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Phylogenetic analysis of Lithuanian tomato black ring virus isolates

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Abstract

Tomato black ring virus (TBRV) was first detected on tomato (*Lycopersicon esculentum* Mill.) plants in Lithuania in 2012. During 2013, additional eleven isolates of this virus were found on ornamental and food plants in different regions of Lithuania. The virus was identified by double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA), electron microscopy and reverse transcription-polymerase chain reaction (RT-PCR). Up to date, full genome sequences of only two different TBRV isolates have been available at GenBank. Comparison of these sequences enabled us to derive PCR primers for amplifying full length copy of TBRV coat protein (CP) gene sequence. With these primers we successfully amplified and sequenced CP gene of all 12 TBRV isolates that we found. The obtained nucleotide sequences have been submitted to the GenBank database. Phylogenetic analysis of CP gene sequence showed Lithuanian TBRV isolates to be more similar to Polish TBRV isolates than to English ones. It also suggests that a few relatively distinct strains of this virus might be present in Lithuania.

Key words: coat protein, phylogeny, plant virus, polymerase chain reaction, sequencing.

Introduction

The *Nepovirus* genus (*ne*matode-transmitted, *po*lyhedral viruses) is a group of viruses that have very unique traits compared to all other plant viruses. All nepoviruses are transmitted through soil by free-living nematodes of *Longidorus* and *Xiphinema* species, feeding on roots. They also have very wide plant host range, which combined with nematode vectors makes them extremely difficult to eradicate since viruses can remain in the soil with the absence of plants. Unlike the vast majority of other plant viruses, nepoviruses can be transmitted by seed and/or pollen and this way uncontrollably cross international borders. Many nepoviruses cause diseases of economic importance in a wide range of cultivated annual, perennial and woody plants, and most of them are of serious concern to quarantine services worldwide (Murant, 1981; Brunt et al., 1997; Card et al., 2007). Moreover, intensity of infection symptoms can range from severe to mild and even asymptomatic infections. And infections that display mild symptoms can often be unnoticed and thus virus can survive and spread unhindered. There are about 46 members in this genus and they are categorized into three subgroups: A, B and C, according to their serological relationships, sequence similarities, and the length and arrangement of RNA-2 (Fauquet, Mayo, 2005; Digiaro et al., 2007; Wei, Clover, 2008). A few nepoviruses (tobacco ringspot virus and tomato ringspot virus) have already been reported in Lithuania (Samuitienė, Navalinskienė, 2001), TBRV was detected in Lithuania for the first time in 2012 (Šneideris et al., 2012).

Tomato black ring virus (TBRV) is known to infect a wide range of important crop species, including grapevine, cherry, apricot, peach, berry-fruits (raspberry, currant, strawberry, blueberry), solanaceous species (potato, tomato, pepper, tobacco), and a number of weed and ornamental species (Brunt et al., 1997; Edwardson, Christie, 1997). Common symptoms of TBRV infection are systemic chlorotic or necrotic ringspot, leaf mottle and deformation (Brunt et al., 1997). Virus is transmitted both through seed and by the nematodes *Longidorus elongatus* and *Longidorus attenuatus* (Harrison et al., 1961; Brown et al., 1989). The virus has been reported in Europe, North and South America, India and Japan (Brunt et al., 1997; Harper et al., 2011).

We discovered TBRV in Lithuania for the first time in 2012 (Šneideris et al., 2012). Up to date, there has been no knowledge about natural spread of this virus (in Lithuania) or damage it might be causing to economically important plants. In 2013, our aim was to acquire more data about the occurrence of TBRV in Lithuania and its genetic variability. Since the first detection of TBRV, we have found eleven other isolates of this virus and this paper presents the data of phylogenetic analysis of all 12 TBRV isolates.

