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The Faculty of Natural Resources and Agricultural Sciences
Department of Plant Biology and Forest Genetics

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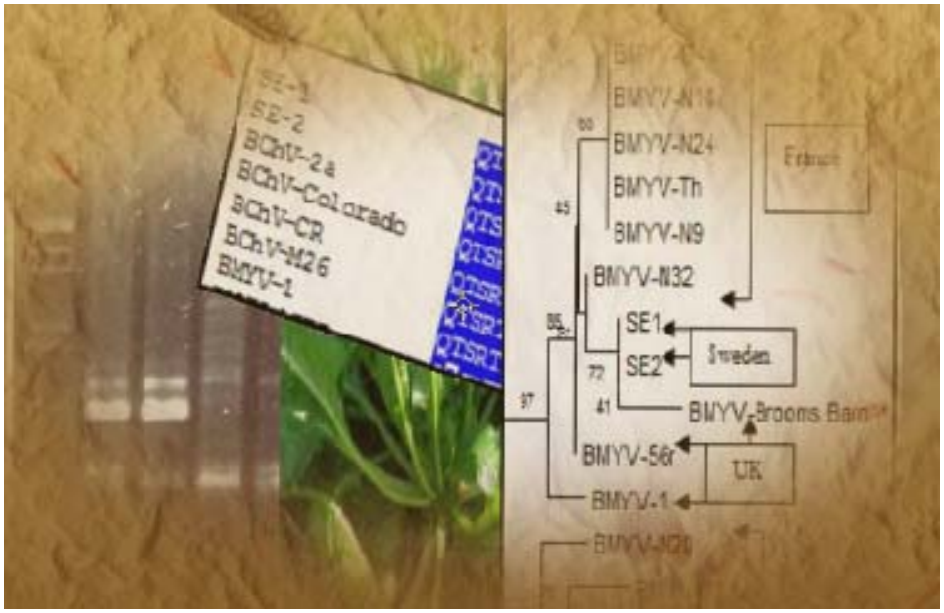

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Abstract

Plants of sugar beet with symptoms of virus infection (mosaic and yellowing) were collected from Skåne in Sweden for virus identification and characterization. To test the presence of *Beet mosaic virus* (BtMV) several detection methods were applied, such as: mechanical inoculation/transmission test with sugar beet (*Beta vulgaris*), red beet (*Beta vulgaris*) cv Rubia, spinach (*Spinacia oleracea*) cv Long Standing Bloomsdale and lettuce (*Lactuca sativa*) cv Sonette; DAS-Enzyme-linked Immunosorbent Assay (DAS-ELISA) with polyclonal antibody; RT-PCR amplification with *Potyviridae*-specific universal primers Sprimer/M4 (Chen et al., 2001), and BtMV-specific primer pairs BM1/BM2 and BM1/BM3 (Glasa et al., 2003). No BtMV infection was observed from the results of the tests mentioned above. To check the possible infection of beet polero/luteovirus, RT-PCR amplification was conducted with *Luteoviridae*-specific universal primers Lu1/Lu4 (Robertson et al., 1991) and the expected band of ca 500 bp was found for the collected samples. The PCR fragments of the isolates were cloned, sequenced and then used for phylogenetic analyses. The sequence information of the two Swedish isolates SE1 and SE2 collected from Skåne revealed a sequence of 505 nucleotides and they were found to be 100% identical to each other. Comparison to the sequences in GenBank using BLASTn search showed 99% nucleotide identity and 100% amino acid identity (using BLASTp & BLASTx) with the Coat Protein encoding region of *Beet mild yellowing virus* (BMYV) isolates. Phylogenetic analyses with different evolutionary best-fit models showed that both of the isolates are very closely related to the British isolate BMYV-Broom's Barn. The sequence information of isolate SE1 was submitted to GenBank and the accession number FN827048 was obtained. To our knowledge, this is the first molecular identification and sequence information for BMYV from Sweden.

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Introduction

The Crop and Diseases

Sugar beet (*Beta vulgaris*), a major source of world's sugar, second only to sugarcane, is widely grown in Europe, Asia, North Africa and some parts of North and South America. In Sweden, the sugar beet cultivation of Skåne is very important since that produces almost enough sugar to make Sweden self-sufficient. Beet was grown in Europe as a fodder crop in the seventeenth century and after discovering the sugar in the beet in Germany (in 1747 by the chemist Andreas Marggraf) more research was continued (by the chemist Achard) to develop the sugar extraction and purification process, which eventually resulted in the first erection of a sugar beet factory in Silesia in 1802 (Lennefors, 2006). Due to the British blockade that cut off the French Empire's raw sugar supply from the West Indies, Napoleon became interested in production of sugar beet in 1811 and established 40 factories in France. In Europe, large scale sugar factories were operated during the second half of the nineteenth century after significant improvement of the sugar beet crop by systematic breeding (Lennefors, 2006). In some parts of Europe, sugar beet is usually grown as part of a rotation with other crops, such as wheat, barley, peas etc, so that the field can be used every year without a major drop in fertility.

