatting was applied. The color bar is under the table.

	1	2	3	4	5	6	7	8	9	10	11
1. RaMV–1		94.7	94.2	74	73.5	74.2	73.8	73	72.5	73.1	73
2. RaMV–CA			95.3	74.5	73.4	74.3	73.7	73.1	72.9	73.8	73.5
3. RaMV–J				74.4	73.2	73.7	72.7	72.9	71.6	72.6	72.2
4. TuRSV–246					97.3	91.2	97.1	94.6	96	97.1	96.6
5. TuRSV–247						91.1	98.1	94.7	95.5	96.6	96.2
6. TuRSV–250							90.8	90.6	89.6	91.4	90.3
7. TuRSV–594								94.6	97.3	96.8	97.6
8. TuRSV–CH210									93.1	93.9	93.8
9. TuRSV–M12										97.3	98.4
10. TuRSV–Toledo											97.9
11. TuRSV–Vl98											
			70	80	90	100					

Table 8. Comparison of intra– and interspecies identity (%) of comoviruses including the RaMV/TuRSV group.

group	RNA1		RNA2		CPL	CPS	Both CPs	Pol	ProPol
	nt	aa	nt	aa	aa				
Comoviruses without TuRSV	55.8	45.6	51.7	38.6	48.7	35.2	43.1	55.2	61.5
TuRSV/RaMV	67.7	73.2	64.2	65	78.9	65.1	73.3	75.6	79.7
RaMV	87.9	96	83.5	93.1	97.7	89.9	94.7	96.3	97
TuRSV	88.3	95.2	85.9	93.4	95.9	93.2	94.8	95.9	97.3
BPMV	90.8	97.5	90.5	98	99.3	96.8	98.4	97.1	97.4
SqMV	89.1	96.7	89.9	91.9	97.3	97.4	97.3	97.3	97.5

1. Complete genomic sequences of RaMV1 and TuRSV–M12, –CH210 were obtained. The polymerase, ProPol, and CPs regions of TuRSV–246, –247, –250, –594, –Vl98 isolates were sequenced.
2. Taking into account the large number of identity values, comparison data are gathered in Table 8. Intra–group diversity of TuRSV is similar to that observed for RaMV, BPMV, and SqMV. Nevertheless RaMV and TuRSV are more similar than any other comoviruses; their intra–group identity is distinctly lower than intra–species numbers.
3. RaMV and TuRSV form a separate lineage regardless of the gene sequence used for phylogenetic analysis (CP–L, CP–S, or Pol)
4. The obtained data are in agreement with the proposal (Sanfaçon et al., 2009) to use (a) the ProPol region, (b) combined CPs to the taxonomic analysis, (c) 80% of ProPol and 75% of CP aa identities as species demarcating criteria.
5. Cross–protection between RaMV1 and TuRSV–CH210 isolates was absent. However, TuRSV showed a higher speed of accumulation, possibly due to the unequal concentrations of the inoculums.

6. Thus, TuRSV can be distinguished as a distinct species in the *Comovirus* genus on the basis presented molecular and biological criteria.

7. RaMV– and TuRSV–specific Taqman probes were designed for further identification/detection analyses.

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SUPPLEMENTS

1. Petrzik, K., & **Koloniuk, I.** (2010). Emerging viruses in the genus Comovirus. *Virus Genes*. doi: 10.1007/s11262-009-0443-1.

Virus Genes
DOI 10.1007/s11262-009-0443-1

Emerging viruses in the genus Comovirus

Karel Petrzik · Igor Koloniuk

Received: 1 June 2009 / Accepted: 21 December 2009

Abstract The intraspecies variability of capsid proteins of five viruses of the genus Comovirus was established. Inclusion of both capsid proteins to the sequence analysis reduces some uncertainties about species/strains demarcation criteria in the Comovirus genus. New approach is proposed for discrimination of Turnip ringspot virus as a separate species.

Keywords Capsid proteins · Plant virus · Radish mosaic virus · Turnip ringspot virus

Only half of the 16 present-day known Comovirus species were at least partially sequenced, compared, evaluated phylogenetically and clearly discriminated quantitatively as a distinct species [1]. The threshold set for quantitative species demarcation is 75% identity in the CP-L gene sequence [2]. All but Radish mosaic virus (RaMV) and Turnip ringspot virus (TuRSV) are far below the limit showing identity in a wide range 34–64%. RaMV and TuRSV, both infecting the same host species, are the only pair of viruses with identity about 79% in the CP-L. However, lower identity along the whole genome predisposes that they represent distinct species [3]. In order to address that problem, we made out sequences of the CP-L gene of additional two isolates of RaMV, five newly identified isolates of TuRSV, two isolates of Red clover mosaic virus (RCMV) and calculated variability and discussed the results for discrimination of species.

