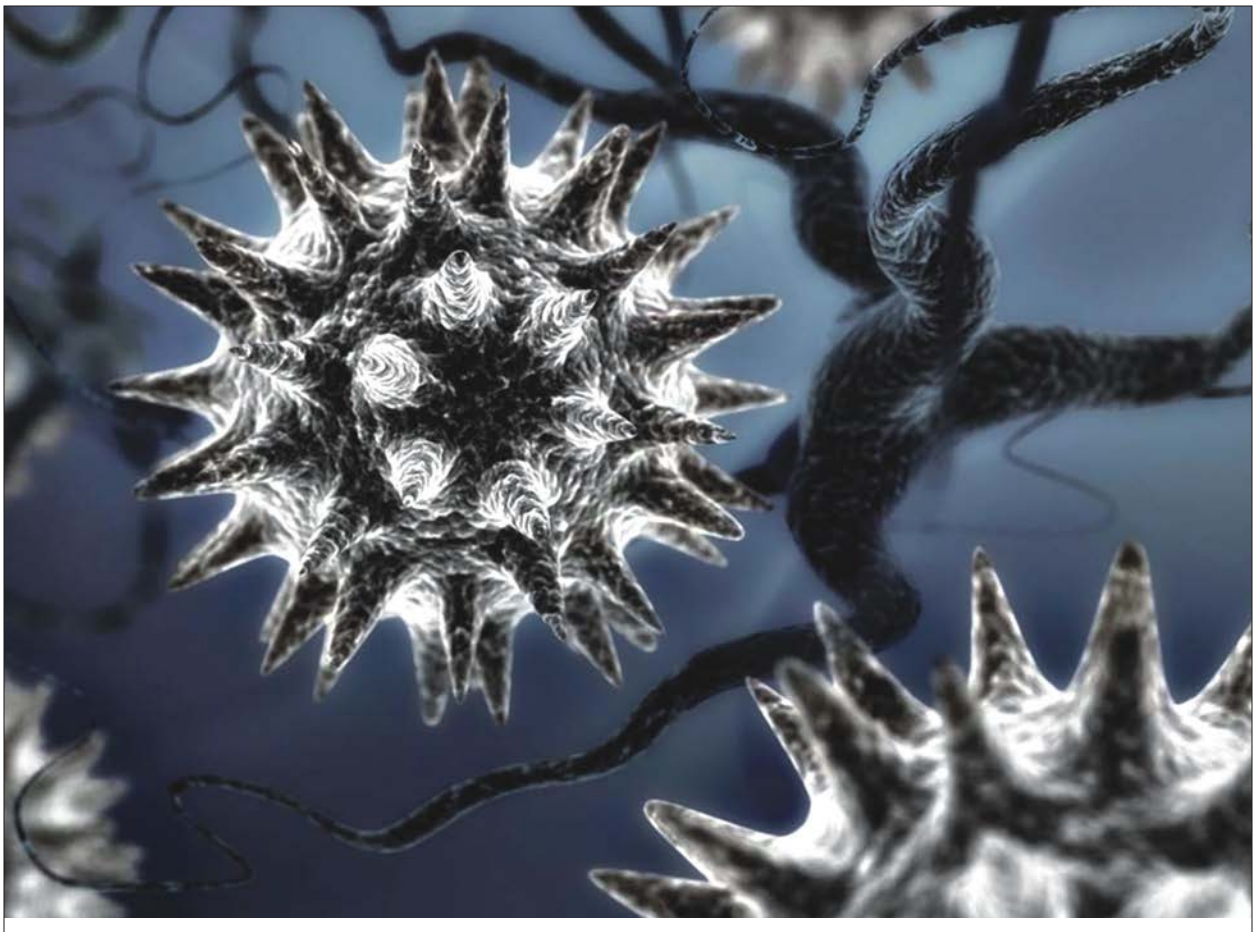

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Sequence Differences between *Grapevine fanleaf virus* and *Arabis mosaic virus* Isolates in RNA2 Encoding the Central Part of Movement Protein

