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Molecular characterization of *Grapevine Pinot gris virus* in Poland

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DIPLOMA THESIS TOPIC

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Guides to writing a thesis:

1. Study recent publications about GPGV.
2. Describe the genome of GPGV.
3. Obtain GPGV isolates from Poland and describe the symptoms. Sequence the replicase gene and make a phylogenetic tree with the other GPGV isolates from the Europe/world. Use the NCBI database.

Mendel University in Brno

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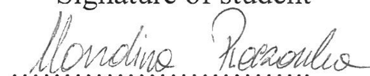

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Karta dyplomowa

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Master of Science
/ Nadawany tytuł /

Tytuł pracy w języku polskim	Molecular characterization of Grapevine Pinot gris virus in Poland
Słowa kluczowe / maksymalnie 5 słów /	wirusologia, RT–PCR, sekwencjonowanie, filogenetyka, winorośl
Streszczenie pracy / maksymalnie 1200 znaków /	Próby różnych odmian winorośli z Małopolski i Podkarpacia zostały zebrane wiosną 2016 zostały sprawdzone na obecność wirusa Grapevine Pinot gris virus (GPGV) przez RT–PCR. 16 z 65 badanych roślin dało pozytywny wynik na obecność GPGV. Do detekcji użyto dwa zestawy primerów amplifikujących część wirusowego białka płaszcza i białka transportowego, oraz część domeny RdRp, następnie oba rejony zostały zsekwencjonowane. Analiza filogenetyczna była podstawą do przyporządkowania polskich izolatów do grupy GPGV bezobjawowej. Wszystkie próby pozytywne na obecność GPGV zostały także przetestowane reakcją multiplex na obecność innych wirusów, 18,5% wszystkich przebadanych roślin było zainfekowanych GPGV i GFkV. Przedstawione badania są pierwszym doniesieniem o obecności GPGV w Polsce.
Tytuł pracy w języku angielskim	Molecular characterization of Grapevine Pinot gris virus in Poland
Słowa kluczowe / maksymalnie 5 słów /	virology, RT–PCR, sequencing, phylogenetics, grapevine
Streszczenie pracy / maksymalnie 1200 znaków /	Samples of different varieties grapevine from Małopolska and Podkarpacie collected on spring 2016 were tested for Grapevine Pinot gris virus (GPGV) by RT–PCR. 16 out of 65 plants were found positive for GPGV. Two set of primers were used for detection, targeting partial movement protein and coat protein, and RdRp domain, both of those regions were sequenced. Phylogenetic analyses settled the Polish isolates to described before asymptotic group. Also GPGV positive samples were tested by multiplex and simplex RT–PCR for multiple infections, and 18,5% of all samples were GPGV and GFkV positive. This was first survey of GPGV in Poland.

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zrealizowanej w: Mendeleum - Institute of Genetics and Plant Breeding Faculty of Horticulture Mendel University in Brno

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1. INTRODUCTION

Grapevine is one of the most wide spread cultivated plant, whether is grown for fresh consumption or for wine production. It has long tradition of cultivation and has high range of available cultivars in both groups. Nowadays Polish viticulture is experiencing come back and each year the number of vineyards is growing. The climate change, change in Polish market and in lifestyle of Poles is more favourable for cultivating grapes.

The range of grapevine pathogens is also wide, there is very many different fungal and bacterial diseases which may occur in vineyard. Those kind of pathogens may be managed by certain plant protection programs. Unlikely, as goes for viruses disease is incurable and grapevine as plant propagated mostly vegetatively is continuously at the risk of accumulating viruses.

There is many methods detecting viruses in plant cells, they are divided into three types: biological indexing, serological method and molecular assays. Nowadays molecular approach is widely used, because due its accuracy. Mostly virus detection is done by polymerase chain reaction (PCR) with specific primers.

Phylogenetic is studying the relations between organisms, and building cladograms which graphicly present the kinship based on differences in sequence. Analyses of this type may determine basic features of the, in this case, viruses. Knowledge of this matter is allowing to track down the so called ‘genetic history’ and way of spreading of virus.

Grapevine Pinot gris virus (GPGV) is new discovered grapevine pathogen. Was found in Trentino region (Italy) in 2012 by deep sequencing of small RNAs. GPGV is transmitted by grafting and recent studies confirm spreading also by mites. Phylogenetic studies divide GPGV isolates in two groups, symptomatic and asymptotic. The disease caused by symptomatic GPGV has several symptoms like chlorotic mottling, leaf deformations and shoot stunting. So far none of those were described as symptoms of GPGV or other unknown disease occurring in Polish vineyards.

2. REVIEW OF LITERATURE

2.1. VITICULTURE IN POLAND

Polish wine growing started in Middle Ages, it was added value to taking Christianity. First reports says wine was made for liturgical purpose [Tarko *et al.* 2010, on–line source: Enoturystyka]. The climatic conditions were never in much favour for growing wine in Poland. Those are just few reason why Poland has never been traditionally wine country. However present climate change, social phenomena in consumers preferences and popularity of so called “slow food” movements may change image of Poland in the winegrowing map of Europe in just few decades. [Bisson *et al.* 2002, Seguin & de Cortazar 2005, Tarko *et al.* 2010, Pink 2015].

Currently in Poland there is 376 vineyards with total areal over 400 ha and predictions are that those numbers will grow over next the years. [Tarko *et al.* 2010, on–line source: WinOgrodnicy.pl]. Most of Polish vineyards are located on south or south–east of Poland, especially regions Małopolska and Podkarpacie have the best climate conditions and therefore the highest number of vineyards. Especially Małopolska is historically wine region. Range of available and grown cultivars is very wide, but most often chosen varieties for red wine are ‘Regent’, ‘Pinot noir’, ‘Rondo’, ‘Cabernet Cortis,’ ‘Zweigelt(rebe)’, ‘Maréchal Foch’, as for white cultivars: ‘Riesling’, ‘Solaris’, ‘Seyval blanc’, ‘Johnniter’, ‘Hibernal’, ‘Chardonnay’, ‘Pinot gris’ ‘Muscat’, ‘Bianca’, ‘Jutrzenka’, ‘Traminer’ [on–line source: WinOgrodnicy.pl/].

2.2. GRAPEVINE VIRUS DISEASES IN POLAND

Evolving viticulture in Poland means growing area of vineyards as well number of grapevine pathogens, especially viruses. Free market, international trading of infected plantings is causing spreading of viral agents, therefore each year new reports appear with information about new region covered by already known virus. Also continuously studies discover new viruses in old vineyards [Giampetruzzi *et al.* 2012, Komorowska *et al.* 2014, Pleško *et al.* 2014, Maliogka *et al.* 2015, Eichmeier *et al.* 2016, Reynard *et al.* 2017].

Nowadays there is 64 different viruses belonging to different genera and families which have been reported as grapevine pathogens and in future is high probability of describing new virus species. Vital for protection against spreading of new viruses is knowledge about their vectors like mealybugs, scale and soft scale insects or dagger nematodes. On the other hand vegetative propagation of fruit crop, such as grapevine, is usually causing accumulation of numerous viruses, therefore not only single viruses but often combinations are responsible for the viral diseases. Serious negative economic affects in vineyards have viral pathogens causing symptoms as namely leafroll, rugose wood and infectious degeneration. Leafroll symptoms are associated mainly with viruses from family *Closteroviridae*. It is probably the most widespread symptomatic complex around the most profitable grapevine growing areas. Symptoms starts occurring in late summer as reddening of the leaves of red–berried and yellowing of white–berried cultivars, with later rolling leaf margin downward while veins stay green. This cause great loss in assimilation surface and effects by decreasing of fruits quality and delaying harvest maturity. Rugose wood (RW) symptoms are developing due to infection of few viruses belonging to family *Betaflexiviridae*, like *Grapevine virus A, –B, –D* (GVA, GVB GVD respectively) and *Grapevine rupestris stem pitting–associated virus* (GRSPaV). RW syndrome cause degeneration of phloem in grapevine shoots, effecting great loss in yield. Infectious degeneration syndrome, collectively known as fanleaf, is result of infection by viruses from *Secoviridae* family, genus *Nepovirus*. Fanleaf degeneration complex occurs on shoots by shortening of internodes or zig–zag growth and double nodes, on leaves by deformed margins and veins pattern that leaves may resemble a fan and finally discoloration of leaf blade. Symptoms of infectious degeneration are developing in spring starting on disfiguration of leaves [Maliogka *et al.* 2015].

Recent research done by Komorowska *et al.* [2014] shows that already there is wide range of grapevine viruses present in Polish vineyards, 23 vineyards were surveyed for occurrence of virus disease and tested by RT–PCR. Presence of *Grapevine leafroll–associated virus* (GLRaV)–1, –2, –3, *Grapevine virus* A (GVA), *Grapevine virus* B (GVB), *Grapevine virus* E (GVE), *Grapevine fanleaf virus* (GFLV), *Grapevine fleck virus* (GFkV) and *Grapevine rupestris stem pitting–associated virus* (GRSPaV) was confirmed. Viruses like GLRaV–1, –2, –3, GVA, GVB, GFLV and GFkV were also tested by ELISA and results were the same. From 460 tested plants – 172 had symptoms which later were confirm as caused by virus disease. Out of the rest 288 tested symptomless plans 171 were positive for virus presence and 37 samples showed mixed infection (at least two viral agents). The highest infection rate has *Grapevine rupestris stem pitting–associated virus*, second in order was *Grapevine fleck virus*, moreover the mix of those two viruses was in majority of samples. Overall level of viral infection in tested plants was 82.6%. According to Komorowska *et al.* [2014] this high rate and presence of double or triple infections is due to trading with initially infected plant material and uncontrolled vegetative propagation of grapevine plants.

Polish law does not allows trading of infected by any virus seedlings, but also Regulation of Ministry of Agriculture and Rural Development from day 15 July 2015 does not lists grapevine viruses by names.

2.3. METHODS FOR DETECTION OF VIRUSES

For detecting the infection of viruses there are three main methods biological indexing, serological assays and molecular assays. Any diagnostic method must take into consider uneven spreading of virus inside the host and seasonal fluctuation inside hosts tissues [Bustin & Nolan 2004]. Knowledge about biology of virus is vital, to choose proper plant body part for diagnostics, but it may be any part like: buds, leaves, stems, roots, bark scrapings and etc.