TuRSV isolates from Germany (CH246) and Croatia (CH594) were provided by Dijana Škorić, University of Zagreb, Croatia. These isolates were maintained there as RaMV and are identical with isolate DT4 [4] and HZ [5], respectively. The Russian TuRSV isolates M3 and M12 were collected in Moscow and previously identified by diffusion test as RaMV [6]. The Toledo isolate of TuRSV was obtained from S.M. Leisner, University of Toledo, OH, USA. RCMV isolates Sl9 and Sl12 were collected from red clover plants on different localities in the Czech Republic. Czech RaMV isolates CB3 and CB9 were obtained from winter turnip rape and characterised in [6]. RaMV and TuRSV isolates were maintained by mechanical inoculation on white mustard plants.

The RNA for RT-PCR was isolated from 0.1 g of infected leaves with RNeasy Plant mini Kit (Qiagen, Germany) according to the manufacturer's recommendations and eluted with 30 µl of water. Complementary DNA was synthesised from 7 µl of RNA with iScript cDNA synthesis kit (Bio-Rad) in 10 µl reaction volume. Fragments, encompassing complete CP genes of RaMV, TuRSV and RCMV were amplified with species-specific primers designed according to the published sequences.

Sequences used in the pairwise comparison are listed in Table 1. The CP genes were translated in silico and the identity values were extracted using the MatGAT program [7].

Comparison of the aa sequences of CP-L only resulted in around 98% intraspecies identities in all five (BPMV, RaMV, RCMV, SqMV and TuRSV) evaluated Comoviruses (Table 2). Moderately lower value observed for RCMV (94.8 ± 4.9% SD) is affected by the RCMV-O strain that shares about 90% identity with the other RCMV

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Published online: 01 January 2010

2. **Koloniuk, I.,** & Petrzik, K. (2009). Complete genome sequence of turnip ringspot virus. *Archives of Virology*. doi: 10.1007/s00705-009-0511-9.

Arch Virol (2009) 154:1851–1853
DOI 10.1007/s00705-009-0511-9

ANNOTATED SEQUENCE RECORD

Complete genome sequence of turnip ringspot virus

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Received: 30 June 2009 / Accepted: 1 September 2009 / Published online: 23 September 2009

Abstract Here we present the complete genome sequences of two TuRSV isolates. They are 90–100% identical in distinct genes, but reasonably less identical with RaMV isolates. Regarding the CPs, TuRSV and RaMV have an aa sequence identity of 72–74% among all isolates and the proposed cut-off level is 75%. For the proteinase–polymerase region, the average value between the two isolates of TuRSV and three isolates of RaMV is 79.8% and the cut-off level is 80%. At the moment, TuRSV and RaMV are the two identified species most closely related within the genus Comovirus.

Until 2006, when partial sequences of turnip ringspot virus (TuRSV, accession numbers: DQ665367, EF191015) were deposited in the GenBank database, radish mosaic virus (RaMV) was the only comovirus known to infect various wild as well as cultivated *Brassicaeae* plants. This is uncommon, as the host range for 11 of the 15 members of the genus is limited to a few species of the family *Leguminosae*. Members of the genus have a bipartite +ssRNA genome (RNA1 and RNA2) encapsidated separately in 30-nm isometric virions. Both genome segments are translated into discrete polyproteins; functional proteins are produced via subsequent cleavage by a virus-specific protease [17]. TuRSV was found in Toledo (OH, USA) and named for the chlorotic ringspot and line pattern symptoms in turnip [12]. A virus with high sequence identity to TuRSV was isolated from Chinese cabbage (*Brassica rapa*) in Russia [6]. The relationship between TuRSV and RaMV is unclear, as TuRSV reacts with an antiserum against RaMV. Additionally, the amino acid (aa) sequence differences between them in the RNA-dependent RNA polymerase (RdRp) and the large capsid protein (CP-L) are at the species demarcation cut-off level [6]. Recently, the complete sequences of the Californian RaMV neotype (RaMV-CA) and Japanese (RaMV-J) isolates have been published. The two RaMV isolates share 90–100% aa sequence identities in distinct proteins [8].