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ABSTRACT

In this study, we reported sequencing and molecular analysis in detail of central part of $2B^{MP}$ gene (approximately 270 nts) in case of *Grapevine fanleaf virus* (GFLV) and *Arabis mosaic virus* (ArMV). For more complex analysis of genetic variability we added reference sequences of evaluated nepoviruses and also sequence of phylogenetically very similar *Nepovirus* GDefV. Range of sequence analysis showed 74.5-99.3% identity at the nucleotide level and 86.5-100% identity at the amino acid level for partial gene $2B^{MP}$ among Czech, Italian, German, Swiss sequences and references of all isolates. In this study eleven isolates were sequenced consisting of 5 GFLV and 6 ArMV isolates. As expected, GFLV and ArMV sequences were distinguished based on sequence identities but differences were not significant. Surprisingly, GDefV sequence was almost identical with the majority of GFLV sequences, even more than some GFLV sequences between each other. Local complexity of nucleotide sequences was also evaluated and it was in average of 0.530 for GFLV and GDefV, for ArMV the average value was 0.533. This study provides detail insights for the genetic variability of GFLV, ArMV and GDefV in the central part of the $2B^{MP}$ coding region.

Key words: RNA2, movement protein, sequencing, identity, complexity

INTRODUCTION

Grapevine fanleaf virus (GFLV) is one of the oldest known viruses on the grapevine (*Vitis vinifera* L.) (Martelli, 1986). It is thought that GFLV has coexisted with grapes since their earliest cultivation and has spread with vegetatively propagated crop. The GFLV disease is spread less in the Czech Republic than another *Nepovirus*, family Secoviridae, ArMV (Kominek and Holleinova, 2003). The ArMV is assigned as quarantine virus in the Czech Republic but GFLV not yet. The GFLV and ArMV induce significant yield reduction and lowering of the quality of grapevine fruit and must. They cause leaves, shoots and fruits malformation, whereas some strains cause yellow discoloration of the leaves. Plant-to-plant spread of GFLV in the vineyard occurs only by the ectoparasitic dagger nematode *Xiphinema index* Thorne and Allen (Esmenjaud *et al*., 1993), vector of ArMV is *Xiphinema diversicaudatum*. Viruses are also transmitted efficiently by grafting and via distribution of infected vegetative propagation materials.

GFLV and ArMV virus particles are very similar on the molecular level. These viruses are typical for their segmented genome. The genome is the main component of virus particle. Virus particles have angular outline roughly 30 nm in diameter, containing a single protein species of Mr 56,000. The genome consists of two positive-sense ssRNA molecules (RNA1, RNA2) which are encapsidated separately. Both genomic RNAs are covalently linked to their 5' ends to small Viral Protein (VPg) and they are polyadenylated in their 3' ends. The 3'-NCR of both RNAs are identical for many nepoviruses (Le Gall *et al*., 1995). The first complete sequenced macromolecule of GFLV

RNA1 and RNA2 belonged to F13 strain (Serghini *et al.*, 1990; Ritzenthaler *et al.*, 1991). The final length was 7342 and 3774 nucleotides for RNA1 and RNA2, respectively.

RNA2 of ArMV was completely sequenced for the first time in 2001 by Wetzel *et al.* (2001) and RNA1 of ArMV was completely sequenced in 2008 (Dupuis *et al.*, 2008).