Biological indexing as the cheapest method is used as first during detection of virus presence. Also used in certification programs or in new diseases studies. This method is done by grafting tested material on the indexing susceptible variety or species (for viruses with many hosts). As an example for GFLV (*Grapevine fanleaf virus*) and GFkV (*Grapevine fleck virus*) indicator is *Vitis rupestris* 'St Gorge', for GCMV (*Grapevine chrome mosaic virus*) detection can be used *V. vinifera* 'Pinot noir' and 'Jubileum 75' and for AMV (*Alfalfa mosaic virus*) *V. rupestris* or *V. vinifera* 'Chardonnay', 'Zweigelt(rebe)', 'Mission', 'Pionot noir' [Martelli 1993]. There is also possibility to use inoculation on herbaceous hosts, widely used are species from genus *Chenopodium* and *Nicotiana*. Indexing virus is done by symptoms and keys for indexing on those plants are developed. Of course there is need to take into consideration how uncertain results may be.

More accurate but still with some marginal mistake is serological method. It is based on specific recognition of virus proteins by mean of homologous antibodies. Most widely and commercially used is the enzyme–linked immunosorbent assay (ELISA). For detecting most of grapevine viruses both direct (Double antibody sandwich, DAS) and indirect (Triple antibody sandwich, TAS) ELISA assays are possible [Martin *et al.* 2000, Maliogka *et al.* 2015].

Highest accuracy of detecting presence of virus have molecular assays. This method allows to detect viruses which may not give any symptoms during biological tests. Also is more accurate with diagnosing of more than one viral agent causing disease (several viral diseases may be caused by more than one virus). As for the advantage from serological test, it is its sensitivity, much less concentrations of viral agents may be detected. Genome of almost all grapevine viruses consist of single–strand RNA and isolation of nucleic acid for those methods is a crucial part. Grapevine contains high amounts of polysaccharides and polyphenolic compounds those substances are inhibitors of enzymes used in this method. Minimizing influence of inhibitors can be achieved by proper isolation. The most used ones are extraction of all RNA by silica–capture method, immunocapture, plant extract dilution and

filtration of sap on nylon membrane after thermal treatment. Taking into consideration specific characteristic of viral genome it is necessary of using reverse transcription–polymerase chain reaction (RT–PCR), this is the most frequently used method. During recent years several versions of RT–PCR were developed and used for detecting grapevine viruses, such as nested RT–PCR, multiplex and real–time RT–PCR (qRT–PCR) [Bustin & Nolan 2004, Maliogka *et al.* 2015]. Each of those before mentioned RT–PCRs are based on use of specific primers designed to amplify viral genome or part of it. Nested RT–PCR is based on using two set of primers, one pair used in first run covers bigger fragment of target genome, product is a template for second run. Then second nested primers set is amplifying inner part of firstly obtained fragment. That approach limits non–specific products and allows to detect smaller quantities of target gene or genome. Multiplex polymerase chain reaction use few pairs of primers during one run, it is commonly used in commercial kits. The advantage is quite cheap and quick way of obtaining information about presence of several viruses. Important is to establishing the same melting temperature for all used primers pairs. Real–time RT–PCR allows to monitor quantity of amplicons during run. The biggest advantage of qRT–PCR is ability of establishing the initial amount of template. All described variants of RT–PCRs allows indirectly confirm presence of viral genome and infection of plant. Direct confirmation is by sequencing final PCR product and then comparing it with database. Sequencing for example can be done using Sanger method [Sanger *et al.* 1977]. Moreover, micro– and macroarrays are used for detection of wide spectrum of viruses simultaneously [Thompson *et al.* 2012]. Lastly, next–generation sequencing is becoming more frequently used as a method of diagnostics of grapevine viral diseases [Giampetruzzi *et al.* 2012, Eichmeier *et al.* 2016]

2.4. GRAPEVINE PINOT GRIS VIRUS

2.4.1. Taxonomy of GPGV

Grapevine Pinot gris virus (GPGV) is classified to order *Tymovirales*, family *Betaflexiviridae*, subfamily *Trivirinae*. GPGV belongs to genus *Trichovirus* with six other species as: *Apple chlorotic leaf spot virus*, *Apricot pseudo–chlorotic leaf spot virus*, *Cherry mottle leaf virus*, *Peach mosaic virus*, *Phlomis mottle virus* and *Grapevine berry inner necrosis virus* (GINV) [Maliogka *et al.* 2015, on–line source: ICTV]. Family of *Betaflexiviridae* consist of 87 different species of mainly plant viruses associated with symptoms like mosaic and ringspots, as grapevine pathogen is related to rugose wood syndrome. All representatives of this family have one positive–sense single–strand RNA, and genome is closed in coat protein, called capsid. The shape of whole virus is filamentous, with length from 600 nm to 1000 nm or even more, with diameter 12–13 nm (fig. 1) [on–line source: ExPASy].

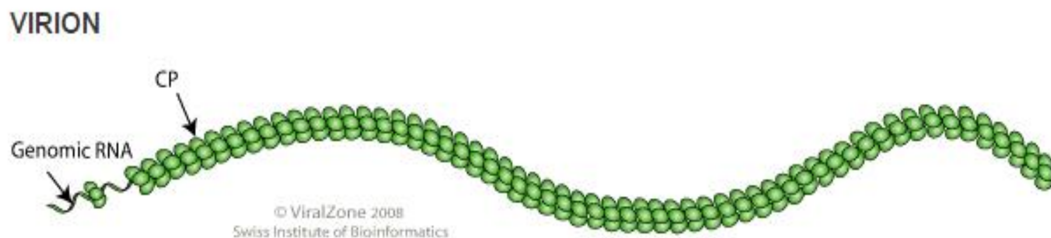

Fig 1. Demonstration of exemplary virus from the family *Betaflexiviridae*. CP – coat protein. [On–line source: ExPASy]

2.4.2. Symptoms

GPGV is one of many grapevine pathogens. Discovered by deep sequencing of small RNAs during study on 'Pinot noir' and 'Pinot gris' grapevine cultivars in the Trentino region (Italy) described by Giampetruzzi *et al.* [2012]. GPGV from the beginning was divided into symptomatic and asymptomatic groups. Symptoms, if they do, may occur on leaves by chlorotic mottling and deformations or shoot stunting (fig. 2.). Biological indexing by Saldarelli *et al.* [2015] was carried out on 100 plants and by four years observation on indicator species *Vitis vinifera* 'Cabernet franc' and *Vitis rupestris* grafted in symptomless and symptomatic plants of 'Pinot gris' vine, did not shown any symptoms in both cases. On the other hand using of 'Pinot gris' or 'Traminer' as an indicators allows symptoms of GPGV infection occur on first year after grafting on symptomatic tested plants.

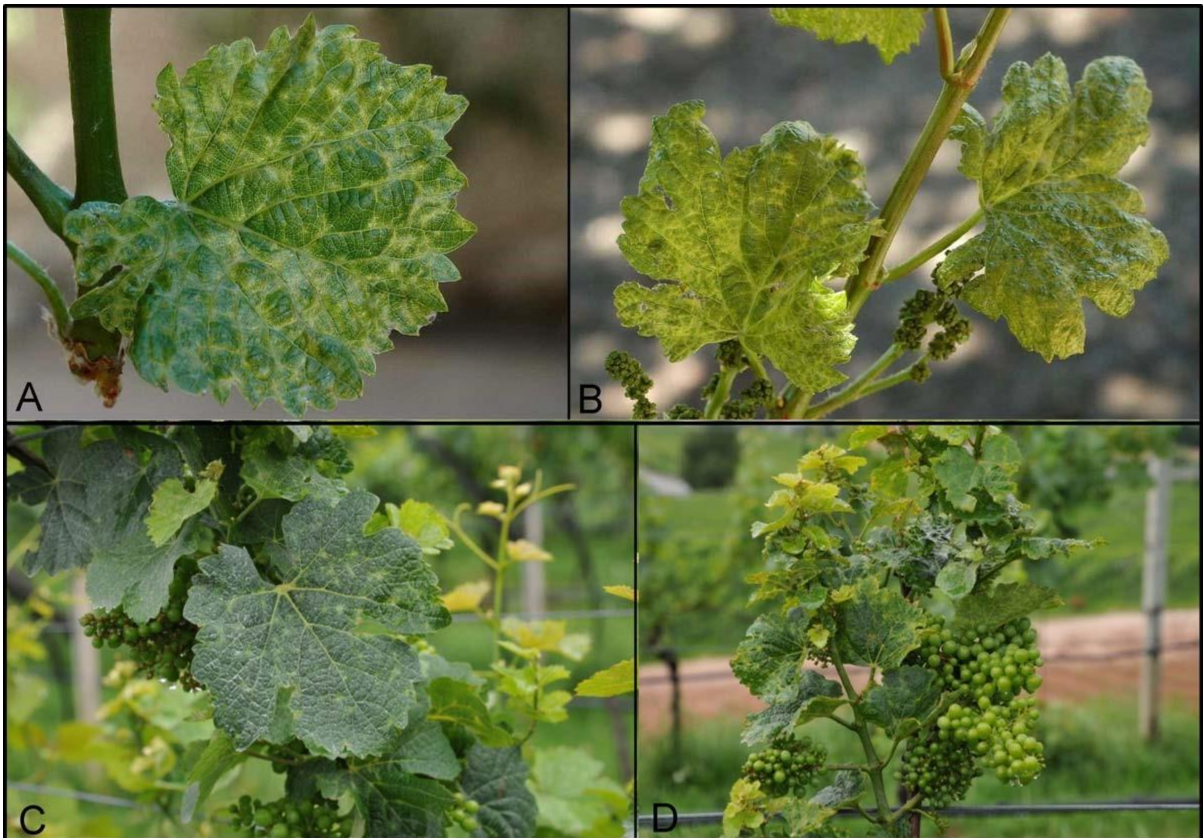

Fig. 2. Symptoms of GPGV in *V. vinifera* 'Pinot gris' – chlorotic mottling and leaf deformations (A and B) and chlorotic mottling and shiit stunting in 'Traminer' (C and D) [Giampetruzzi *et al.* 2012].