Materials and methods

Virus isolates and their cultivation. Material for investigation was collected from private and commercial greenhouses, farm fields, city parks and botanical gardens in 2012 and 2013. Nearly 170 samples of plants exhibiting virus-like disease symptoms were collected and tested for tomato black ring virus (TBRV) infection. Viruses were identified by electron microscopy negative staining technique, DAS-ELISA tests and RT-PCR method. Isolates were maintained and propagated in tobacco (*Nicotiana rustica* L.) and Jimson weed (*Datura stramonium* L.) plants. Viruses were mechanically inoculated to leaves of young test-plants using carborundum powder as an abrasive. Research and experiments were carried out in Nature Research Centre, Institute of Botany, Phytovirus Laboratory.

Double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA). ELISA tests were performed using Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) immunological kits according to manufacturer's instructions. Tests were considered to be positive if the ultraviolet (UV) absorbance of investigated sample was equal or greater than three times the absorbance of negative (healthy plant) control.

RNA purification and reverse transcription-polymerase chain reaction (RT-PCR). Total ribonucleic acid (RNA) extraction was performed by cetrimonium bromide (CTAB) method (Chang et al., 1993). Extracted RNA was stored at −20°C in diethylpyrocarbonate (DEPC) water. Extracted RNA was used for virus detection and identification by RT-PCR as well as coat protein (CP) gene region amplification. Extracts from healthy plants were used as negative control. Primers for TBRV detection and identification were F: 5'-TCTGGITTTGCYTTRACRGT-3' and R: 5'-CTTRTCACTVCCATCRGTAA-3' (Wei, Clover, 2008). Our derived primers for CP gene amplification were TBRV-CP-F: 5'-GGCAGACGGGGATTTTGC-3' and TBRV-CP-R: 5'-CTATGCRGGAATAGTWARAGG-3'. Amplification was performed in "TProfessional Thermocycler" ("Biometra", Germany) using two-step RT-PCR method. At first step reverse transcription was performed for 60 min at 42ºC in mixtures containing 2 µl of reverse primer, 4 µl 5X buffer, 2 µl deoxyribonucleotide triphosphate (dNTP) mix (10 mM), 0.5 µl RiboLock™ RNase inhibitor, 1 µl RevertAid™ reverse transcriptase (200 u μl^{-1}) ("Thermo Scientific", Lithuania), 4 µl RNA and DEPC H_2O up to a total volume of 20 µl. Reverse transcription was terminated by heating at 70ºC for 10 min. PCR was performed in mixtures containing 2 µl of each primer (25 µM), 7.5 µl 10X *Taq* buffer, 3.5 µl $MgCl_2$ (25 mM), 1 µl dNTP mix (10 mM), 0.25 µl *Taq* polymerase (5 u μl^{-1}) ("Thermo Scientific"), 4 µl cDNA and DEPC H_2O up to a total volume of 50 µl. The following cycling scheme for both TBRV identification and CP gene amplification was used: 94ºC – 5 min, 40 cycles (94ºC – 1 min, 53ºC – 1 min, 72ºC – 1 min) and 72ºC – 10 min. Amplified DNA fragments were analyzed by electrophoresis in 1.5% agarose gel, stained with ethidium bromide and visualized in "BioDocAnalyze" ("Biometra") gel documentation system using GeneRuler™ 50 bp DNA ladder and GeneRuler™ 100 bp DNA ladder plus markers depending on PCR product size ("Thermo Scientific").

PCR product purification and in silico analysis. When needed, specific 1537 bp length PCR products of CP gene locus were excised from gel and purified using a DNA extraction kit ("Thermo Scientific") according to manufacturer's instruction before sequencing at "Macrogen" (Korea). To reduce the impact of sequencing errors, sequencing of each isolate was carried out twice in each direction, giving up to four crossovers covering most part of the CP gene. Nucleotide sequences were compared with sequences available in the GenBank using basic local alignment search tool (BLAST). Sequence analysis, consensus building and alignments were performed using DNASTARv7 and MEGA4 computer programs. Phylogenetic analysis was carried out by neighbour-joining method with bootstrap values based on 1000 pseudo replicates. Coat protein gene sequences of TBRV isolates from Poland (AY157994) and England (X80831) were acquired in GenBank (Le Gall et al., 1995; Jonczyk et al., 2004).