The RNA1 and RNA2 are monocistronic and each encodes a single polyprotein that is processed proteolytically into functional proteins required to complete the virus life cycle. The RNA2-encoding P2 polyprotein which contains (from the N-to C-terminus) the domains for the homing protein ($2A^{HP}$), the movement protein ($2B^{MB}$) and the coat protein ($2C^{CP}$) (Margis *et al.*, 1993). The $2A^{HP}$ is localized in the replication site and has been implicated in RNA1-dependent replication of RNA2 (Gaire *et al.*, 1999). The $2B^{MP}$ is a movement protein and is found in tubules observed in the plasmodesmata (Ritzenthaler *et al.*, 1995). The $2C^{CP}$ is a multifunctional coat protein, important in specific transmission by *X. index* Thorne and Allen, encapsidation of genomic RNA and systemic spread in plants (Hewitt *et al.*, 1958; Belin *et al.*, 2001; Callaway *et al.*, 2001; Andret-Link *et al.*, 2004). The GFLV was observed in various molecular variants in many countries of Europe, Africa, Middle East, North and South America (Naraghi-Arani *et al.*, 2001; Vigne *et al.*, 2004; Fattouch *et al.*, 2005; Bashir *et al.*, 2007; Pompe-Novak *et al.*, 2007; Liebenberg *et al.*, 2009; Mekuria *et al.*, 2009), occurence of ArMV is also global (Wetzel *et al.*, 2002; Lockhart, 2006; Rakhshandehroo *et al.*, 2006). The GFLV was described in the Czech Republic only by Kominek *et al.* (2006) who described the mild isolate HV5 (Eichmeier *et al.*, 2010, 2011). The ArMV is a quarantine virus in relation to species *Fragaria* L. and *Rubus* L. Molecular information about ArMV as viral agent of grapevine has not been published yet in the Czech Republic.

The majority of the studies listed above are focused on special regions on the RNA2 molecule of these two nepoviruses. Studies focused on characterisation of gene variability localizated on RNA1 molecule are still rather exceptional (Ritzenthaler *et al.*, 1991; Wetzel *et al.*, 2004; Dupuis *et al.*, 2008; Mekuria *et al.*, 2009; Oliver *et al.*, 2010; Eichmeier *et al.*, 2011; Lopez-Fabuel *et al.*, 2013). The spatial spread of both viruses in the Czech Republic is not the same. The GFLV is not so frequent as ArMV (Kominek and Holleinova, 2003). Thereby ArMV is a bigger threat than GFLV in the Czech Republic. It can be caused by the fact that vector of ArMV *X. diversicaudatum* is natural in the Czech Republic but the vector of GFLV *X. index* Thorne and Allen hasn't been discovered in the Czech Republic yet (Kumari *et al.*, 2005). We suppose that spread of GFLV in the Czech Republic is realized mainly by vegetative propagation. Another theoretical danger can be the fact of possible existence of recombinants between ArMV and GFLV (Mekuria *et al.*, 2009) which may be spread by nematodes that are natural in the Czech Republic. Nucleotide sequence coding $2B^{MP}$ gene is the most homologous genomic portion of the RNA2 strand between GFLV and ArMV (Wetzel *et al.*, 2002). Mekuria *et al.* (2009) further stated that $2B^{MP}$ sequences seem to be "hot spots" for interspecies genetic exchanges between closely related grapevine-infecting nepoviruses.

Recombination can be an important factor in viral evolution (Garcia-Arenal *et al.*, 2001; Moury *et al.*, 2006) and in the case of examined nepoviruses, recombination has been reported to occur within RNA2 of ArMV and GFLV among distinct genetic variants (Vigne *et al.*, 2004; Boulila, 2007; Pompe-Novak *et al.*, 2007; Mekuria *et al.*, 2009; Vigne *et al.*, 2009) and *Grapevine deformation virus* (GDefV) (Mekuria *et al.*, 2009).

MATERIALS AND METHODS

Plant material: The isolates were collected since 2008 according to screening tests by RT-PCR. Five infected grapevines of two grape cultivars (Kodrjanka and Pamjati Negrula)

Table 1: RNA2 sequences of *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV) and *Grapevine deformation virus* (GDefV) used in this study