Sugar beet suffers from a number of pathogens and pests such as beet nematode (punctures the plant cells opening for fungal or bacterial invasion), Cercospora (causes defoliation, reduced tonnage, lower sucrose content and high impurities), *Beet necrotic yellow vein virus* (soil-borne virus vectored by the protist *Polymyxa betae*), Fusarium Yellows (causing leaves to die and lay in a clump around the crown of the beet), Rhizoctonia (causing root and crown rot diseases), powdery mildew (causes powdery film or mould starting on the backside of leaves), *Beet curly top virus* (transmitted by sugar beet leafhopper), *Beet yellows virus* (BYV), *Beet mild yellows virus* (BMYV), *Beet western yellowing virus* (BWYV), *Beet cryptic virus* (BCV), *Beet chlorosis virus* (BChV) and *Beet mosaic virus* (BtMV).

Potyviruses and Poleroviruses of Sugar beet

Potyvirus

Classification, Nature of Virus and Diseases

The genus *Potyvirus* belongs to one of the largest known families of plant viral pathogens: *Potyviridae* (Ward et al., 1992). Within this family, the genus – species concept is very well developed and is used as the main system of classification (Barnett, 1991). Six genera have been established (Hasan, 2004) in the family *Potyviridae: Bymovirus* with its type species *Barley yellow mosaic virus* (BaYMV), *Ipomovirus* with its type species *Sweet potato mild mottle virus* (SPMMV), *Macluravirus* with its types species *Maclura mosaic virus* (MacMV), *Rymovirus* with its type species *Ryegrass mosaic virus* (RGMV), *Tritimovirus* with its type species *Wheat streak mosaic virus* (WSMV) and *Potyvirus* with its type species *Potato virus Y* (PVY). Among them, the genus *Potyvirus* has a large

number of economically important viruses – *Bean yellow mosaic virus* (BYMV), *Plum pox virus* (PPV), PVY etc.

Although most of the potyvirus species have a relatively narrow host range, their cumulative host range covers a large number of economically important plant species from several families such as Chenopodiaceae, Cucurbitaceae, Fabaceae, Poaceae and Solanaceae. They are frequently present as part of the natural pathogen spectrum and cause chronic reductions in yield and quality of seed stocks, stems, bulbs or seeds (Hasan, 2004). Moreover, cosmetic damages by decreasing crop value such as fruit distortion in cucumber can be observed by a potyvirus infection (*Zucchini yellow mosaic virus,* ZYMV*)*, and growth of the infected plants is often reduced (Lisa & Lecoq, 1984; Hasan, 2004).

Potyviruses are transmitted naturally by aphids in a non-persistent (non-circulative) manner and aphids usually lose the ability to transmit the virus after the first or second probe or penetration of the plant cell by stylet (Hasan, 2004). Two virus-encoded proteins, Coat protein (CP) and the helper component proteinase (HC-Pro), are responsible for virus transmission by the aphids. Virus can be acquired by the aphids after only a brief feeding contact with an infected host plant and they normally retain the virus for less than an hour although some virus particles retain infectivity up to 40 hours (Hasan, 2004). Moreover, the spread of viruses depends on the interaction between the host plant, the vectors, the virus sources and the environmental conditions.

In the past, host range and symptomology played a great role to identify and classify potyviruses (Hasan, 2004). However, sometimes different potyviruses caused similar symptoms in the hosts, also different climatic conditions and host genotypes can have vast effects on disease susceptibility. Therefore, from the symptomology it was often very difficult to compare results observed in different laboratories (Bos, 1970). Moreover, symptomology may not be a reliable marker of genetic relatedness and it was found that a single mutation in the CP of *Tobacco mosaic virus* (TMV) (Knorr & Dawson, 1988) or *Cucumber mosaic virus* (CMV) (Hasan, 2004) totally changed the symptom phenotype from a systemic mosaic to appearance of local lesions.