Saldarelli *et al.* [2015] explains this fact due to its close relationship to *Grapevine inner necrosis virus* and variability in symptoms occurrence may be related to frequent recombination events between both viruses. As the newest studies shows occurrence of symptoms is not related only to genetic variability of GPGV strain but as well to concentration of viral agent inside host cells. Bertazzon *et al.* [2016] study six grapevine plants from variety 'Glera', all infected by GPGV. Three had different intensification of symptoms, and three symptomless. Monthly leaves were sampled and tested by qPCR for GPGV presence. Results shows the relative higher concentration of GPGV is closely related with occurring symptoms of disease. Moreover, during season concentration was decreasing in both groups of tested plants.

Fig. 3. Symptoms of inner necrosis of berries caused by GPGV found on *Vitis viniferax x V. labrusca* variety 'Tamnara' in Korea [Cho *et al.* 2013]

In Korea observations on hybrid grapevine *Vitis viniferax x V. labrusca* variety 'Tamnara' shows symptoms of inner necrosis of berries (fig. 3) and poor yielding, characteristic to GINV. However later tests by RT–PCR targeting coat protein of GPGV confirm those symptoms are due to *Grapevine Pinot gris virus* infection. [Cho *et al.* 2013].

Research done by Malagnini *et al.* [2016] shows that vector transmitting *Grapevine Pinot gris virus* among the other *V. vinifera* plants is done by eriophyid mite *Colomerus vitis*. Studies confirmed presence of virus RNA in mites and moreover later transmission viral genome to new plants. Study was carried in three experiments on varieties 'Traminer', 'Pinot gris' and 'Glera', from plants infected by GPGV and infested by *C. vitis*, mites were removed and relocated closely to heathy shoot tips. Later observations did not report occurring symptoms of GPGV infection, nevertheless later PCR analyse confirm that on five out of 24 plants virus was transmitted to new host. *Colomerus vitis* is mite which also is vector of other grapevine virus from genus *Trichovirus*: *Grapevine berry inner necrosis virus* (GINV).

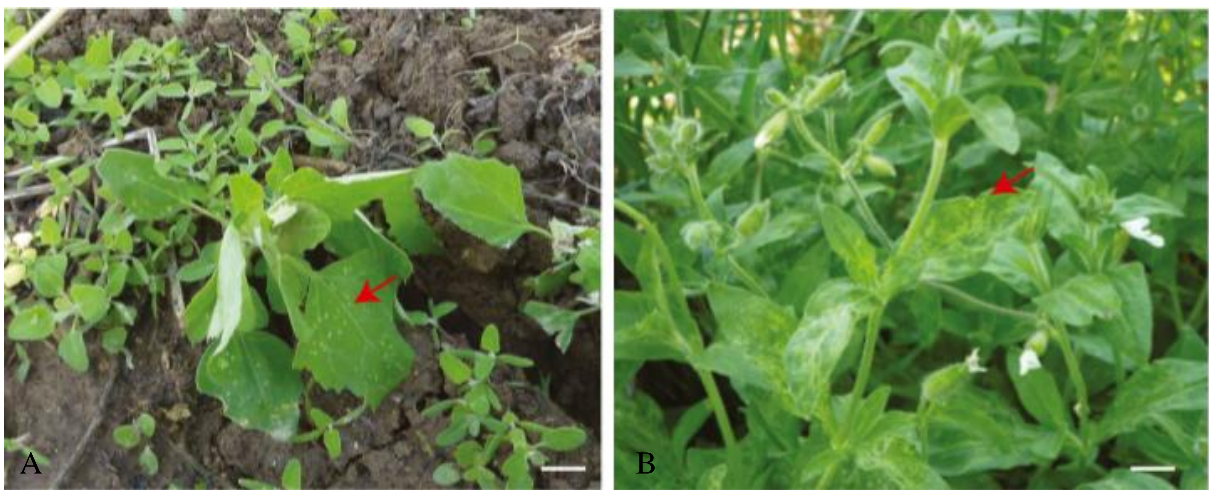

Fig 4. Symptoms of virus–related infection of GPGV on *Chenopodium album* L. (A) and *Silene latifolia* subsp. *Alba* (Mill.) (B). Arrow shows chlorotic and mottling spots (respectively). Bar = 1 cm. [Gualandri *et al.* 2016]

Grapevine may be not only host of GPGV. Researchers, Gualandri *et al.* [2016], spoted herbaceous plants of *Silene latifolia* subsp. *Alba* (Mill.) (bladder campion) and *Chenopodium album* L. (white goosefoot) showing symptoms like chlorotic and mottling spots (fig. 4). Those weeds were growing between the rows in vineyard, therefore later studies were targeting grapevine viruses, especially GPGV. Further analyses confirm that bladder campion and white goosefoot are infected by *Grapevine Pinot gris virus*. This was first report of GPGV infecting non–*Vitis* host. So far, among other grapevine viruses only *Grapevine fanleaf virus* (GFLV) have same ability, it was found on *Cynodon dactylon* [Izadpanah *et al.* 2003].

Studies reveal that currently GPGV reached almost all primary wine producing European countries among others Italy, France, Portugal, Germany, Slovenia, Slovakia, the Czech Republic, Greece, Turkey, Bosnia, Croatia, Romania, Serbia, Spain, and Ukraine [Giampetruzzi *et al.* 2012, Glasa *et al.* 2014, Pleško *et al.* 2014, Beuve *et al.* 2015, Gazel *et al.* 2016, Reynard *et al.* 2016, Ruiz–García & Olmos 2017, Vončina *et al.* 2017]. Also several reports shows that GPGV is widespread worldwide, in countries such as Korea, China, the United States and Canada was confirmed occurrence of GPGV [Cho *et al.* 2015, Fan *et al.* 2016, Rwahnih *et al.* 2016, Xiao *et al.* 2016]. The newest report, where the results of this work are included by Eichmeier *et al.* [2017], confirm presence of GPGV in Poland.

2.4.3. GPGV genome

Genome of *Grapevine Pinot gris virus* is characteristic to its genus *Trichovirus* and has identical to *Grapevine berry inner necrosis virus* (GINV) organization of genome. GPGV consists of one positive–sense single–stranded RNA with three overlapping ORF's (open reading frames). ORF1 encodes the replicase–associated proteins, a viral methyltransferase, a viral RNA helicase and RNA depended RNA polymerase (RdRp), size of complete ORF1 is 1855 amino acids (aa) (214 kDa). Also it consists of the AlkB domain, a partial HxD motif, residues essential for Fe^{2+} coordination (a 2OG-Fe (II) oxygenase). ORF2 consist of 376 aa polypeptide (42 kDa) it codes GPGV movement protein (MP). Lastly ORF3 encodes the 195 amino acids 22kDa in size and coat protein gene (CP) is located there (fig. 5) [Giampetruzzi *et al.* 2012, Beuve *et al.* 2015, Gualandri *et al.* 2016].

AlkB domain is one of members of 2-oxoglutarate- and Fe(II)-dependent oxygenase superfamily. Function of viral AlkB protein is part of protecting virus against the post–transcriptional gene silencing (PTGS) system discovered in plants [Bratlie & Drabløs 2005]. The partial HxD motif in eukaryotic cells is part of functional protein kinases [Kannan & Neuwald 2005]. Viral RNA helicase was found to be involved in remodelling and disassembly of RNP complexes [Kalinina *et al.* 2001]. RdRp – RNA depended RNA polymerase takes part in virus proliferation. Movement protein is responsible for transmitting virus between host cells trough the plasmosesmata. Coat protein (capsid) is covering the genome and protects the viral RNA from mechanical and enzymatical damages.

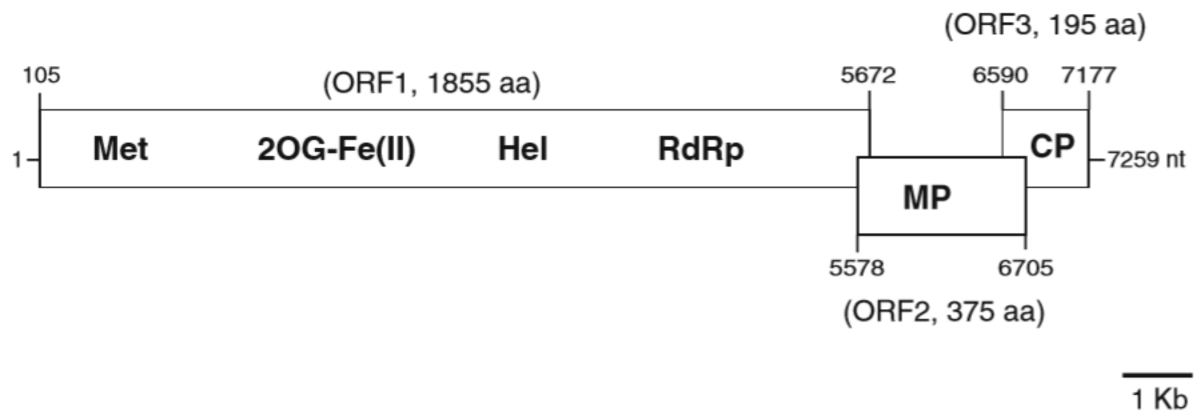

Fig. 5. Organization of GPGV RNA genome based on GPGV_FEM01 clone GenBank accession no KU312039.1 [Gualandri *et al.* 2016]. nt – nucleotides; Met – methyltransferase; 2OG-Fe (II) – 2OG-Fe (II) oxygenase; Hel – viral RNA helicase; RdRp – RNA dependent RNA polymerase; MP – movement protein; CP – coat protein.

2.4.4. GPGV detection

Symptomatic isolates of GPGV may be detected firstly by studying symptoms in host plant or by grafting to the indicator variety such as 'Pinot gros' and 'Traminer' (those cultivars are most susceptible to GPGV infection) [Saldarelli *et al.* 2015]. But this method is not efficient and accurate, mainly because majority of grapevine virus infection are double or triple (sometimes even more) causing several symptoms often covering one another. Furthermore GPGV has the two separate groups of isolates symptomatic and asymptotic, second group is impossible to detect even on susceptible indicators.