Virus	Accession nts	Accession aa	Host	Cultivar	Isolate	Size (nts)	Country of origin	Reference
GFLV	NC_003623	NP_619706	*V. vinifera* L.	Muscat	F13	3774	France	Serghini *et al.* (1990)
GFLV	DQ386866	ABD46755	*V. vinifera* L.	...	HV5	814	Czech Republic	Kominek *et al.* (2006)
GFLV	KF986559	AHL27350	*V. vinifera* L.	Kodrjanka	KO1	288	Czech Republic	This study
GFLV	KF986555	AHL27346	*V. vinifera* L.	Pamjati Negrula	PN35	288	Czech Republic	This study
GFLV	KF986556	AHL27347	*V. vinifera* L.	Pamjati Negrula	PN33	288	Czech Republic	This study
GFLV	KF986557	AHL27348	*V. vinifera* L.	Dimrit	55TK	288	Italy	This study
GFLV	KF986558	AHL27349	*V. vinifera* L.	URS	UR11	288	Italy	This study
ArMV	NC_006056	YP_053924	*V. vinifera* L.	Pinot Gris	NW	3820	Germany	Wetzel *et al.* (2001)
ArMV	KJ137007	AHJ81267	*V. vinifera* L.	...	AR/G4	270	Germany	This study
ArMV	KJ137008	AHJ81268	*V. vinifera* L.	...	AR/G2	267	Germany	This study
ArMV	KJ137009	AHJ81269	*V. vinifera* L.	Pinot Noir	K4	272	Czech Republic	This study
ArMV	KJ137010	AHJ81270	*N. clevelandii*	...	AR/S2	267	Switzerland	This study
ArMV	KJ137012	AHJ81272	*V. vinifera* L.	Pinot Noir	S10	267	Czech Republic	This study
ArMV	KJ137011	AHJ81271	*N. clevelandii*	...	OL1	267	Czech Republic	This study
GDefV	AY291208	AAQ56597	*V. vinifera* L.	Dimrit	N66	3753	Turkey	Ghanem-Sabanadzovic *et al.* (2005)

planted in the South Moravia (Czech Republic) and two infected Italian grapevines of cultivars Dimrit and URS were included to this study. Cultivars Kodrjanka and Pamjati Negrula have origin in Moldova and are grown in the South Moravia (Czech Republic) more than 20 years. Two additional isolates were provided by Mediterranean Agronomic Institute of Bari (Italy) to this collection (URS and Dimrit). Six infected grapevines (Pinot Noir and unknown cultivars) and *Nicotiana clevelandii* plants infected by ArMV were used for this study (Table 1).

RT-PCR: Reaction for reverse transcription consists of water (HPLC purity), 0.25 μg random primer P(dN)6 (Roche, Indianapolis, USA) and 0.1 μg (2 μL) of total RNA in total volume 12.5 μL. This mix was denaturated at 95°C for 5 min at first. Subsequently 5 μL 1×RT buffer (Fermentas, Bourlington, Canada), 1.25 μL 10 mM dNTPs (Invitek, Berlin, Germany) and 0.5 μL 200 U reverse transcriptase M-MLV-RT (Fermentas, Bourlington, Canada) were added. The time for reverse transcription was 60 min at 42°C. The PCR master mixture was prepared according to Wetzel *et al.* (2002).

Sequencing of PCR products: The PCR amplicons of expected lengths were cut from the agarose gel and purified by NucleoSpin Extract II (Macherey-Nagel, Germany). Nucleotide sequencing was done by BigDye® Terminator v3.1 (Applied Biosystems, USA). The products of sequencing reaction were separated using genetic analyser ABI-PRISM 310 (Applied Biosystems, USA). The sequencing was done in 5'-3' direction for each isolate. The amplification primers (Wetzel *et al.*, 2002) were also used as sequencing primers.

RESULTS AND DISCUSSION

The aim of this study was to advance our knowledge of the genetic variability of GFLV and ArMV RNA2 by characterizing the partial nucleotide sequence of $2B^{MP}$ gene of nine viral isolates from Czech Republic, Italy, Germany and Switzerland. For higher relevance, sequences from

database GenBank NC_003623 (GFLV reference sequence), DQ386866 another known Czech isolate, NC_006056 (ArMV reference sequence) and AY291208 (GDefV) (Table 1) were added to sequence analysis. All obtained sequences were submitted to NCBI/GenBank, all accesion numbers are listed in Table 1. The isolates S10 (ArMV) and K4 (ArMV) were the most interesting, obtained ArMV isolates from the view of symptomatology. Symptoms of infections of these two isolates were absolutely indefinable from appearance of GFLV infection. Those isolates were obtained from vine cultivar Pinot Noir in different production vineyards in the Czech Republic, wine region Moravia.