Genome Structure and Organization

In terms of the genomic structure and strategy of expression, potyviruses are similar to the plant bipartite comoviruses and nepoviruses, and to the animal picornaviruses. Moreover, the genome of these viruses have a region of conserved gene order encoding non-structural proteins, which are involved in RNA replication and for this reason it has been proposed that the comoviruses, nepoviruses and potyviruses might be arranged in a supergroup of picorna-like plant viruses. Like most plant viruses, potyviruses have a single-stranded, positive-sense RNA genome and the RNA is approximately 10 kb long with a 5′-terminal genome-linked protein (VPg) and a 3′ poly-(A) tail (Riechmann et al., 1992). It has one long open reading frame (ORF) translated into a large polyprotein (ranging between 340K and 368K depending on the potyvirus), which is subsequently cleaved into smaller polypeptides (Riechmann et al., 1992).

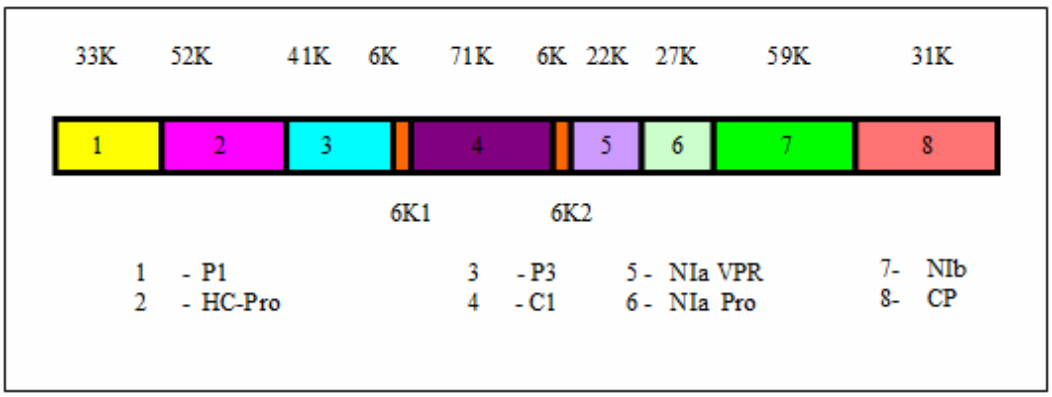


Figure 1: Genome organization of potyviruses displaying mature proteins (modified from Gammelgård, 2007)

Although the processing and function of these proteins are still controversial, it is assumed that they are multifunctional with functions such as proteinase (P1, HC-Pro, NIa-Pro), cell-to-cell movement (HC-Pro, CP), aphid-mediated transmission

Table 1: Mature potyviral proteins and their known functions (Gammelgård, 2007).

NAME	POSSIBLE FUNCTION(S)
P1	Proteinase Accessory factor for suppression of RNA silencing
HC-Pro	Proteinase Cell-to-cell and long-distance movement Suppressor of RNA silencing Aphid transmission Symptom expression Avirulence determinant in SMV(*Rsv*) Interaction with calmodulin-like protein Interaction with ring-finger protein and HIP2
P3	Avirulence determinant
6K1	Avirulence determinant
CI	Helicase Avirulence determinant Cell-to-cell movement
6K2	Long-distance movement
VPg	Cell-to-cell and long-distance movement Avirulence determinant Interaction with the eukaryotic translation factor eIF4E and eIF(iso)4E Interaction with PVIP
NIa-Pro	Proteinase Elicitor of Ry-mediated resistance
NIb	RNA-dependent RNA polymerase Interaction with poly(A) binding protein (PABP)
CP	Encapsidation of viral RNA Cell-to-cell and long-distance movement Aphid transmission

(HC-Pro, CP), avirulence determinant (6KI), long distance movement (6K2)(Table 1). By looking at the genetic diversity of the full genome sequences of distinct potyvirus species, it was found that the first protein (P1), the third

protein (P3) and the N-terminal domain of the CP are the most variable regions of the potyviral polyprotein, while the replicase (nuclear inclusion b, NIb) is the most conserved protein (Figure 1) (Aleman-Verdaguer et al., 1997).

Beet Mosaic Virus

Beet mosaic virus (BtMV) (genus *Potyvirus*; family *Potyviridae*) is a worldwide occurring pathogen causing mosaic disease of sugar beet and significant yield losses in beet (Shepherd et al., 1964; Mali et al., 2000). It has been reported that early infection by BtMV results in a higher yield reduction, and considerable reduction of yield can occur when disease incidence is high early in the season (Dusi, 1999; Hasan, 2004). However, when late infection occurs, the crop has already leaf material enough to sustain yield at commercial levels (Hasan, 2004). Infection with BtMV reduced the maximum rate of leaf gross photosynthesis in mature leaves by 16% and increased dark respiration by 85 to 90% for young as well as matured leaves (Dusi, 1999; Hasan, 2004).