Table. Locations and plant hosts of tomato black ring virus (TBRV) isolates

Isolate	Plant host	Region	Year of discovery
Lt-1	*Phlox* sp.	Širvintos distr.	2013
Lt-2	*Viola* sp.	Klaipėda	2013
Lt-3	*Phlox* sp.	Kaunas	2013
Lt-4	*Lycopersicon esculentum* Mill.	Kėdainiai distr.	2013
Lt-5	*Hosta* sp.	Vilnius	2013
Lt-6	*Lamprocapnos spectabilis* L.	Širvintos distr.	2013
Lt-7	*Rheum rhabarbarum* L.	Anykščiai distr.	2013
Lt-8	*Clematis* sp.	Kelmė distr.	2013
Lt-9	*Hosta* sp.	Klaipėda	2013
Lt-10	*Lycopersicon esculentum* Mill.	Kaunas distr.	2012
Lt-11	*Fragaria vesca* L.	Panevėžys distr.	2013
Lt-12	*Phlox* sp.	Vilnius	2013

Results and discussion

Leaves of diseased plants exhibiting chlorotic and necrotic ringspot, leaf mottle and deformation were collected from greenhouses, farm fields, city parks and botanical gardens in Lithuania (Fig. 1). Collected samples were primarily tested for TBRV infection by ELISA and electron microscopy. Isomeric virus particles of ~30 nm diameter were often detected in ELISA positive samples (data not shown). Virus infection was further confirmed by RT-PCR using detection primers which yield 221 bp size product (data not shown). In total, 12 TBRV isolates

were obtained (including one previously reported in 2012, Šneideris et al., 2012) from different plants (Table): two isolates were identified in tomato (*Lycopersicon esculentum* Mill.) plants (Lt-4, Lt-10), three in phloxes (*Phlox* sp.) (Lt-1, Lt-3, Lt-12), two in hostas (*Hosta* sp.) (Lt-5, Lt-9) and remaining five in bleeding hearth (*Lamprocapnos spectabilis* L.) (Lt-6), rhubarb (*Rheum rhabarbarum* L.) (Lt-7), viola (*Viola* sp.) (Lt-2), wild strawberry (*Fragaria vesca* L.) (Lt-11) and clematis (*Clematis* sp.) (Lt-8). Though TBRV was mostly found in ornamental plants, considering its wide plant host range, impact of this virus on economically important plants should not be disregarded. This only proves how many ornamental plants and wild weeds may be natural sources for virus propagation and spreading.

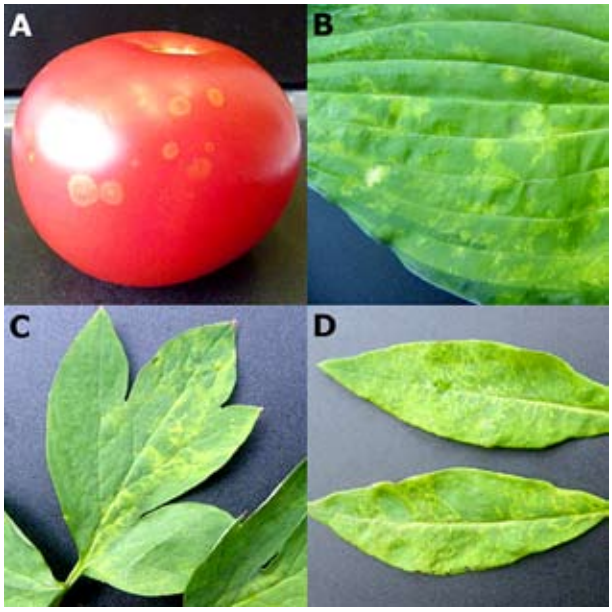


Figure 1. Symptoms of tomato black ring virus (TBRV): A – tomato fruit (*Lycopersicon esculentum*), B – hosta (*Hosta* sp.), C – bleeding hearth (*Lamprocapnos spectabilis*) and D – phlox (*Phlox* sp.)