Therefore GPGV detection is done by polymerase chain reaction (PCR) with specific primers and its variations. The most commonly used set of primers is targeting most homogenic sequence covering end of movement protein and partial coat protein sequence (MP/CP fragment). Other set of primers, often used in GPGV detection, is targeting partial sequence of viral RNA polymerase domain (RdRp). This section of GPGV genome is less homogenic then MP/CP region. Sequence of those amplicons are widely used in phylogenetic studies on *Grapevine Pinot gris virus* [Saldarelli *et al.* 2015, , Malagnini *et al.* 2016, Eichmeier *et al.* 2017]. Based on studies by Bianchi *et al.* [2015], GPGV can be easily detected by real–time RT–PCR with primers and probes designs for two mentioned before regions. The advantage with this approach allows to obtain also initial concentration of virus in homogenate.

Lastly novel next generation sequencing (NGS) of libraries of small RNAs may be used for detection of GPGV. Moreover this technic is detecting wide range and multi infections in the same time. GPGV was discovered by usage of NGS by Giampetruzzi *et al.* (2012). Later sequences of all genome were also obtained by this method [Glasa *et al.* 2014, Eichmeier *et al.* 2016].

2.5. PHYLOGENETICS

Term 'phylogenetics' was proposed by Ernst Haeckel in his work Generelle Morphologie der Organismen from 1866. He, as a first, noticed certain traits are primary, and other are occurring during ontogenesis. Therefore is possible to establish timeline on which events of developing new traits are marked. Also this knowledge allows investigating of history and relationships between organisms, groups, species, populations etc. It was start and became a heart of understanding present view of biodiversity and evolution [Edwards & Cavalli–Sforza 1963]. Nowadays correlations of this sort are determine by certain specially designed algorithms. The idea, behind it, is to evaluate relationship by heritable traits – morphological or more homogenic such as DNA or RNA (in case of viruses). Algorithms based on differences in DNA or RNA sequences are calculating the kindship. Results are shown in form of cladogram, called also phylogenetic tree. Each tree consist of tips, nodes and branches. Node is where branches meet and each node is representing the speciation event, time distance between present and speciation event may not be always properly estimated. Nevertheless the oldest node, called the root, is establishing first differentiation of trait, DNA mutation etc. On top of the branches are tips, they are the smallest taxonomic units presented in a phylogenetic tree. Length of branches is often representing the time. Mostly trees are binary which means that no more than two branches are connected in one node. Phylogenetic trees also are determine by their balance – symmetry, properly balanced tree is called pectinate, chained or comblike. However, it is possible to prepare polytomie, tree out of balance which allows to have nodes connecting three or more branches. It means speciation event was much more complex, and the differentiation developed 3 traits out of one. Still disputable is interpretation of presented on tree patterns in evolutionary process, always is possibility of inaccurate estimation. Moreover several studies confirm different methods lead to different estimations, and final results consequently gives trees with different balance [Mooers & Heard 1997].

Study carried out by Chare & Holmes [2005] have conducted phylogenetic survey of recombination frequency in 12 out of 36 positive–sense RNA virus species. They used 975 capsid gene sequences and 157 complete genomes. The most frequent recombination was found in *Potyviruses* (examples of *Potyviruses* are: *Potato virus Y* (PVY), *Plum pox virus* (PPV), *Tobacco vein mottling virus* (TVMV)). Study confirm also the coat protein gene has higher homogeneity in comparison to alignments with whole sequences, 17% and 44% evidences of recombination respectively.

2.5.1 Phylogenetics of GPGV

Grapevine Pinot gris virus is especially interesting in point of phylogenetic studies due to its characteristic division into symptomatic and asymptotic groups of isolates.

Phylogeny presented by Glasa *et al.* [2014] for Slovak isolates, already had unusual patterns of divergence which could not be comparable to other *Trichoviruses*. It was noticed by accumulation of the indel polymorphisms in ORF1 in three short regions. Also was pointed out that amino acid sequences of those regions had higher similarity to GINV than GPGV.

But firstly Saldarelli *et al.* [2015] discovered association between sequences and the occurrence of symptoms on infected plant. Presented by him phylogenetic trees divide symptomatic and asymptotic GPGV isolates. Moreover analyses of MP/CP region and RdRp domain sequence shows almost same results, in case of RdRp the root already isolate all investigated samples, for MP/CP region the division was in second node. This means the symptomatic and symptomless GPGV can be easily distinguish by its sequence.

3. AIM OF THE EXPERIMENT

The aim of presented work is to characterize Polish isolates of *Grapevine Pinot gris virus* and by phylogenetic studies determine symptomatic or symptomless character of this virus in Poland.

4. MATERIAL AND METHODS

4.1. MATERIAL

In April and May 2016 from 12 varieties and 6 unidentified varieties random sampling was carried out. Total number of tested samples was 65 (tab. 1). Origins of tested grapevine plants were three vineyards and two private gardens, all localized in southern Poland near Kraków and Rzeszów (fig. 6). Age of plant was less than 10 years for the samples from Garlica, Srebrna Góra and Smardzowice, plants tested from Jasionka were older, had more than 30 years.

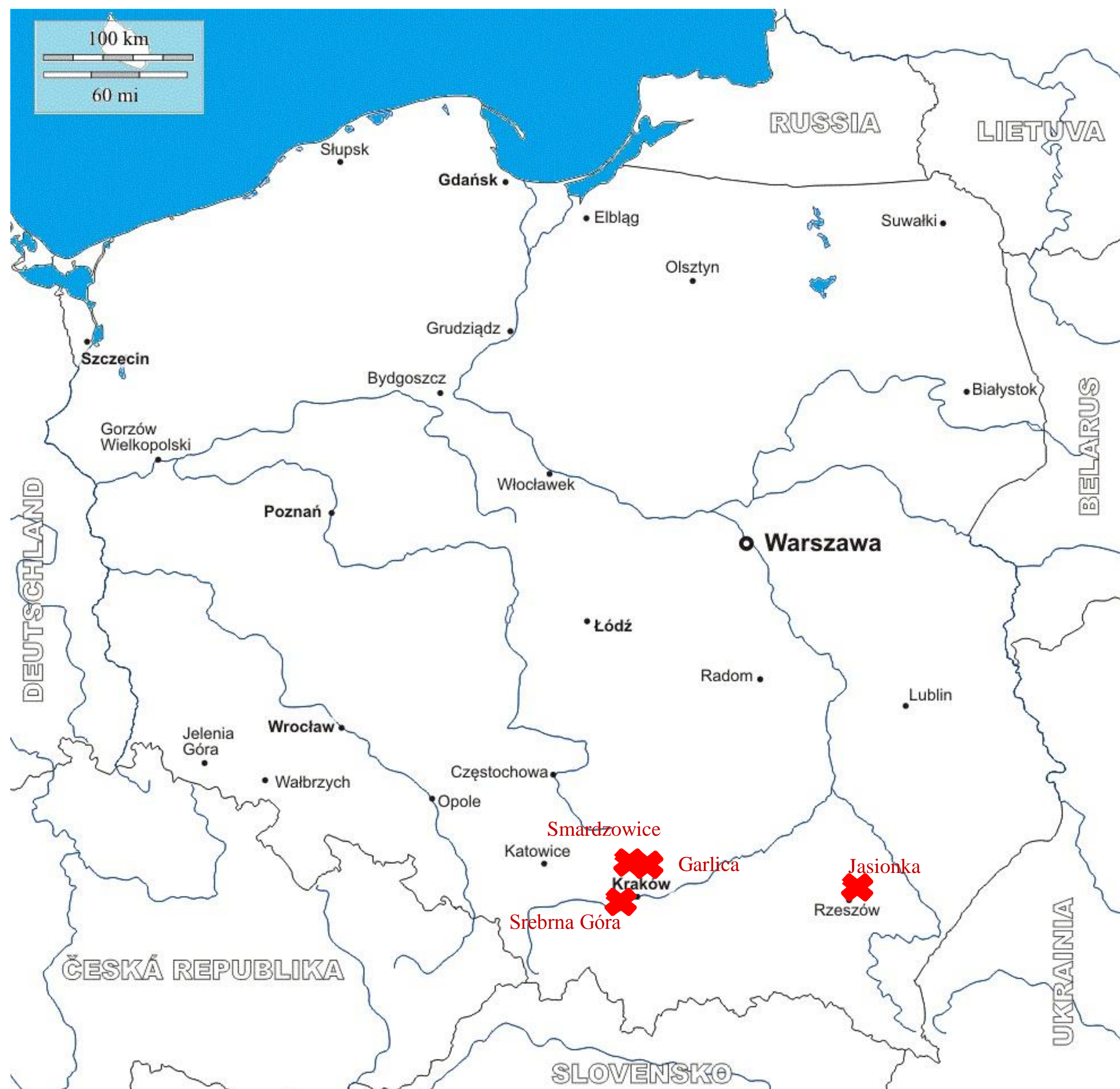

Fig. 6. Map of Poland with marked origin of samples used in research. http://d–maps.com/.

Table 1. List of samples with their origin.