BtMV causes a mosaic disease in sugar beet and spinach with clear symptoms. The host range includes all cultivated sugar beet and BtMV infects mainly plants in the families Chenopodiaceae, Solanaceae and Fabaceae. Generally, the symptoms of BtMV distinctly differ from those evoked by other viruses attacking sugar beet. Early symptoms are numerous small yellow spots and blotches on one or several central leaves followed by a light mosaic and mottle on young leaves as disease develops. Sometimes leaflets can be also stunted with necrosis in the leaf tip; and in several cases, diseased leaves roll into a tubular shape (Hasan, 2004).

Like other potyviruses, it can be transmitted mechanically as well as by several vector species, with varying degrees of efficiency. Although BtMV is transmissible by more than 28 aphid species, *Myzus persicae* and *Aphis fabae* are the principal vectors in the field. Transmission occurs in a non-persistent manner and is improved by starving aphids for 2-5 minutes before acquisition feeding (Watson & Watson, 1953; Hasan, 2004). BtMV can be transmitted by mechanical inoculation utilizing infected plant sap as well as by grafting, but it can not be transmitted by seed, dodder, contact between plants or by pollen (Hasan, 2004). The disease caused by BtMV is polycyclic, and the shorter the latency period, the faster the epidemic can develop. Studies indicate that the length of the latency and incubation period increased with decreasing temperatures and during summer months the latency and incubation period are shorter (Dusi, 1999; Hasan, 2004).

Polerovirus

Classification, Nature of Virus and Diseases

The genus *Polerovirus* belongs to one of three genera in the family *Luteoviridae* and it consists of viruses which were previously included in the genus *Luteovirus.* The three genera are (Stevens et al., 2005): *Enamovirus* (3 ORFs upstream of the CP, with VPg; lacking the functions for systemic movement, which are supplied by a second virus of the genus *Umbravirus*); *Luteovirus* (2 ORFs upstream of the CP; without VPg; with systemic movement) and *Polerovirus* (3 ORFs upstream of the CP; with VPg; with systemic movement) (Figure 2). Within the genus *Polerovirus*, virions are isometric (icosahedral) and non-enveloped with a diameter of 25-30 nm. There are three members of the genus *Polerovirus* that

induce yellowing of sugar beet: *Beet mild yellowing virus* (BMYV), *Beet chlorosis virus* (BChV) and *Beet western yellows virus* (BWYV) (Figure 2). Within Europe non-beet-infecting isolates of BWYV have been found which have now been re-named *Turnip yellows virus* (TuYV) (Stevens et al., 2005).