Here, we present the complete genome sequences of two TuRSV isolates and show that they are highly identical in their aa sequences for various proteins. A comparable level was also calculated between RaMV isolates. The intra-species aa sequence identities are estimated to be less than 74 and 80% for the CPs and protease-polymerase region, respectively.

The Russian TuRSV isolate M12 from Chinese cabbage, identified by previous sequencing [6], and the Croatian TuRSV isolate B from cauliflower, described originally as RaMV [10], were used for determining the complete genome sequence of TuRSV. The B isolate was kindly provided by Prof. Dijana Škorić, University of Zagreb, Croatia, under the code CH-210. Both isolates were maintained and propagated on white mustard plants (*Sinapis alba*). The virus was purified by the PEG-6000/NaCl precipitation/centrifugation method [4]. The viral RNA was isolated from the purified virus using the RNeasy Plant Mini kit (Qiagen), and cDNA synthesis was done using an iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's recommendations. A PCR-based primer-walking approach was used to amplify the majority of the genome, and the 5′ends were obtained using a 5′-RACE protocol. The amplification products were either

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3. **Koloniuk I.,** Petrzik K. Taxonomic intrigue in Comovirus genus: TuRSV – a new member of the genus or a species-breaker of RaMV? XVIII. Česká a slovenská konference o ochraně rostlin, Brno, Czech Republic, 2-4 September, 2009. p.23. (oral presentation)

Taxonomic intrigue in Comovirus: TuRSV – a new member of the genus or a species-breaker of RaMV?

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Two isolates of *Turnip ringspot virus* (TuRSV) has been described as closely related to *Radish mosaic virus* (RaMV) recently. However, TuRSV does not have an official taxonomic position yet. For the majority of Comovirus members, natural host-range is narrow and limited by plants from Fabaceae family, whereas both RaMV, and TuRSV infect Brassicaceae plants. Our study focused on Croatian (CH210) and Russian (M) isolates of TuRSV. Altogether, there are three reported isolates – two European and the third American one. This suggests that TuRSV is also a world wide distributed, as RaMV.

Molecular characterization and phylogenetic analysis of the full-length genome sequences confirmed that both TuRSV isolates (M and CH210) are a species-specific RaMV lineage. Moreover, serological analyses have revealed high levels of diversity among European RaMV strains, which also included both of studied here isolates. Pair-wise identities of amino-acid (aa) sequences of the large capsid protein (CP-L) and the RNA polymerase between TuRSV and RaMV isolates are close to the current species demarcation level in the Comovirus genus. Additionally, differences are distributed evenly along the both parts of their genomes; besides, recombination events were not detected by any method. Therefore, there is a possibility that group of TuRSV isolates could taxonomically divide the RaMV species into two subspecies.

Here we present full genomic sequences of two European TuRSV isolates (M and CH210). Phylogenetic trees, based on large capsid protein (CP-L) and polymerase aa sequences were reconstructed. The taxonomic position of TuRSV is discussed. We assume, that another isolates have been also identified falsely in the past and additional isolates of TuRSV group will be discovered among existing RaMV collections.

4. **Koloniuk, I.,** Špak, J., & Petrzik, K. (2008). Turnip ringspot virus recognised on Chinese cabbage in Russia. *European Journal of Plant Pathology*, *122*(3), 447-450. doi: 10.1007/s10658-008-9283-4.

Eur J Plant Pathol (2008) 122:447–450
DOI 10.1007/s10658-008-9283-4

Turnip ringspot virus recognised on Chinese cabbage in Russia

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Received: 21 May 2007 / Accepted: 28 January 2008 / Published online: 22 February 2008

Abstract The nucleotide sequence of the 3′-terminal part of the RNA1 genome segment of the M12 isolate of comovirus *Turnip ringspot virus* (TuRSV) was established. This isolate originated in 1989 in Moscow (Russia) from Chinese cabbage with *Radish mosaic virus*-like symptoms. Comparison of the M12 RNA polymerase amino acid sequence with that of Radish mosaic virus (RaMV) revealed significant differences; these proteins are of different length and are only about 75% identical. On the other hand, the amino acid sequence of the M12 RNA polymerase was more than 94% identical with that of TuRSV recently described in Toledo (USA). We conclude that TuRSV occurs in Europe as well as in America and probably represents a new species of the genus *Comovirus*.