No.	Variety	Vineyard	No.	Variety	Vineyard
1	Aurora	Garlica	40	Regent	Garlica
2	Aurora	Garlica	41	Jutrzenka	Garlica
3	Aurora	Garlica	42	Jutrzenka	Garlica
4	Bianca	Garlica	43	Jutrzenka	Garlica
5	Bianca	Garlica	44	Jutrzenka	Garlica
6	Bianca	Garlica	45	Jutrzenka	Garlica
7	Hibernal	Garlica	46	Jutrzenka	Garlica
8	Hibernal	Garlica	47	Jutrzenka	Garlica
9	Johaniter	Garlica	48	Jutrzenka	Garlica
10	Johaniter	Garlica	49	Jutrzenka	Garlica
11	Johaniter	Garlica	50	Jutrzenka	Garlica
12	Jutrzenka	Garlica	51	Bianca	Garlica
13	Jutrzenka	Garlica	52	Bianca	Garlica
14	Leon Millot	Garlica	53	Bianca	Garlica
15	Leon Millot	Garlica	54	Bianca	Garlica
16	Leon Millot	Garlica	55	Solaris	Srebrna Góra
17	Marechal Foch	Garlica	56	Solaris	Srebrna Góra
18	Marechal Foch	Garlica	57	Solaris	Srebrna Góra
19	Muskat Odeski	Garlica	58	Solaris	Srebrna Góra
20	Muskat Odeski	Garlica	59	Solaris	Srebrna Góra
21	Regent	Garlica	60	Solaris	Srebrna Góra
22	Regent	Garlica	61	Solaris	Srebrna Góra
23	Regent	Garlica	62	Solaris	Srebrna Góra
24	Rondo	Garlica	63	Leon Millot	Smardzowice
25	Rondo	Garlica	64	Marechal Foch	Smardzowice
26	Seyval Blanch	Garlica	65	Bianca	Jasionka
27	Seyval Blanch	Garlica	66	NN* (white table grapes)	Jasionka
28	Seyval Blanch	Garlica	67	NN (white table grapes)	Jasionka
29	Solaris	Garlica	68	NN (white table grapes from greenhouse)	Jasionka
30	Solaris	Garlica	69	NN (red table grapes)	Jasionka
31	Solaris	Garlica	70	NN (blue table grapes)	Jasionka
38	Regent	Garlica	71	NN (blue table grapes)	Jasionka
39	Regent	Garlica			

* – Unknow variety

None of grapevine plants had visible symptoms of GPGV infection (Appendix 1, 2 & 3).

Isolation of RNA was carried out directly from the homogenate from bark tissue scrapings with Spectrum Plant Total RNA Kit (Sigma Aldrich, St. Louis, Missouri, USA).

4.2. REVERSE TRANSCRIPTION

Reverse transcription from RNA to cDNA (RT–PCR) was prepared in two steps. Protocol for First Strand cDNA Synthesis was used, prepared by kit RevertAid™ Reverse Transcriptase (Fermentas, Bourlington, Canada). First step was denaturation in 95ºC for 5 minutes of 2 µl of isolated RNA, 0,5 µ Oligo(dT) primer (10 mM) Roche with water (HPLC purity) up to 12,5 µl volume. Second was reverse transcribing in 42ºC for 60 minutes with previously denatured template. Mix consist of 1× RT buffer (Fermentas, Bourlington, Canada), 0.2 mM dNTPs (AppliChem, Darmstadt, Germany) and 200U reverse transcriptase M–MLV–RT (Fermentas, Bourlington, Canada) were added to final volume 20 µl.

Quality of reverse transcription reaction was made by PCR for internal positive control in this case for partial malate dehydrogenase gene (MDH), by use of C1163 and H968 primers (tab. 2) [Stewart & Nassuth 2001].

4.3. PCR

All isolated samples were tested twice by the PCR, each reaction with different pair of primers (tab. 2). The first primer pair (DetF/DetR) were targeting partial MP/CP genes sequences (primer forward is starting sequence from end of the movement protein (MP) and reverse ends sequence at the beginning of the coat protein (CP) gene [Morelli *et al.* 2014]). Second pair (GPGVRepF/GPGVRepR) covered sequence of RNA–dependent polymerase RNA (RdRp) domain of the GPGV replicase gene and the conditions of reaction were: initial denaturation at 94ºC for 2 minutes; followed by 30 cycles: started by 94ºC for 30 s, 60ºC for 40 s, and 72ºC for 45s, as a final extension 72ºC for 7 min were used.

Table 2. DNA primers used for PCR amplification for internal positive control – MDH, specific targeting sequences of partial MP/CP sequence and RdRp domain of GPGV.

Primer name	Primer sequence	Product size (bp)
MDH–C1163	5’ CCTTTGAGTCCACAAGCCAA 3’	196
MDH–H968	5’ GCATCTGTGGTTCTTGCAGG 3’	
DetF	5’ TGGTCTGCAGCCAGGGGACA 3’	588
DetR	5’ TCACGACCGGCAGGGAAGGA 3’	
GPGVRepF	5’ TGAGGCATTCGATGTTTCCCA 3’	525
GPGVRepR	5’ ACCCAATCAAGCCATGAACCT 3’	

Mix for polymerase chain reaction consist of water (HPLC purity), 1× DNA polymerase buffer, 25mM $MgCl_2$ Solution, 0.2mM dNTPs, 1U G2 Flexi DNA polymerase (Promega, Madison, Wisconsin, USA) and set of chosen primers 0.1mM of each one (upstream and downstream) [Eichmeier *et al.* 2017].

Separation of PCR products was on a 1.3% agarose gel in 1× TBE buffer.

All samples which were positive for GPGV infection were also tested by Multiplex RT–PCR reaction by QualiPlante Detection Kit for Grapevine virus PCR.7VV–100Liq. This test covered seven most common viruses present in grapevine plants: ArMV (*Arabis mosaic virus*), GFLV (*Grapevine fanleaf virus*), GLRaV–1 (*Grapevine leafroll associated virus 1*), GLRaV–2 (*Grapevine leafroll associated virus 2*), GLRaV–3 (*Grapevine leafroll associated virus 3*), GVA (*Grapevine virus A*) and GFkV (*Grapevine fleck virus*).

4.4. SEQUENCING

Sequencing was done starting with asymmetric PCR prepared with the reverse primers by means dideoxy chain termination method, using the BigDye® Terminator v3.1 (Applied Biosystems, Carlsbad, USA) kit. Conditions for reaction were following initial denaturation 96ºC for 1 min and then 30 cycles of 96ºC for 20 s, 30ºC for 15 s, and 60ºC for 4 min, change of temperature were 1ºC in 1 s. Cleaning from reaction mix was made by BigDye® XTerminatior™ Purification Kit provided by Applied Biosystems. Final step – separation of the fragments was done on an ABI PRISM 310 genetic analyser (Applied Biosystems, Carlsbad, USA).

4.5. BIOINFORMATICS ANALYSIS

Obtained sequences were analysed by BLAST for conformation of affiliation to GPGV. Later nucleotide sequences were treated by means of two programs CLC Genomic Workbench 6.5 (CLC bio, Aarhus, Denmark) and MEGA 7.0.18. Based on each of two genome fragments (MP/CP and RdRp domain) phylogenetic trees were constructed. Isolates from Poland were compared to other available in NCBI gene database and assign to proper group – symptomatic or symptomless.

5. RESULTS

5.1. AMPLIFIABILITY OF RNA EXTRACTS

Firstly all isolates were checked for internal positive control with primers targeting malate dehydrogenase gene (MDH). Amplification was successful in every case (fig . 7). Therefore all samples were taken into further study of virus detection.

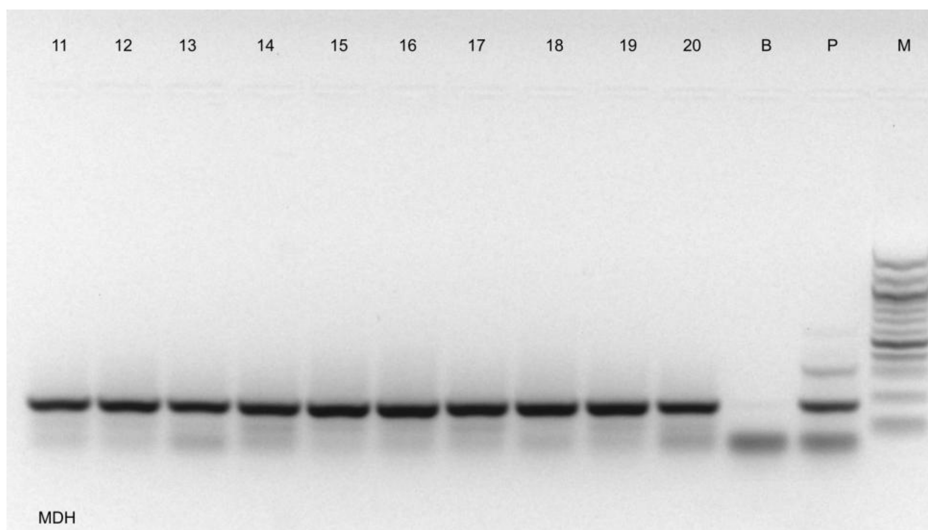

Fig. 7. Internal positive control. Separation of PCR products on 1,3% agarose gel, amplification with primers targeting MDH gene (196 bp) – chosen samples 11–20. (B – blanc, P – positive control, M – size marker). Photography Dr inż. Z. Gajewski.

5.2. DETECTION OF GPGV IN SAMPLES

During research, field observation, none of selected to research plants showed symptoms characteristic to GPGV infection (see Appendix 1, 2 & 3). Nevertheless all samples were tested by two pair of starters for GPGV presence (see Appendix 4). Test by PCR confirmed GPGV presence in 6 isolates amplifying MP/CP region and 16 isolates in sequence of RdRp domain. Amplification with primers MP/CP did not show any result for samples 41–45, 47, 48, 50, 67 and 68 (fig. 8 A) while PCR products for RdRp region were amplified (fig. 8 B, tab. 3). Two samples from unknow variety are positive for GPGV infection but product of amplification was very weak.

All tested plants form variety ‘Jutrzenka’ and two ‘Solaris’ were positive for GPGV infection. Localization of infected plants was in 2 out of 3 wine regions (Garlica, Srebrna Góra and Jasionka), only samples from Smardzowiece vineyard were negative for the GPGV presence.