Nucleotide analysis: Obtained nucleotide sequences in the lenght of 267-288 nts located in position of reference sequence GFLV (1337-1603 bp) were equivalent to position of 1403-1669 bp of ArMV reference sequence. In case of nucleotide chain of reference sequence. The GDefV the region is located in 1335-1601 bp as it is closer to GFLV than ArMV genome. These sequences were subsequently aligned by software CLC Main Workbench 5.0 (CLC bio, Denmark) (Fig. 1).

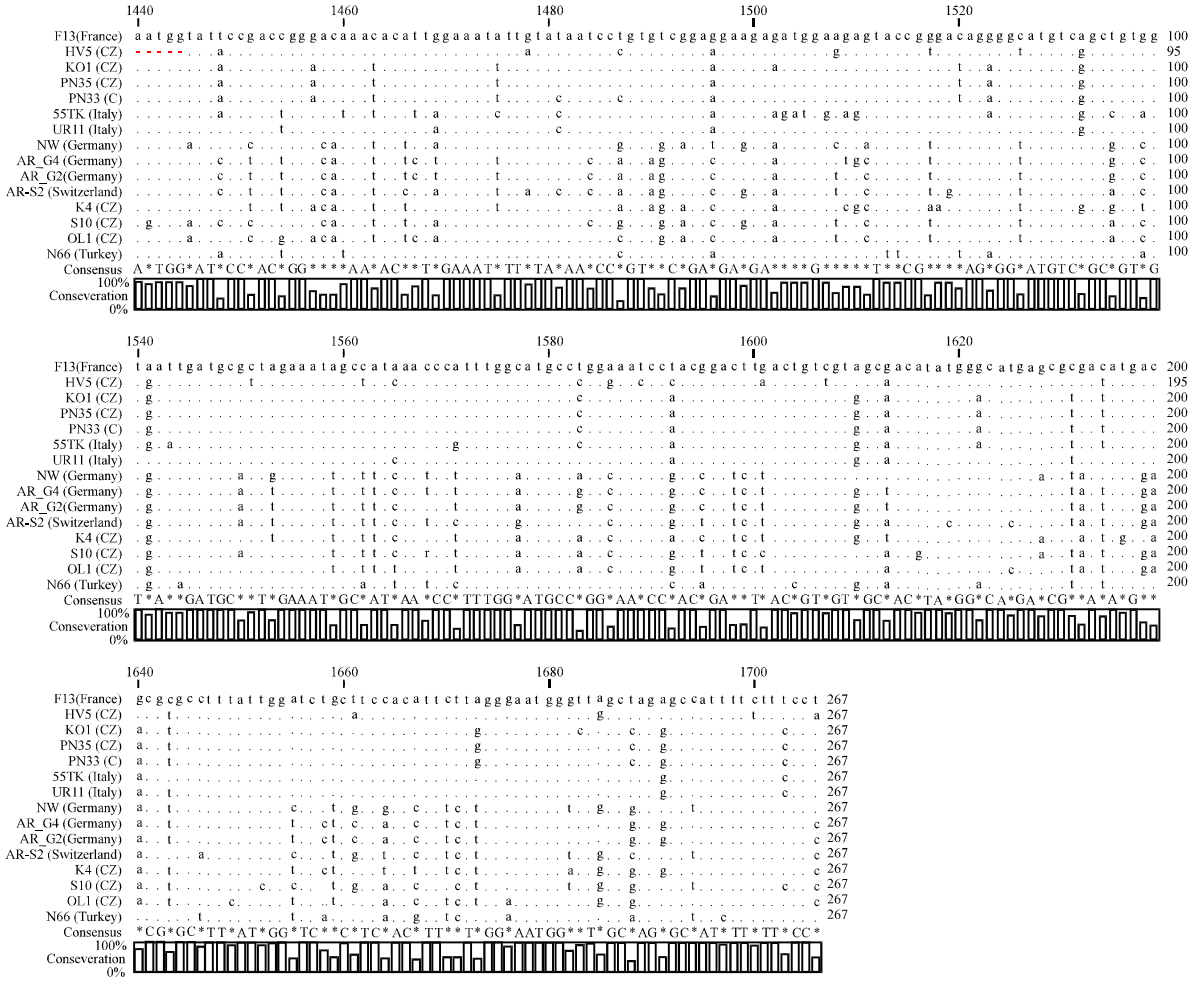

Fig. 1: Multiple alignment analysis of obtained partial sequences of 2B^{MP} GFLV, ArMV sequences supplemented by reference sequence, GFLV, ArMV and GDefV. Dots mean identical nucleotides and the lower line is consensus with an conservation degree