Up to date, only two examples of TBRV RNA-2 sequence have been available at GenBank (Le Gall et al., 1995; Jonczyk et al., 2004). Sequence comparisons of these two isolates showed adequately conservative regions flanking coat protein gene which allowed us to choose degenerate PCR primers capable of amplifying full length copy of CP gene from our TBRV isolates. Apart from expected size products (~1500 bp), PCR also produced nonspecific bands in almost all virus positive samples (Fig. 2). As such bands were also amplified in negative controls (healthy plant tissue); nonspecifics probably were amplified from plant RNA. Nonspecific products are usually also induced by degenerated primers. CP gene was sequenced of all twelve TBRV isolates in our possession. However, after sequence comparison only eight distinctive groups could be distinguished as sequences of other four isolates were nearly identical to one or another of those eight. And these differences of few nucleotides most likely occurred due to errors of polymerase during PCR. Sequences of these eight genetically different TBRV isolates (from Lt-1 to Lt-8) were submitted to GenBank (accession numbers of isolates from Lt-1 to Lt-8, respectively: KF678365, KF678366, KF678367, KF678368, KF678369, KF678370, KF678371 and KF678372).

CP gene nucleotide and amino acid sequence comparisons show that all Lithuanian TBRV isolates are more closely related to Polish isolate than the one from England (Fig. 3). And that seems logical given geographical locality of our countries. As already mentioned, 8 different groups could be distinguished out of all 12 isolates. Isolate Lt-9 was identical to Lt-2, Lt-10 to Lt-6, Lt-11 to Lt-1 and Lt-12 to Lt-5. Differences among Lithuanian isolates seem to be based on geographical regions where they were found and not the plant host in which they were found. Isolates found in the same plant species but in different places had significant CP gene

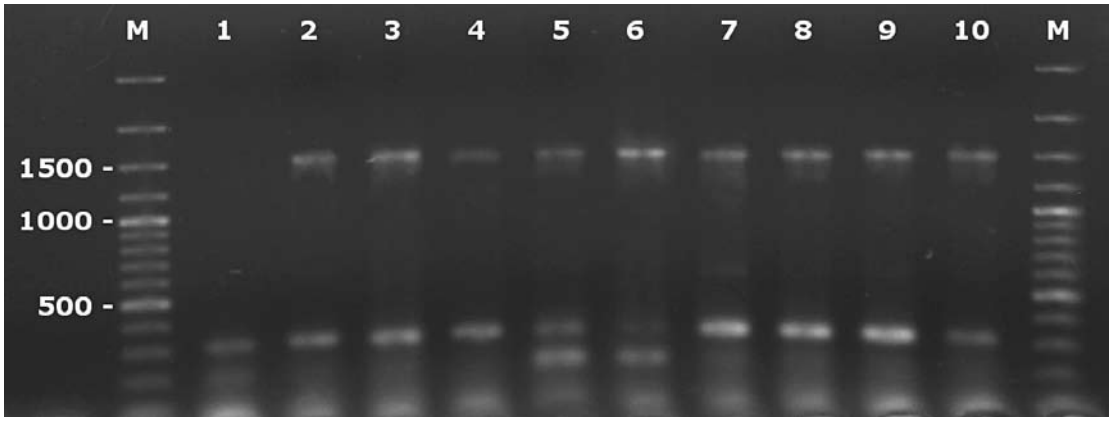


Figure 2. Tomato black ring virus (TBRV) specific 1537 bp PCR product amplified with TBRV-CP-F/TBRV-CP-R primer pair from naturally infected plants: M – marker (GeneRuler™ 100 bp DNA Ladder Plus), 1 – negative control (healthy plant), 2 – *Clematis* sp. (Lt-8), 3 – *Phlox* sp. (Lt-3), 4 – *Phlox* sp. (Lt-12), 5 – *Hosta* sp. (Lt-5), 6 – *Hosta* sp. (Lt-9), 7 – *Lycopersicon esculentum* (Lt-4), 8 – *Lamprocapnos spectabilis* (Lt-6), 9 – *Viola* sp. (Lt-2), 10 – *Rheum rhabarbarum* (Lt-7)