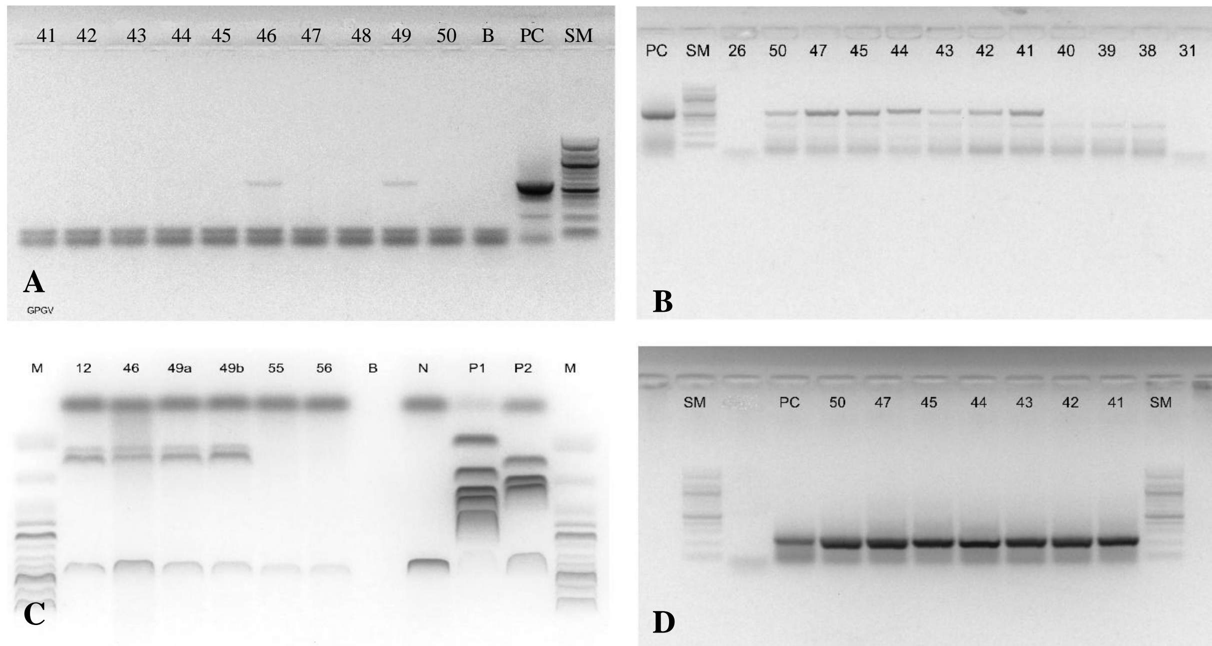

Fig 8. Separation of PCR products. M or SM – size marker; B – blanc; PC – positive control; A – after amplification with primers targeting MP/CP fragment (588bp). Photography Dr inż. Z. Gajewski; B – amplification with primers targeting partial RdRp domain (525 bp); C – multiplex reaction with starters targeting ArMV, GFLV, GLRaV–1, –2, –3, GVA and GFkV (N – negative control, P1 – positive control (from the bottom respectively) GLRaV–2, ArMV, GLRaV–3, GLRaV–1,GFLV, P2 – GLRaV–3, GVA, GFkV; D – amplification with specific primers for GFkV.

Samples positive for GPGV presence were also tested for other viruses infection by multiplex reaction designed to detect ArMV, GFLV, GLRaV–1, –2, –3, GVA and GFkV (fig. 8 C), 12 out of 16 gave positive result for *Grapevine fleck virus* (GFkV) (tab. 3). All samples positive for GFkV also showed second product slightly shorter than expected for GFkV. Therefore second PCR with specific primers targeting GFkV was done to validate previous results (fig. 8 D).

Table 3. Characteristics of isolates positive for *Grapevine Pinot gris virus* presence.

No. of sample	Variety	Vineyard	Origin of plants	GPGV detection MP/CP	GPGV detection RdRp domain	Other present viruses
12	Jutrzenka	Garlica	Comercials plantations near Jasło (Poland)	+	+	GFkV
13	Jutrzenka	Garlica	Comercials plantations near Jasło (Poland)	weak PCR product	+	GFkV
41	Jutrzenka	Garlica	Comercials plantations near Jasło (Poland)	–	+	GFkV
42	Jutrzenka	Garlica	Comercials plantations near Jasło (Poland)	–	+	GFkV
43	Jutrzenka	Garlica	Comercials plantations near Jasło (Poland)	–	+	GFkV
44	Jutrzenka	Garlica	Comercials plantations near Jasło (Poland)	–	+	GFkV
45	Jutrzenka	Garlica	Comercials plantations near Jasło (Poland)	–	+	GFkV
46	Jutrzenka	Garlica	Comercials plantations near Jasło (Poland)	+	+	GFkV
47	Jutrzenka	Garlica	Comercials plantations near Jasło (Poland)	–	+	GFkV
48	Jutrzenka	Garlica	Comercials plantations near Jasło (Poland)	–	+	GFkV
49	Jutrzenka	Garlica	Comercials plantations near Jasło (Poland)	+	+	GFkV
50	Jutrzenka	Garlica	Comercials plantations near Jasło (Poland)	–	weak PCR product	GFkV
55	Solaris	Srebrna Góra	Comercials plantations in Geisenheim region (Germany)	+	+	
56	Solaris	Srebrna Góra	Comercials plantations in Geisenheim region (Germany)	+	+	
67	NN* (white table grapes)	Jasionka	unknown	–	weak PCR product	
68	NN (white table grapes from greenhouse)	Jasionka	unknown	–	weak PCR product	

* – Unknown variety

Therefore 22% of all tested plants were GPGV positive and 75% out of them had double infection, this gives 18,5% double infections among all samples.

5.3. SEQUENCING

Amplified fragments of GPGV genomic RNA regions 5 isolates (MP/CP) and 13 isolates (RdRp domain) were sequenced. All of them are available in GenBank with accession Nos KX611835–KX611846 and KX674682–KX674687 [Eichmeier *et al.* 2017]. At nucleotide level identity between sequences was from 98 to 99% for the MP/CP gene and from 94 to 99% of the RdRp domain.

5.3.1. Phylogenetic analysis

Analyse of two sequenced fragments of GPGV genome was done with MEGA 7. Five sequences of MP/CP fragment were compared to chosen sequences from Saldarelli *et al.* [2015] and all of Polish are within asymptotic group (in box fig. 9).

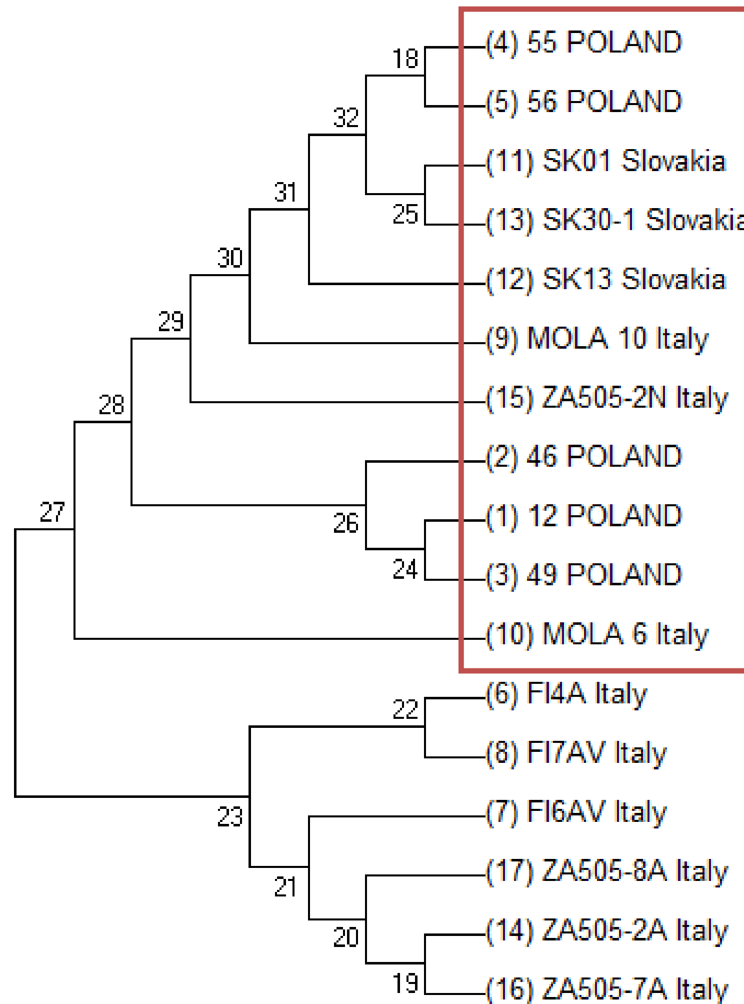

Fig. 9. Cladogram of Maximum Likelihood of MP/CP sequence (280 bp). Polish isolates (tab. 3) and isolates published by Saldarelli *et al.* [2015] were determined asymptotic (in box) or symptomatic. Analyse was done by MEGA 7.