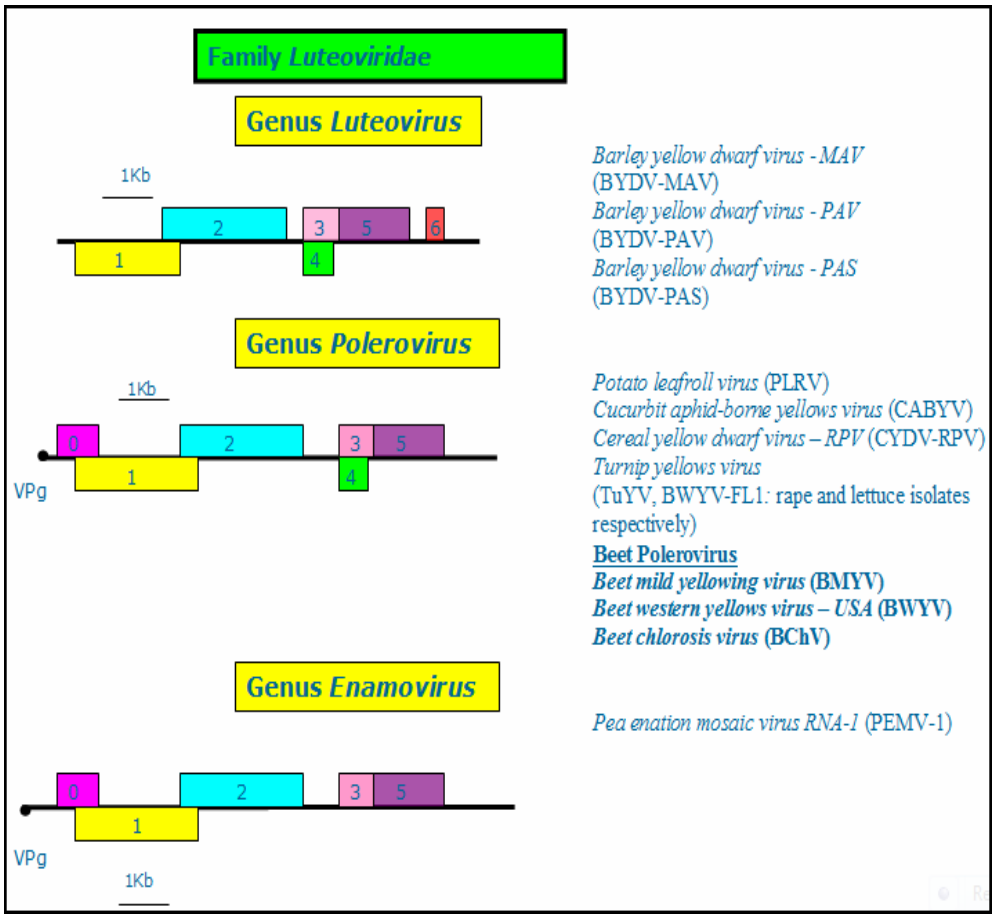


Figure 2: Taxonomy and genome organization of members of the family *Luteoviridae*. Virus species (in bold type) that induce yellowing of sugar beets are termed as ´Beet Polerovirus'. Schematic diagrams of genome organization of the three *Luteoviridae* genera are shown (modified from Stevens et al., 2005).

Beet poleroviruses are transmitted in a persistent (circulative, non-propagative) manner by several different aphid species. Generally, sugar beet infected with beet poleroviruses will have patches of chlorosis on the older leaves 4-6 weeks post-infection and these areas expand until the whole leaf becomes yellow and older leaves then tend to thicken as well as becoming brittle (Stevens et al., 2005).

Genome structure and organization

The polerovirus genome is monopartite, linear, single-stranded, positive-sense RNA, and 5.3-5.7 kb long. The 3´ terminus has neither a poly-(A) tract nor a tRNA–like structure and the 5´ terminus has a genome-linked protein (VPg).

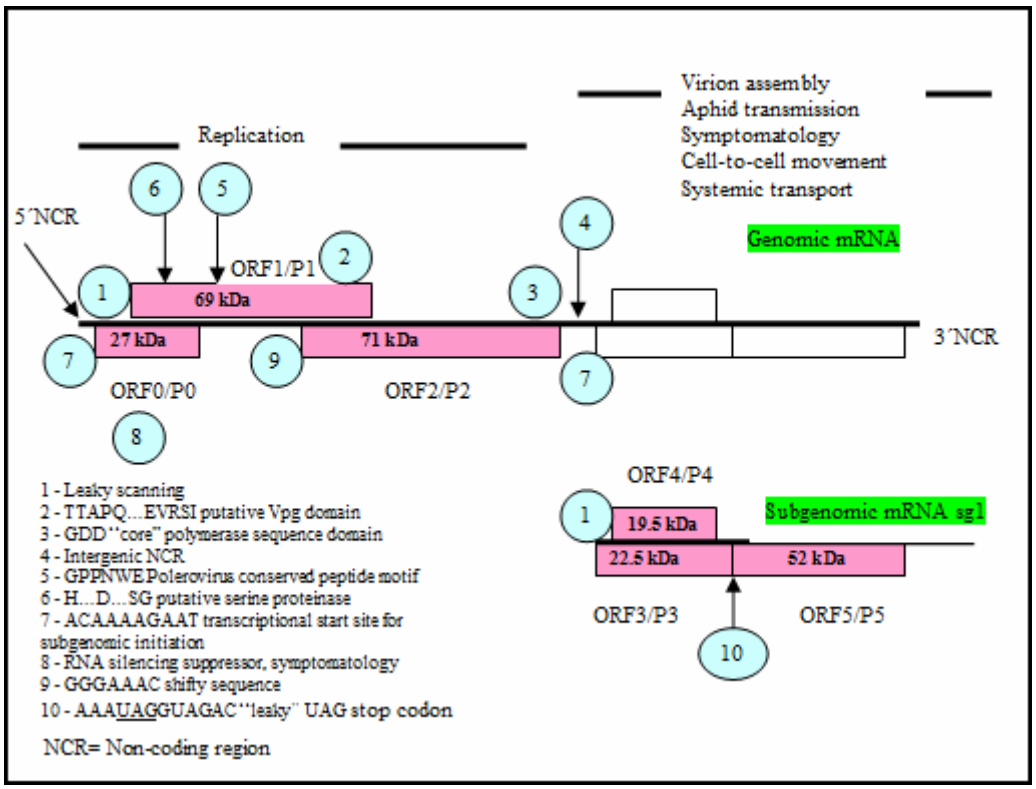


Figure 3: Organization and expression of a representative beet polerovirus genome. The essential putative and deduced functions of the encoded proteins are indicated (Stevens et al., 2005).