Figure 1 shows very close relations among members of monitored nepoviruses but some differences are clear. Particularly distinct is proportion between GFLV and ArMV sequences, GDefV sequence is more similar to GFLV sequences of monitored nucleotide region. Pairwise analysis was applied and proved interesting results, all sequences together provided percent identity from 74.5-99.3% and no gaps with the exception for sequence of HV5 where 5 nucleotides of the 5' end were missing in the alignment. Monitored sequence coding $2B^{MP}$ of GFLV is slightly more conservative than the same $2B^{MP}$ portion of ArMV as showed in Fig. 1.

Pairwise analysis of GFLV isolates was done. The highest percent identity was detected between sequences of isolates PN33 and KO1 relative to PN35, 99.25%. The lowest percentage identity 82.4% was detected between sequences of 55TK and HV5 isolates which is distorted because HV5 isolate contains 5 gaps in the alignment. The GDefV sequence is more similar to GFLV than to ArMV in the alignment of sequenced $2B^{MP}$ part. Percent identity of GDefV sequence and GFLV isolates consensus was estimated as 87.64%, GDefV and ArMV as 77.9%. Consensus of GFLV and ArMV sequences were compared and estimated percent identity was 80.2%. Pairwise analysis of ArMV isolates proved percent identity of 83.9% between sequences of S10 and K4 isolates. This is an interesting fact because both isolates come from Moravia (Czech Republic) showed the highest differences in $2B^{MP}$ region among all isolates. Maximal identity of 98.5% was between sequences AR/G2 and AR/G4 which come from the same vineyard in Germany.

The relative information content of DNA can be described by its sequence complexity, a quantity obtained from DNA reassociation kinetics. It can be measured by software CLC Genomics Workbench 6.5.1 (CLC bio, Denmark). The local complexity is a measure of the diversity in the composition of nucleotides within a given range of $2B^{MP}$ sequence. The K2 algorithm was used for calculating local complexity (Wootton and Federhen, 1993).

Based on results of pairwise analysis GFLV sequences were analyzed together with GDefV sequence mainly because of close sequence similarity. Local complexity of GFLV reference sequence was calculated from minimal value 0.254 to maximal value 0.596 (NC_003623), average value of local complexity for this sequence was 0.530. Average value of all GFLV and GDefV sequences based on monitoring of $2B^{MP}$ portion was estimated 0.530.

Local complexity of ArMV nucleotide sequences was calculated from 0.330 to 0.596 (NC_006056) of sequenced 267 nts. Average local complexity for 267 nts of all ArMV sequences was calculated as 0.533 for all ArMV sequences was estimated.

Thus, local complexity of obtained ArMV sequences is higher by 0.003 than local complexity of average of GFLV and GDefV sequences.

Amino acids analysis: Putative amino acid chains were derived from nucleotide sequences. These chains were analyzed in detail at the level of divergence for one amino acid.

Multiple alignment (Fig. 2) depicted amino acid sequences derived from nucleotides. The GFLV sequences are very conservative apart from sequence of 55TK isolate, sequence of this Italian GFLV isolate is different in 4 amino acids, in position 132 Met it is substituted by Asp, in position 133 Glu is substituted by Gly, in position 134 Glu is substituted by Arg and in position 145 Ile is subtituted by Asn. Interesingly, sequence of GDefV is different from GFLV sequences only by one amino acid in position 135 where Tyr is substituted by Phe.