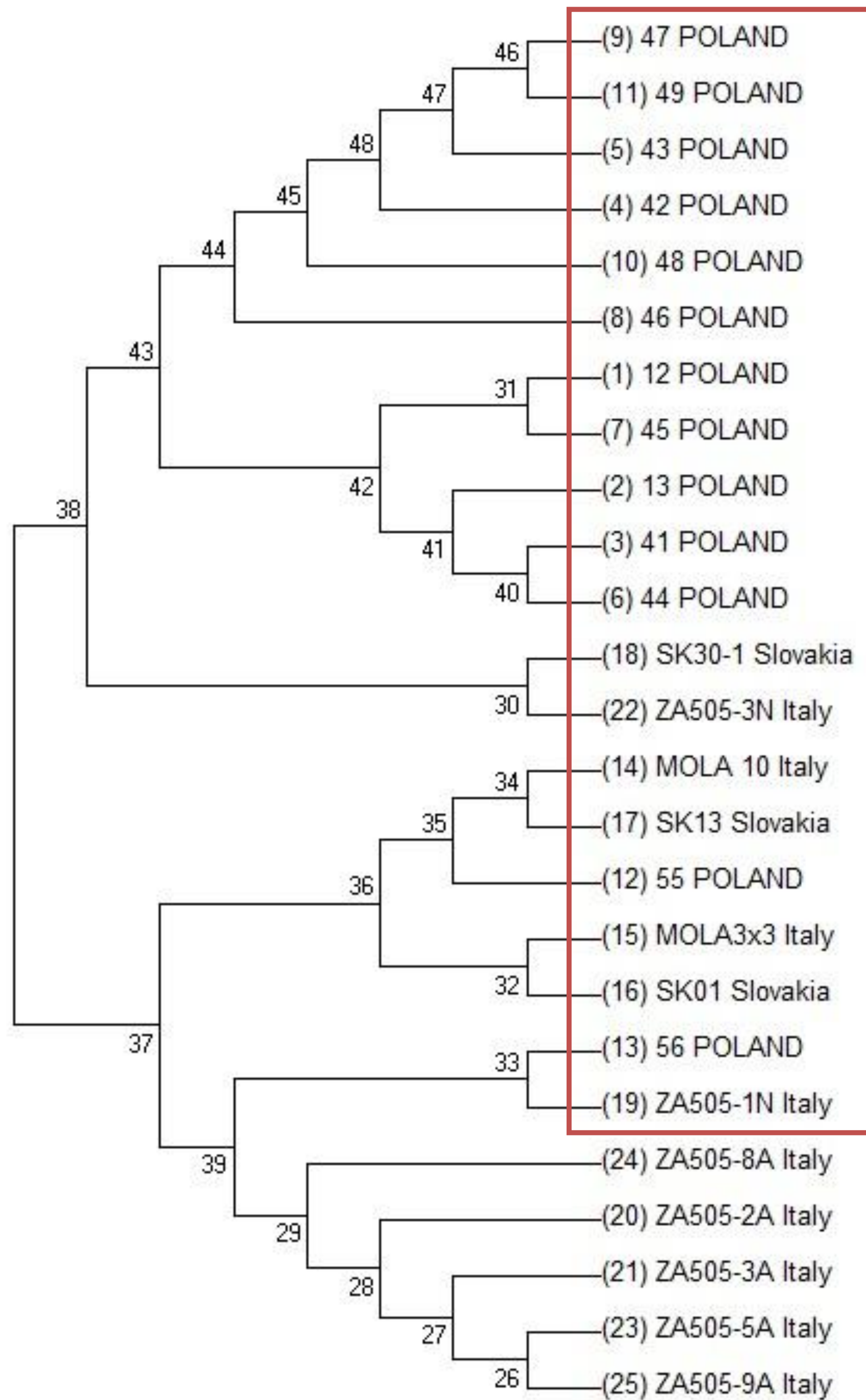

Fig. 10. Cladogram of Maximum Likelihood based on RdRp sequence (430 bp). Polish isolates (tab. 3) and isolates published by Saldarelli *et al.* [2015] were determined asymptotic (in box) or symptomatic. Analyse was done by MEGA 7.

Subsequently evaluation based on sequence of partial RdRp domain of Polish isolates and chosen sequences from Saldarelli *et al.* [2015] gave same results. All obtained sequences from Poland are in asymptomatic group (in box fig. 10).

This phylogenetic analyse is conformation of field observation (see Appendix 1, 2 & 3), which did not report any symptoms characteristic to GPGV infection among tested plants.

6. DISCUSSION

Those results are first report of *Grapevine Pinot gris virus* occurrence in Poland [Eichmeier *et al.* 2017]. Meaning that GPGV is spreading quickly among new countries. Each year researchers discover the presence of this virus in new vineyards and the range is growing. Not only Europe but also North America and Asia already has problem with this pathogen [Giampetruzzi *et al.* 2012, Glasa *et al.* 2014, Pleško *et al.* 2014, Beuve *et al.* 2015, Cho *et al.* 2015, Fan *et al.* 2016, Gazel *et al.* 2016, Rwahnih *et al.* 2016, Xiao *et al.* 2016, Ruiz–García & Olmos 2017]. Confirmation of presence of GPGV in Polish vineyards is alarming information. This issue in near future may cause great economic impact in Polish winemaking industry.

Main reason of spreading is due to trading with already infected plants [Maliogka *et al.* 2015, Eichmeier *et al.* 2017]. And by Polish law seedlings, as a trading material, should be virus free [Regulation of Ministry of Agriculture and Rural Development from day 15 July 2015], therefore GPGV should be added as a new pathogen, and propagating virus–free seedlings should be tested also on this virus. Presented study confirms that either way GPGV is constantly spreading among European countries. Taking into consideration the origin of plants, both Poland and Germany, occurrence of GPGV in those countries was confirmed in 2016 and 2017 [Reynard *et al.* 2016, Eichmeier *et al.* 2017]. Due to this fact, so far it was possible of international trading of infected material with no knowledge about it. Also, in favour of spreading GPGV is vegetative propagation of grapevine, not just in nurseries but also in vineyards, when growers propagate their own material. Practise of exchanging plants among growers only helps to place the virus in new vineyard. Finally not always proper protection against mites (like *Colomerus vitis*) may cause transmitting GPGV to other grapevine plants in vineyard [Malagnini *et al.* 2016]. However GPGV is also spreading to other herbaceous plants, like weeds between the rows and transmitting way has not yet been described. In case of viruses from *Trichovirus* genus mites are the main vector, nevertheless *C. vitis*, as vector of GPGV, has been reported as monophagus species and attack only grapevine plants [Gualandri *et al.* 2016]. This indicates the GPGV epidemiology is much more complex and further studies on air– and soli–born vectors are needed.

Based on results, it is vital mentioning, that from one vineyard Garlica, tested samples of several varieties of grapevine, only plants from variety 'Jutrzenka' were infected by GPGV, this may indicate its susceptibility for the infection. Hitherto have not been any specific conclusion that any variety may be more likely infected by this virus. For that reason

studies in this topic are recommended. Between samples taken from the Srebrna góra vineyard just two out of eight from same variety ‘Solaris’ were positive for GPGV presence, this knowledge should be important to grower that vineyard is in risk of spreading virus among other plants. Age of infected plants were different, samples collected from Jasionka were from grapevine plants over 30 years old. This fact gives suspicion that GPGV may be present for a longer time in Poland.

Results show there is difference in efficiency of, selected in this study, primers. Primers targeting RdRp domain had higher score and confirmed infection in more cases then primers targeting MP/CP gene. This may indicate Polish isolates had changed sequence in primer attachment site. This assumption is due to having result as a weak PCR products or none for MP/CP primers, while indisputable primers targeting RdRp domain confirm GPGV infection. In support of this thesis research of Saldarelli *et al.* [2015] and Bertazzon *et al.* [2016] are assuming GPGV has high variability and evolutionary dynamic. Nevertheless during their study, they are obtaining more sequences of MP/CP fragment than RdRp domain. Studies done by Bianchi *et al.* [2015] suggest the MP/CP region is much more homogenic and by real–time RT–PCR method were able to detect more samples positive for GPGV. Due to this fact it is vital usage of both primers for conformation of GPGV presence in samples, especially during certification of virus–free plantings and propagation material.

In this case sequencing of GPGV fragments was by dideoxy chain termination method [Sanger *et al.* 1977]. Sequencing was for preparing phylogenetic analyse, moreover also conformation that it is actually GPGV present in Polish vines. High identity percentage among Polish isolates, at the nucleotide level, may confirm close relationship between GPGV occurring in Poland. Therefore high similarity among sequences in groups, MP/CP or RdRp domain, might indicate possibility of existence Polish GPGV strain or strains.

Nowadays Next Generation Sequencing (NGS) is much help and is widely use in virology study [Giampetruzzi *et al.* 2012, Glasa *et al.* 2014, Saldarelli *et al.* 2015, Eichmeier *et al.* 2016]. Based on this is recommended to use NGS in future study to obtain the first full length genome sequence from Polish GPGV isolate. Afterwards, it might confirm suspicion of existing characteristic Polish GPGV strains, as before it was suggested in other countries [Glasa *et al.* 2014, Eichmeier *et al.* 2016].

Later in phylogenetic studies it would be possible of understanding better what is the reason of having symptomatic and symptomless isolates. As well would be possible to find the path how GPGV is spreading among the countries, and how and when came to Polish vineyards. The relations between isolates could be helpful to determine the reason and mechanism of this high variability of GPGV. Possibilities are growing. So far results, shown in this work, are supporting thesis about two groups of GPGV isolates, one with latent variants and second asymptomatic. The cause, why GPGV is divided into two strongly diverse groups, is still yet to discover. Now phylogenetics studies and field observations are classifying Polish GPGV isolates to asymptotic group, but the growers have to be cautious. Study of Saldarelli *et al.* [2015] and Galasa *et al.* [2014] are dividing GPGV into symptomatic and symptomless groups, but it may not to be exclude possibility of moving between those two groups. Obtained Polish isolates are closely related to Slovaks [Galasa *et al.* 2014] and this may cause a suspicion there is one common ancestor for Polish and Slovak GPGV. On the other hand, there is suspicion by Bianchi *et al.* [2015], that the presence of the GPGV in a high number asymptomatic plants is quite disputable if GPGV is clearly the etiologic agent of the new syndrome. Commonly multiple infections of one plant, cause that symptoms are multiplying. Also there is probability of recombination inside the host cells [Chare & Holmes 2005]. Research by Bertazzon *et al.* [2016] suggest that not just the general viral variants but also concentration of virus in titre is responsible for symptom occurring. Nevertheless, concentration of viral agent may be strictly related to sequence. Due to this observation, they implicate that isolates of GPGV with higher multiplication rate may more likely manifest symptom of disease. Taking all that into consider the studies on GPGV are needed, also like establishing climate and environmental influence.

Overall, phylogenetic studies have great tools for analysing the evolution of each species. In studies on viruses it has great importance, it allows to trace relatively common recombination events. In studies done by Chare & Holmes [2005], they establish by studies on alignments, that in more than one in three positive–sense plant RNA viruses sequences occur recombination. At the molecular level this frequent events are mostly deleterious, this process clearly leads to mutations. Negative effect of those kind of recombination is actually undetectable due to natural selection processes. Nevertheless, statistically with rather high frequency of recombination, and therefore mutations, this process enhance the evolutionary change, rise the variability and virulence. GPGV with characteristic division for symptomatic and symptomless isolates is very interesting from point of view further studies. Its rather short

genome and NGS becoming more frequent use in studies on viruses may bring in future very innovative perception on all viruses. Polish GPGV isolates so far were strictly asymptotic, the field observation combined with phylogenetic studies, may be a reason of using them in later to study of reason why part of GPGV isolates give symptoms and other part not.