Since viruses of the genus *Polerovirus* share the same basic genome structure, it is presumed that the expression strategy and gene functions identified for one species will apply to all other within the genus (Stevens et al., 2005). The genome of the polerovirus BMYV has six ORFs. The three 5´-proximal ORFs are expressed directly by translation from the genomic RNA (gRNA) while the ORFs downstream of a non-coding region (ca. 200 nucleotides) are translated from a subgenomic RNA (Figure 3). The initiation of the translation of ORF0 begins after a short leader sequence at the first AUG codon of the genome although leaky scanning allows ribosomes to bypass this site in order to initiate translation at the start codon of ORF1 (Figure 3). The translation of ORF2 is achieved via a ribosomal frame shift from ORF1 and through mutational analysis it was found that P1 and P2 are essential for infection as well as having a role in replication (Figure 3). Genes of the 3´-proximal cluster (ORFs 3, 4 and 5) are translated following the synthesis of subgenomic RNA which is thought to depend on the initiation of the viral RdRP at internal promoter sites on the minus strand synthesized during genomic RNA replication. ORF5 is expressed as a consequence of translational read-through by suppression of the amber stop codon of ORF3 and P5 is therefore found only as minor fusion protein (P3 + P5) (Stevens et al., 2005). The protein is involved in symptom induction and plays a key role in transmission efficiency and specificity, as well as virus persistence within aphid vector. P3 is the CP and it is assumed that the structural protein P3 and the P5 read-through domain (RTD) have roles in systemic movement (Stevens et al., 2005) (Figure 3). Also these ORFs are numbered consistently with other members of the family *Luteoviridae*.

***Beet Western Yellows Virus* (BWYV)**

BWYV was first characterized in the western United States (Duffus, 1960) initially called radish yellows and later, *Beet western yellows virus* (BWYV) (Beuve et al., 2008). BWYV causes stunting and chlorosis of a wide range of dicotyledonous species including sugar beet, red beet, spinach, lettuce, broccoli, cauliflower, radish, turnip and flax. It is transmitted readily by aphids but not by sap inoculation. Transmission occurrs in a persistent (circulative) manner and the virus persists in the vector for over 50 days. Vectors retain the ability to transmit after molting although the virus is not transmitted to their progeny. However, the virus is not transmissible through seed or by dodder.

Although polyclonal antisera raised against either BMYV or European BWYV do not discriminate between these poleroviruses, a monoclonal antibody (Mab) raised against *Barley yellow dwarf virus-PAV-IL-1* (BYDV-PAV-IL-1) enables this serological distinction (D'Arcy et al., 1989).

Beet Mild Yellowing Virus (BMYV)

BMYV, first characterized in England in 1958, can cause a severe yellows disease of sugar beet in Europe (Russell, 1958; Guilley et al., 1995). BMYV is very closely related serologically to BWYV (Guilley et al., 1995). The complete nucleotide sequence of the genomic RNA of BMYV, isolate 2ITB showed that the RNA consists of 5722 nucleotides and has six ORFs (Guilley et al., 1995). The three 3′-proximal ORFs, which encode the viral CP, a putative movement protein and the Readthrough Domain (RTD), show high sequence identity to the corresponding genes of BWYV while the three 5′-proximal ORFs are more closely related to the corresponding genes of *Cucurbit aphid-borne yellows virus* (CABYV) (Guilley et al., 1995).

Beet Chlorosis Virus

BChV, first discovered in 1989 both in USA and the UK (Stevens et al., 2005) was considered as a distinct strain of BMYV (Stevens et al., 1994), which did not react with the monoclonal antibody to BYDV-PAV-IL-1 as well as failed to infect the traditional indicator species *Capsella bursa-pastoris* or *Montia perfoliata*. However, after analyzing biological properties and genome organization (Hauser et al., 2001) it was included as a new distinct species of the genus *Polerovirus*.