Keywords New virus · Phylogeny · RNA polymerase · Sequence comparison

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The genus *Comovirus* comprises fifteen definitive virus species with the last one added more-than 20 years ago (Brunt et al. 1982). Comoviruses are characterised by a bipartite + ssRNA genome encapsidated separately in isometric virions constructed from two polypeptide species. Most Comoviruses have a narrow host range with 11 of the 15 species being restricted to a few species of the family *Leguminosae*. The exceptions are *Andean potato mottle virus* infecting *Solanaceae*, *Squash mosaic virus* infecting *Cucurbitaceae*, *Ullucus virus C* infecting *Basellaceae* and *Radish mosaic virus* (RaMV) infecting different wild as well as cultivated *Brassicaceae* plants.

In 2006, partial sequences of another comovirus that infects *Brassicaceae* occurred in GenBank with the provisional name *Turnip ringspot virus* (TuRSV, accession numbers: DQ665367, EF191015). This virus found in Toledo (OH, USA), produced chlorotic ringspot and line pattern symptoms in turnip and was mechanically transmissible to plants in the *Brassicaceae* (Khandekar et al. 2007). Initially, it was considered to be a strain of RaMV. However, we revealed, that the nucleotide sequence of the published partial RNA polymerase gene (RdRp) differed significantly from that of the Czech RaMV1 isolate (Petrzik et al. 2005). In addition, hybridisation of TuRSV with many RaMV-specific primers failed, which indicated considerable genomic differences. We hypothesise that the Toledo isolate represents either a distant (recombinant) strain of RaMV or a new virus species.

5. Petrzik K., **Koloniuk I.,** Špak J. Turnip Ringspot Virus and Radish Mosaic Virus – closely related comoviruses infecting Brassicaceae. 9th International congress of plant pathology, Torino, Italy, 24-29 August, 2008. p.436. (poster)

Turnip ringspot virus and Radish mosaic virus - closely related comoviruses infecting *Brassicaceae*

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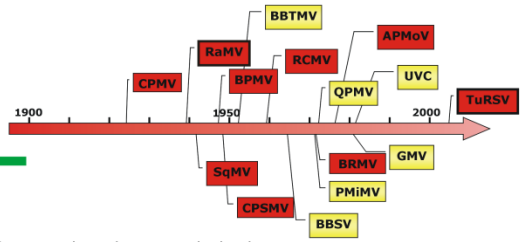
Two comoviruses naturally infect cruciferous plants - *Radish mosaic virus* (RaMV) and recently described *Turnip ringspot virus* (TuRSV). **Taxonomic status** of TuRSV was not resolved yet, as the large capsid protein (CP-L) identity (78%) and the RNA polymerase protein identity (75%) is just on the border of the comovirus species demarcation criteria (less than 75% homology). We revealed that RaMV and TuRSV **differ serologically**: In double diffusion test the TuRSV isolate from Toledo (OH, USA) and from Moscow (Russia) did not react with antisera against the RaMV isolates. Comparison of nucleotide sequences of the large and the small capsid protein genes of several TuRSV isolates with those of RaMV isolates showed, that the **intra-species variability** was lower than 3% in both viruses, but it was about 25% between the species in the CP-L and CP-S genes. We concluded that TuRSV and RaMV have the **closest relationship** among comoviruses. Randomly distributed variability along the whole genome proved that RaMV and TuRSV are **not recombinants**. Maybe, that we notice **speciation** of two viruses...?

Discovery of species

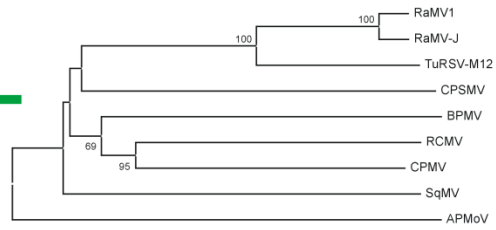


Sequenced species are marked red

Symptoms on *N.occidentalis* 37B

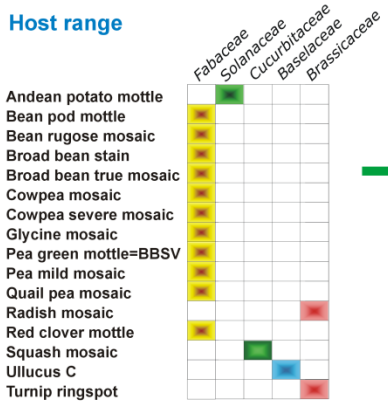
RaMV TuRSV

Phylogenetic relationships

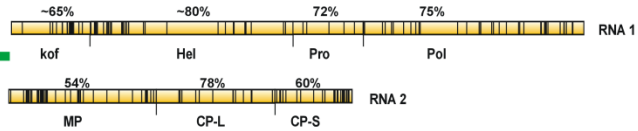


Phylogenetic tree based on amino acid sequence comparison of RNA polymerase. Bootstrap values higher than 50 are denoted in the tree.