This study is gaining new information about GPGV that in future may be used for developing virus–free plants or helps breeding cultivars resistant to virus infections. Possibilities for developing virus–free plants continuously grow, like in–vitro propagation by meristem cultures [Wang *et al.* 2003, Maliogka *et al.*2009]. Recent studies by Kurth *et al.* [2012] shows already developing vector which might be use for silencing viruses and be use as plant vaccine. They present two kind of approach to use a technology of plant vaccination, one for enhancing expression of chosen protein and second for knocking down gene expression via RNA interference. Vector was designed based on GLRaV–2 genome. The gene expression cassette harbouring the reporter endoplasmic reticulum (ER) – targeted green fluorescent protein (GFP). This allowed to track virus infection, and brought new light on localization and flow of virus inside the host. Combining this studies with discovery of what is reason why part of GPGV isolates are symptomless could be adapted and used for silencing the symptomatic GPGV. Furthermore, if the mechanism would be universal this could be the breakthrough in plant virology. With help of plant engineering it would be possible to achieve not virus–free plants but, possible, plants with no symptoms of virus infection. The genetic engineering is continuously developing wide range of tools which can be used in future plant breeding programs. Nevertheless, firstly it is necessary to know what we are dealing with.

7. CONCLUSION

Presented research confirm occurrence *Grapevine Pinot gris virus* in Poland [Eichmeier *et al.* 2017]. Provides information about present localization of plants infected by GPGV. Due to this fact GPGV should be added as a next pathogen occurring in vineyards in Poland and Polish growers should me more cautious.

This studies shows primers my not always work properly, especially with virus know for high variability, like GPGV. Previous studies by Saldarelli *et al.* [2015] and Bianchi *et al.* [2015] obtained more MP/CP fragments than RdRp sequence, while this research had opposite results. It is necessary to test samples with more than just one pair of primers. It seems there is need to obtain the universal pair of primers.

Presented work show that Polish isolates are symptomless by observation of plants in vineyard and as well by the phylogenetic analyse, which is classifying them into asymptotic group. Further studies in this matter may confirm existence of Polish strain of GPGV. Regardless, if the asymptomatic character of presented isolates is related to its genetic character, low multiplication rate or environmental influence, it is no questionable that *Grapevine Pinot gris virus* is present in Polish vineyards and should be taken as a serious threat for growers in future.

8. SUMMARY

Topic: Molecular characterization of Grapevine Pinot gris virus in Poland

Text: Samples of different varieties grapevine from Małopolska and Podkarpacie collected on spring 2016 were tested for Grapevine Pinot gris virus (GPGV) by RT–PCR. 16 out of 65 plants were found positive for GPGV. Two set of primers were used for detection, targeting partial movement protein and coat protein, and RdRp domain, both of those regions were sequenced Phylogenetic analyses settled the Polish isolates to described before asymptotic group. Also GPGV positive samples were tested by multiplex and simplex RT–PCR for multiple infections, and 18,5% of all samples were GPGV and GFkV positive. This was first survey of GPGV in Poland.

Key words: virology, RT–PCR, sequencing, phylogenetics, grapevine

Téma: Molecular characterization of Grapevine Pinot gris virus in Poland

Text: Vzorky různých odrůd révy vinné z Malopolského a Podkarpatského vojvodství odebrané na jaře 2016 byly testovány na přítomnost viru Grapevine Pinot gris virus (GPGV) přes RT-PCR. 16 ze 65 zkoumaných rostlin bylo pozitivních na přítomnost GPGV. Na detekci byly použity dvě sestavy primerů aplifikujících část obalového a transportního proteinu viru, i část domény RdRp, potom byly obě oblasti zsekvencované. Filogenetická analýza byla základem přiřazení polských izolatů do skupiny GPGV. Všechny vzorky pozitivní na přítomnost GPGV byly také testovány multiplex reakcí na přítomnost jiných virů, 18,5 % ze všech probádaných rostlin bylo infikovaných GPGV a GFkV. Tento výzkum byl první zprávou o přítomnosti GPGV v Polsku.

Klíčová slova: virologie, RT–PCR, sekvenování, fylogenetika, vinná réva

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10. ANNEX

10.1. List of Tables

Table 1. List of samples with their origin.

Table 2. DNA primers used for PCR amplification for internal positive control – MDH, specific targeting sequences of partial MP/CP sequence and RdRp domain of GPGV.

Table 3. Characteristics of isolates positive for *Grapevine Pinot gris virus* presence

10.2. List of Figures/Graphs

Fig 1. Demonstration of exemplary virus from the family *Betaflexiviridae*.

Fig. 2. Symptoms of GPGV in *V. vinifera* 'Pinot gris' – chlorotic mottling and leaf deformations (A and B) and chlorotic mottling and shiit stunting in 'Traminer' (C and D).

Fig. 3. Symptoms of inner necrosis of berries caused by GPGV found on *Vitis viniferax x V. labrusca* variety 'Tamnara' in Korea.

Fig 4. Symptoms of virus–related infection of GPGV on *Chenopodium album* L. (A) and *Silene latifolia* subsp. *Alba* (Mill.) (B). Arrow shows chlorotic and mottling spots (respectively). Bar = 1 cm.

Fig. 5. Organization of GPGV RNA genome based on GPGV_FEM01 clone GenBank accession no KU312039.1.

Fig. 7. Internal positive control. Separation of PCR products on 1,3% agarose gel, amplification with primers targeting MDH gene (196 bp) – chosen samples 11–20. (

Fig 8. A. Separation of PCR products after amplification with primers targeting MP/CP fragment (588bp).

Fig. 8. B. Separation of PCR products – amplification with primers targeting partial RdRp domain (525 bp);

Fig. 8. C. Separation of PCR products – multiplex reaction with starters targeting ArMV, GFLV, GLRaV–1, –2, –3, GVA and GFkV

Fig. 8. D. Separation of PCR products – amplification with specific primers for GFkV.

Fig. 9. Cladogram of Maximum Likelihood of MP/CP sequence (280 bp). Polish isolates (tab. 3) and isolates published by Saldarelli *et al.* [2015] were determined symptomatic (in box) or asymptomatic. Analyse was done by MEGA7.

Fig. 10. Cladogram of Maximum Likelihood based on RdRp sequence (430 bp). Polish isolates (tab. 3) and isolates published by Saldarelli *et al.* [2015] were determined symptomatic (in box) or asymptomatic. Analyse was done by MEGA7.

10.3. APPENDIX

Appendix 1. Symptoms of the other pathogens or pests on plants GPGV positive from Jasionka. Numbers of samples 67 and 68.

Appendix 2. Symptomless plants positive fog GPGV from Garlica vineyard variety Jutrzenka. Numbers of samples 12 and 46.

Appendix 3. Asymptomatic plant positive fog GPGV from Srebrna Góra vineyard. Numbers of samples 55 and 56.

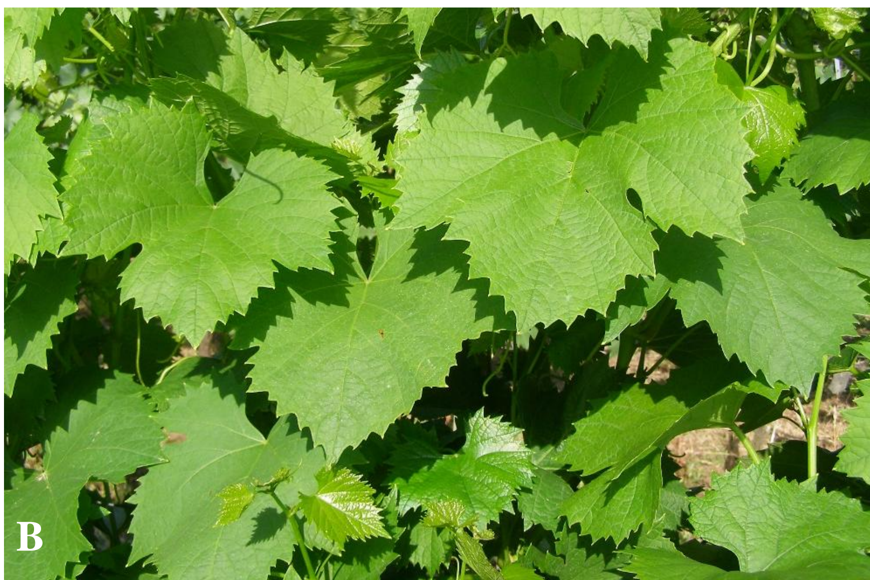
Appendix 4. Separation of PCR product targeting GPGV RdRp partial domain sequence.

11. ABBREVIATIONS

2OG-Fe (II) – 2OG-Fe (II) oxygenase,
aa – amino acid
ArMV – *Arabis mosaic virus*,
bp – base pairs,
cDNA – complementary deoxyribonucleic acid,
CP – coat protein,
DNA – deoxyribonucleic acid,
GINV – *Grapevine berry inner necrosis virus*
GFkV – *Grapevine fleck virus*,
GFkV – *Grapevine fleck virus*,
GFLV – *Grapevine fanleaf virus*,
GLRaV–1, –2, –3 – *Grapevine leafroll associated virus* 1, –2, –3
GPGV – *Grapevine Pinot gris virus*,
Hel – viral RNA helicase,
HxD – His-x-Asp catalytic core of kinases
kDa – kilo Dalton
MDH – malate dehydrogenase gene
Met – methyltransferase,
MP – movement protein,
nt – nucleotides,
ORF – open reading frames
PCR – polymerase chain reaction,
qPCR - real–time polymerase chain reaction
RdRp – RNA dependent RNA polymerase,
RNA – ribonucleic acid,
RT–PCR – reverse–transcription polymerase chain reaction.

Appendix 1. Symptoms of the other pathogens or pests on plants GPGV positive from Jasionka. Numbers of samples 67 (A, B) and 68 (C, D).

Appendix 2. Symptomless plants positive fog GPGV from Garlica vineyard variety Jutrzenka. Numbers of samples 12 (A, B) and 46 (C).



Appendix 3. Asymptomatic plant positive fog GPGV from Srebrna Góra vineyard. Numbers of samples 55 (A, B) and 56 (C, D).

Appendix 4. Separation of PCR product targeting GPGV RdRp partial domain sequence. SM –size marker, PC – positive control.