Sugar beet infected with BChV showed interveinal chlorosis symptom and BChV displayed a narrower host range compared to BMYV and BWYV (Hauser et al., 2001). Although BChV showed a genetic organization typical of other polerovirus members including 6 ORFs, interspecific and intraspecific phylogenetic studies suggested that BChV arose by recombination events between a polerovirus-like ancestor donating P0 and the replicase complex; and either BMYV or a BWYV progenitor providing the 3′ ORFs 3, 4 and 5 (Hauser et al., 2001).

Plant Virus Detection Methods

By combination of one or more of the following methods (Christie et al., 1995; Sharma, 2004; Agrios, 2005; Makkouk & Kumari, 2006) it might be possible to diagnose a virus infection of a plant:

a) Inclusion
Staining Properties: Some of the virus groups and individual viruses can be reliably diagnosed by using differential stains (Christie et al., 1995). There are two stains used for identification of viral inclusions: Azure A (AA) and Orange–green stain (OG). AA stains RNA pink and DNA blue and OG is used for staining proteins. For example, some inclusions, such as the cylindrical inclusions of most potyviruses, stain only in OG.

Locations: Sometimes the location of the inclusion also helps to narrow down the type of virus infecting the plant. For instance, many inclusions are found in the epidermis but others can only be found in the vascular tissue (e.g., *Citrus tristeza virus*) or nucleus (geminivirus and rhabdovirus).

b) Electron Microscopy
Plant sap containing virus particles can be prepared and put into an electron microscope (EM) and examined for viral particle structure. For example, tobamoviruses such as TMV have rigid rod shape whereas *Potato yellow dwarf virus* and *Lettuce necrotic yellow virus* have a spherical shape (Sharma, 2004). Therefore, by viewing the particle in the EM it is possible to narrow down the type of virus.

c) Host Range Inoculations and Symptomatology
Some viruses are not mechanically transmissible and the host range is determined using vectors. Since plant virologists have inoculated plant viruses to many different plants, each known plant virus has a list of susceptible and insusceptible plant species. Therefore, by grinding a sample of an infected plant in a buffer it is possible to manually inoculate other plants and those that become infected can be compared with the list of plants susceptible to a suspected virus (Agrios, 2005). If the host range of an unknown virus is the same as that of a known virus, this may be another clue for virus diagnosis.

d) Serological Tests
Virus can be purified and injected into a mammal (such as a rabbit) and the inoculated animal will produce antibodies to the protein coat of this virus. Then the animal is bled and the serum (antiserum) that results can be used to detect plant viruses either by ELISA (Enzymed-Linked Immunosorbent Assay) or Immunodiffusion Test.

During the last three decades ELISA has been widely used for the detection of viruses due to its high sensitivity, simplicity and ability to quantify virus content in plant tissue (Makkouk & Kumari, 2006). The binding of virus and specific antibody is made visible through an antibody tagged with an enzyme, which can react with a substrate to produce a coloured, water-soluble product. For example

in a double-antibody sandwich ELISA (DAS-ELISA) test, the antibody is bound to the solid phase (e.g., Polystyrene microtiter plate). Then the test sample, enzyme-labelled antibody and the substrate are added sequentially, with unbound material removed by washing steps. In a positive test, the substrate solution turns coloured (whereas a negative test remains colourless) and the colour intensity, which is proportional to virus concentration can be measured spectrophotometrically (Makkouk & Kumari, 2006).

However, like other diagnosis methods (inclusions, electron microscopy, inoculation, etc) serological tests are not always reliable for identifying the specific species of a virus family. For instance, the close serological relationship between BMYV and BWYV, and the similarities in host range had led to claims that they were all strains of one virus although later a monoclonal antibody originally raised against BYDV-PAV-IL was able to distinguish BMYV and BWYV (Stevens et al., 2005).

e) Molecular Nucleic Acid Based Methods

Recent development in molecular detection technology helps scientists not to rely exclusively on symptomology and/or time-consuming diagnostic procedures, and permits early detection of viral infection (Makkouk & Kumari, 2006). Nucleic acid-based virus detection systems make use of cloned DNA probes in a dot-blot assay or specifically designed primers in polymerase chain reaction (PCR)/reverse transcription polymerase chain reaction (RT-PCR)/real-time PCR where it is possible to detect single nucleotide differences.