ArMV sequences are more variable in monitored $2B^{MP}$ region. There is an interesting point in position 133 where sequences contain 2 types of amino acids, Glu and Asp (both amino acids have negative electric charge and are acidic), this can be supposed as a point of recombination events

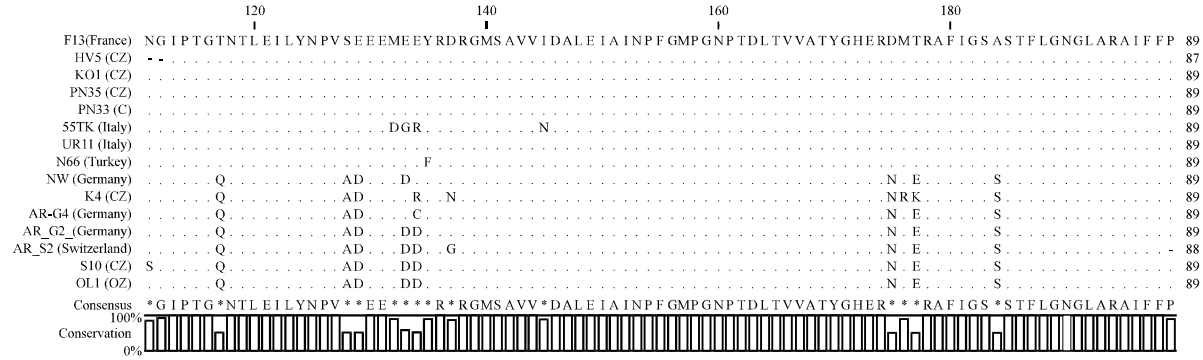

Fig. 2: Multiple alignment of deduced amino acid sequences derived from nucleotide sequences coding $2B^{MP}$

between mentioned nepoviruses. Position 134 also show similar variability level where the majority of ArMV sequences show Asp, majority of GFLV sequences Glu, AR/G4 show Cys and interesingly K4 (ArMV) and 55TK (GFLV) show Arg. Moreover, in this case there are described 2 aminoacids which are contiguous and this spot is very variable in the set of monitored sequences.

Isolate K4 and AR/S2 sequences show differences from the other GFLV and ArMV sequences also in position 137 where K4 shows Asn and AR/G2 Gly.

Positions 117, 175 and 184 are typical by their amino acids of monitored nepoviruses, according to these positions GFLV and ArMV sequences can be clearly distinguished. Only the sequence of isolate K4 shows a different amino acids in position 176 and 177.

According to this study it is possible to determine the sequences of K4 (ArMV) and 55TK (GFLV) are most distinct in the entire test set.

Pairwise analysis was done on all samples and the range of identity was from 86.5-100% (CLC Genomics Workbench 6.5.1), between GFLV and GDefV 93.3-100% and between ArMV 93.3-100%.

Phylogenetic study: Phylogenetic analysis (Fig. 3) declassifies the relationship within the monitored isolates at the amino acid level. Phylogenetic tree is divided into 2 main clusters and clearly uncovers the basic differences between GFLV and ArMV sequences also in the set of relatively short sequences. The top cluster with GFLV contains 8 sequences which are grouped of closely identical sequences of isolates from the Czech Republic (PN35, KO1, HV5 and PN33), France (F13) and Italy (UR11). Interestingly, according to phylogenetic analysis of sequences the sequence of the isolate GDefV N66 from Turkey is more similar to sequences of GFLV isolates than sequence of 55TK isolate from Italy and also than ArMV sequences. This is a clear evidence of very variable and unstable coding region from the phylogenetic viewpoint. Second cluster contains sequences of ArMV isolates which come from various plant hosts. Identical sequences have isolates OL1 from the Czech Republic and AR/G2 from Germany followed by reference sequence of isolate NW from Germany, sequence of isolate S10 from Moravia-Czech Republic comes from naturally infected grapevine planted in production vineyard, these sequences are followed by AR/S2 from Switzerland and AR/G2 from Germany. Phylogenetically, sequence of K4 from Moravia which also come from naturally infected grapevine is the most diverse at the amino acid level.