Host range



Differences between RaMV and TuRSV polyproteins



Positions of amino acid differences are marked.
Percent identity of genes are above the scheme.

References

Koloniuk I, Špak J, Petrzik K (2008) Turnip ringspot virus recognized on Chinese cabbage in Russia *Eur. J. Plant Pathol.* DOI: 10.1007/s10658-008-9283-4

This research was granted by GACR No 522/07/0053.

6. Petrzik K., **Koloniuk I.,** Franova J., Špak J. Taxonomic position of the comovirus species. XIV. International Congress of Virology, Istambul, Turkey, 10-15 August, 2008. (poster)

Taxonomic position of the comovirus species

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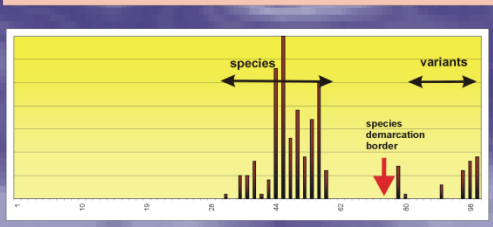
The **genus Comovirus** was established from the Comovirus group and fourteen virus species were included there according to type of genome, shape and size of virus particles, serological relationships etc. At present, 12 of the original 14 species are classified in that genus and another four were included in the last years. The species demarcation criteria in the genus are less than 75% nucleotide identity treshold of large capsid protein and polymerase amino acid sequences. However, eight species were sequenced till now. To solve unclear taxonomic position of newly described virus, we performed numerical taxonomy analysis of that genus and compared the results with related genera **Fabavirus** and **Nepovirus**. A large capsid protein gene sequence of several isolates of *Radish mosaic virus*, *Turnip ringspot virus*, and *Red clover mosaic virus* were included in the analysis.

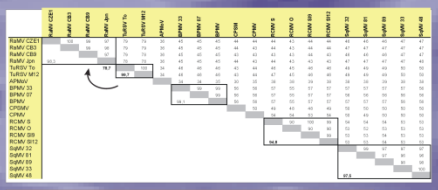
Taxonomy lineage of the genus Comovirus

Order: Picornavirales
Family: *Cheravirus*
Dicistroviridae
Iflavirus
Marnaviridae
Picornaviridae
Sadwavirus
Sequiviridae
Comoviridae
Genus Comovirus
Fabavirus
Nepovirus

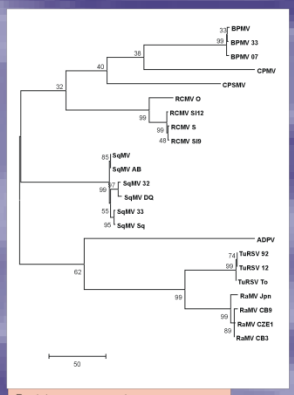
Results

Comparison of large capsid protein gene amino acid sequence identity of five comovirus species represented with three isolates at least, showed, that **intraspecies** identity is always higher than **94% in comoviruses.** However, distribution of pairwise sequence analysis identity percentages between CP-L sequences clearly distinguished **three peaks:** one at 35-60%, the second at 92-100%, and the third at 78-80%. The first of these recognised distinct species, and the second includes sequence variants of species isolates. The third peak arose with comparison of RaMV and TuRSV and could represent yet unknown category analogous to subspecies category in genus Potyvirus. Similar position just on the species demarcation border were found for two *Raspbery ringspot virus* isolates and one *Arabis mosaic virus* isolate from the related genus *Nepovirus*.
Randomly distributed variability along the whole genome proved that RaMV and TuRSV are not recombinants. Maybe, that we notice speciation of two viruses...?





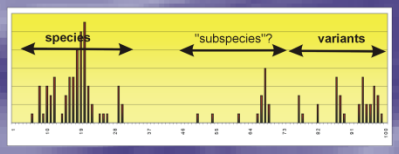
At the lower left end side of the species boxes is indicated the intraspecies pairwise percentage identity, the RaMV-TuRSV inter-species pairwise percentage identity is indicated between species boxes



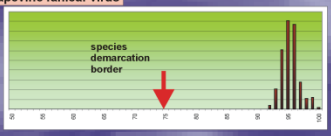
Bootstrap consensus tree
Maximum parsimony, 1000 replicates

Similarity with the genus Nepovirus

Very divergent genus with distantly related species: Inter-species identity is in range 5-30%, but the intra-species identity is above 90%. However, there aretwo isolates of *Raspberry ringspot* and one isolate of *Arabis mosaic* viruses, that are significantly less identical, than the "true" isolates - 68-70% and 77-78%, respectively.