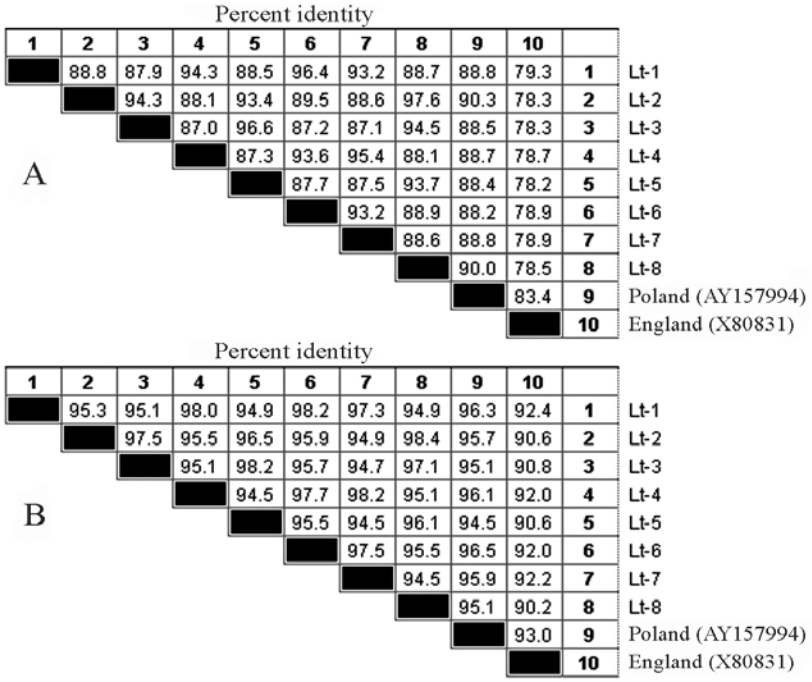
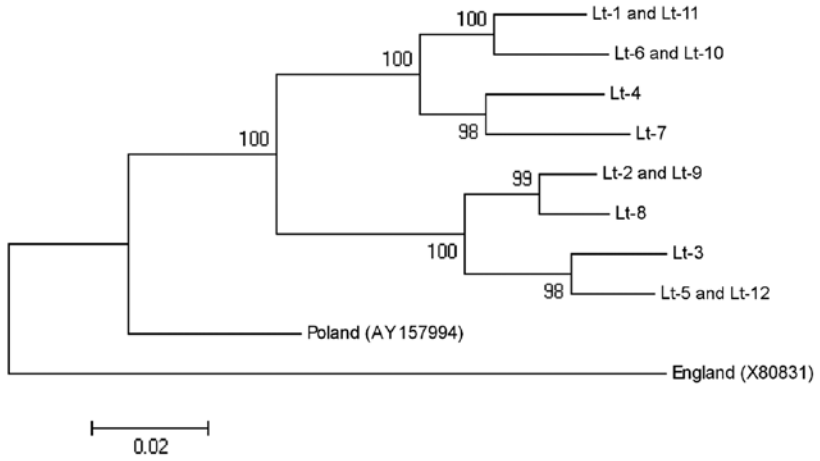


Figure 3. Tomato black ring virus (TBRV) coat protein gene nucleotide (A) and translated amino acid (B) sequence similarity comparisons

nucleotide sequence differences, for example isolates Lt-5 and Lt-9 both found in hostas. While isolates from different plants like Lt-5 (hosta) and Lt-12 (phlox) found in the same botanical garden were identical.