In molecular epidemiology of infectious diseases, phylogenetic inference is also an important tool. The very fast substitution rate of RNA viruses, means that these show substantial genetic

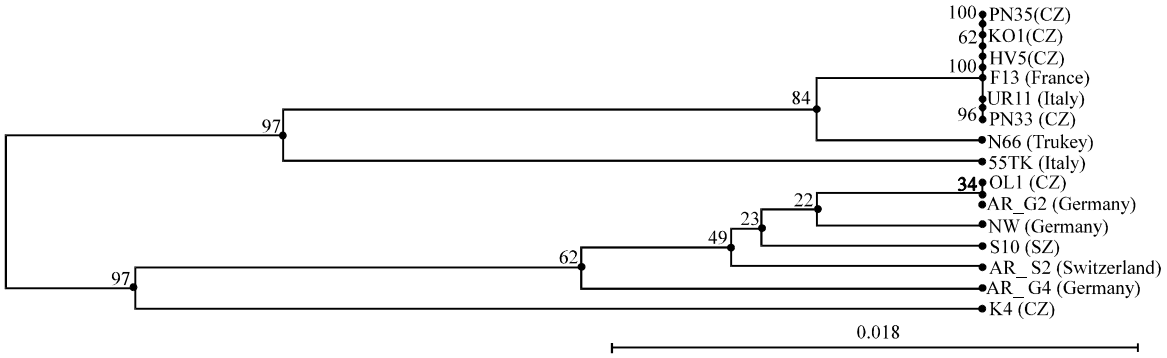

Fig. 3: Phylogenetic tree of analyzed isolates. Algorithm UPGMA, distance measure Jukes-Cantor and bootstrap 100 replicates parametres were used (CLC Genomics Workbench 6.5.1). Scale bar represents a genetic distance of 0.018

divergence over the time-scale of months and years. Therefore, the phylogenetic relationship between the pathogens from individuals in an epidemic can be resolved and contributed valuable epidemiological information about transmission chains and epidemiologically significant events (Leitner and Albert, 1999; Forsberg *et al*., 2001). Very high percent identity at nucleotide level in the study for examined partial $2B^{MP}$ gene of nepoviruses GFLV, ArMV and GDefV was approved. Partial gene $2B^{MP}$ of all examined sequences (Table 1) at the amino acid level together showed 74.5-99.3% (GFLV and GDefV) and 86.5-100% (ArMV) percent identity. Basically, the central part of the $2B^{MP}$ coding region was monitored in this study. There is a very interesting fact that sequences of GFLV and GDefV shared 93.3-100% and ArMV sequences together 99.3-100% which showed absolutely same values. Results of this study confirmed the results of Ritzenthaler *et al.* (1995) which indicated high percent identity of GFLV and ArMV amino acids in $2C^{CP}$ coding region (69%) and in $2B^{MP}$ (88%), which confirmed higher variability in coding sequence $2C^{CP}$. RNA-2-encoded proteins of GFLV and ArMV share 68-78 % similarity at the amino acid level (Vigne *et al*., 2008) which confirmed very close phylogenetic relationship of both examined nepoviruses. Percent identity of partial $2C^{CP}$ nucleotide sequences described earlier ranged from 83-86% and from 81-91% for the amino acid residues, respectively (Eichmeier *et al*., 2010). Variability of $2B^{MP}$ gene can also play an important role in transmissibility by *X. index* (Belin *et al*., 2001; Andret-Link *et al*., 2004). This is approved by the fact that chimeric virus with protein $2B^{MP}$ of GFLV origin was transmitted by *X. diversicaudatum* as well as constructs with protein $2B^{MP}$ of ArMV origin which indicates that protein $2B^{MP}$ is not involved in transmission specificity (Belin *et al*., 1999). Belin *et al.* (1999) supposed that the nine C-terminal residues of $2B^{MP}$ must be of the same virus origin as the proteinase for efficient proteolytic processing of polyprotein P2 and from the same virus origin as the $2C^{CP}$ for systemic virus spread. It would mean that recombinations could be followed by new quasispecies only in the case that all described proteolytic activities of nucleic acids were in a proper relation to species of virus. This is the main reason why it is so important to know the variability of coding sequences where recombinations can occur. The central part of the $2B^{MP}$ coding region which was desribed in this study at the molecular level clearly belongs to mentioned types of sequences with recombination potential.

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