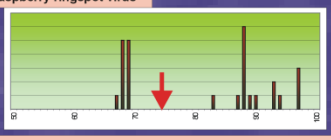


Grapevine fanleaf virus



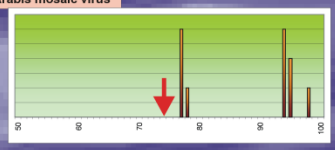
Distribution of similarity among 178 isolates of "good" species formed single maximum.

Raspberry ringspot virus



Two isolates (EF534293, AY310445) are so distant, that could represent different species

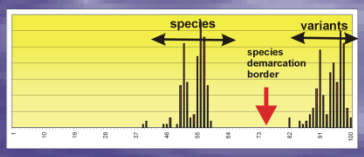
Arabis mosaic virus

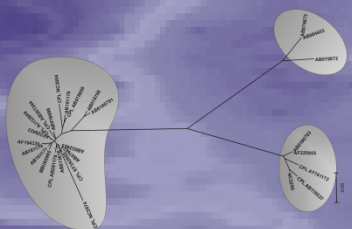


Isolate AB279739 could represents subspecies

Similarity with the genus Fabavirus

Three clearly separated branches represents three species. The similarity distribution plot showed 39-60% identity between species and 82-100% identity between variants. No subspecies were detected.





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This research was supported by the Ministry of Agriculture of the Czech Republic project No.: QH71145 and by the Grant Agency of the Czech Republic project No.: 522/07/0053

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Genetic comparision of two comoviruses infecting brassicas

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An isometric virus was isolated from a sample of Chinese cabbage (*Brassica pekinensis*) plant with mosaic symptoms about 15 years ago in Moscow (Russia). The virus cross-reacted weakly with Radish mosaic comovirus (RaMV) antiserum (Spak, 2000). Until now it has been supposed to be its isolate called M12.

Comoviruses are characterized by their bipartite, single-stranded RNA genomes, expressed as polyproteins, one from RNA-1 (ca. 5900 nt), and two (inframe, overlapping and sharing a common termination codon) from RNA-2 (ca. 3600 nt) (Goldbach & Wellink, 1996). The RNA-1-encoded polyprotein yields the RNA-dependent RNA polymerase (RdRp), a helicase and a protease, as well as a protease cofactor and the genome-linked viral protein (VPg), while the RNA-2-encoded polyproteins yield the movement protein (MP), large capsid protein (CP-L) and small capsid protein (CP-S). Each RNA is 3´-polyadenylated, is linked to a 5´-VPg and is individually encapsidated by 60 copies each of the two capsid proteins (CPs), to form 28 nm diameter, icosahedral particles, with a pseudo T=3 structure (Goldbach & Wellink, 1996).

Recently several RT-PCRs were performed with RaMV-specific primers and it was not successful. The M12 genome was partially sequenced by means of primer-walking strategy. 3´-regions of RNA-1 with 2673 nt and RNA-2 with 2000 nt lengths were obtained. They consisted of complete 3´-UTR, CPs and RdRp sequences and partially protease gene. It turned out there is only 72% identity in RdRp amino acid sequence between M12 virus and RaMV1 isolate. There is one unique insertion in aa sequence of M12 RdRp.

The RaMV is the most related one to M12 within the genus: the amino acid sequence of the CP-L and CP-S genes is 78,7% and 63,5% respectively identical. The highest identities of the RaMV CPs are about 46% (with CP-L of Squash mosaic virus) and about 33% (with CP-S of Red clover mosaic virus). The variable position in CP-L are distributed evenly along the gene, in CP-S most of the variable positions is concentrated in the last third of the protein.

BLAST search showed 100% identity of received RdRp sequence with 55% sequence coverage of Turnip Ringspot Virus RdRp gene. The last one is mentioned only in GenBank as tentative member of Comovirus and has its origin in the USA accordingly to the submitted data. It is highly possible that we have deal with the same virus isolate.

Received data leads us to propose to discriminate the virus as a new species of the genus Comovirus and give it the name “Turnip ringspot virus”.

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