Phylogeny analysis shows that Lithuanian isolates tend to cluster into two groups, one containing isolates Lt-1, Lt-4, Lt-6, Lt-7 and the other – Lt-2, Lt-3, Lt-5, Lt-8 (Fig. 4). In some cases nucleotide sequence similarities among Lithuanian isolates from different groups (88.5% between Lt-1 and Lt-5 or 88.1% between Lt-2 and Lt-4) are lesser than similarities of these Lithuanian isolates to Polish isolate (88.8% between Lt-1 and Polish or 90.3% between Lt-2 and Polish). It is likely that at least two different virus strains are present in Lithuania. These results coincide with recent discoveries in Poland, where it was shown that Polish TBRV isolates also differ from each other and do not form a monophyletic cluster (Rymelska et al., 2013). It is not possible to suggest different virus strains based on only one gene sequence analysis, full genome sequence would be needed for this. However, the virus coat protein gene sequence usually represents the whole virus genome well enough to make phylogeny analysis because it is one of the most conservative virus genes. We make assumption that in the future Lithuanian TBRV isolate might be shown to be significantly different from Polish isolate



Notes. Subjected to 1000 bootstrap replicates, numbers at branching points indicate bootstrap values (only values >90% are shown). The horizontal branch lengths are proportional to the genetic distances.

Figure 4. Phylogenetic tree of tomato black ring virus (TBRV) based on coat protein full gene sequences, constructed with MEGA4.1 using neighbour-joining algorithm

based on sequences of other genes and those differences might be sufficient to distinguish Lithuanian TBRV as a separate strain. Moreover, maybe even a few different Lithuanian TBRV strains might be distinguished.

Conclusion

A closer survey showed that tomato black ring virus is more common in Lithuania than expected. In total, twelve separate isolates of TBRV were found to infect tomato (*Lycopersicon esculentum* Mill.), phlox (*Phlox* sp.), hosta (*Hosta* sp.), bleeding hearth (*Lamprocapnos spectabilis* L.), rhubarb (*Rheum rhabarbarum* L.), viola (*Viola* sp.), wild strawberry (*Fragaria vesca* L.) and clematis (*Clematis* sp.). Coat protein gene nucleotide and amino acid sequences of Lithuanian TBRV isolates were more similar to Polish TBRV isolate than English isolate. There also were significant differences among Lithuanian isolates which suggests that possibly a few different strains of this virus might be present in Lithuania.

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Lietuvoje aptiktų valgomojo pomidoro (*Lycopersicon esculentum* Mill.) juodojo žiediškumo viruso izoliatų filogenetinė analizė

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Santrauka

Valgomojo pomidoro (*Lycopersicon esculentum* Mill.) juodojo žiediškumo virusas Lietuvoje pirmą kartą buvo aptiktas 2012 m. 2013 metais vienuolika papildomų šio viruso izoliatų aptikta dekoratyviniuose ir maistui naudojamuose augaluose iš įvairių Lietuvos regionų. Virusas buvo identifikuotas nuo fermentų priklausomos imuno-sorbensijos (DAS-ELISA) ir elektroninės mikroskopijos bei polimerazinės grandininės reakcijos (RT-PGR) metodais. Iki šiol Genų banke buvo tik dviejų skirtingų juodojo žiediškumo virusų nusekvenuotų genomų pavyzdžiai. Palyginus šių genomų sekas, buvo parinkti PGR pradmenys, skirti amplifikuoti juodojo žiediškumo viruso apvalkalo baltymo geno seką. Su šiais pradmenimis amplifikuotos ir nusekvenuotos visų dvylikos Lietuvoje rastų juodojo žiediškumo viruso izoliatų apvalkalo baltymo geno sekos. Nusekvenuotos geno nukleotidinės sekos buvo atiduotos į Genų banką. Apvalkalo baltymo geno sekų analizė atskleidė, kad lietuviški juodojo žiediškumo viruso izoliatai yra artimi Lenkijoje rastiems atitinkamo viruso izoliatams. Pagal filogenetinės analizės duomenis Lietuvoje gali būti daugiau nei viena šio viruso grupė.

Reikšminiai žodžiai: apvalkalo baltymas, augalų virusai, filogenija, polimerazinė grandininė reakcija, sekoskaita.