Nowadays PCR-based diagnosis methods are becoming popular. In PCR a pair of synthetic oligonucleotides or primers (virus-species specific or genus specific) is used, each hybridizing to one strand of a double-stranded DNA target, with the pair spanning a region that will exponentially be reproduced. Here the hybridized primer acts as a substrate for a DNA polymerase, which creates a complementary strand via sequential addition of deoxynucleotides. RT-PCR is used for RNA viruses where the RNA viral genome is transcribed to its complementary DNA (cDNA) using reverse transcriptase (RT) enzyme. With real-time PCR it is possible to visualise the progress of amplification in a quantitative manner by the labelling of primers, probes or amplicon with fluorogenic molecules. The amplified DNA fragment from PCR is then separated by agarose gel electrophoresis and the bands are visualized by staining with ethidium bromide in ultra-violet light. PCR products can be cloned and sequenced to find the nt (nucleotide) identity to similar sequences from sequence databases such as GenBank.

Phylogenetic Analyses

Phylogenetic analysis is the process to determine the evolutionary relationship within a gene family and the results of an analysis can be drawn in a hierarchical diagram called a cladogram or phylogram (phylogenetic tree). In the phylogenetic trees various diagrams used for depicting evolutionary relationships resemble the structure of a tree (Figure 4), and the terms referring to the various parts of these diagrams (e.g., root, branch, node, clade) are also reminiscent of trees (Vandadamme, 2003).

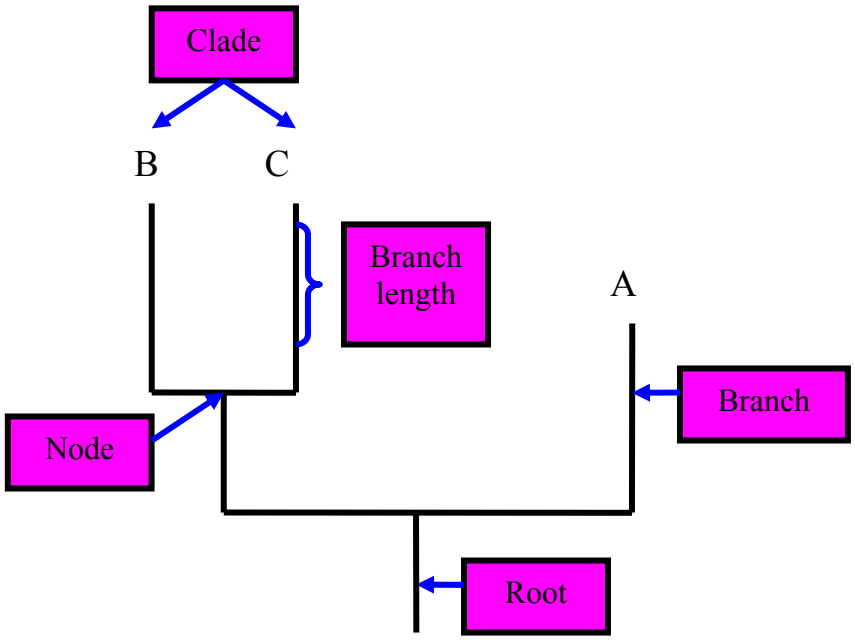


Figure 4: Sample model organization of a phylogenetic tree.

The branch length usually represents the number of changes that has occurred in the branch and the branching pattern of the tree is called topology (Figure 4). In rooted phylogenetic trees, a particular node called *root* represents a common ancestor from which a unique path leads to any other node; whereas an unrooted tree only specifies the relationship among species without identifying a common ancestor or evolutionary path. In the tree a group of two or more taxa or DNA sequences that includes both their common ancestor and all other descendents is termed *clade* (Figure 4). Originally, phylogenetic trees were created using morphology, but now, determining evolutionary relationships includes matching patterns in nucleic acid and protein sequence.

Molecular phylogenetics attempts to determine the rates and patterns of change occurring in RNA (or DNA) or proteins, and to reconstruct the evolutionary history of genes and organisms. Generally, a phylogenetic analysis consists of five steps (Baldauf, 2003): **assembling a dataset**- finding and retrieving sequences from public domain; **multiple sequence alignment-** by using sequence alignment programme (e.g., Clustal X / W); determining the substitution **model** (eg amino acid substitution matrix, gamma correction etc) and **tree building methods** (distance matrix or clustering/algorithmic method – e.g., UPGMA, NJ; Discrete Data or tree searching method- e.g., parsimony, maximum likelihood, Bayesian method); **test the phylogenetic accuracy** (e.g., bootstrapping) and **data presentation.**

Distance-Based Methods for Phylogenetic Prediction

In distance-based tree-building methods, the degrees of differences between pairs of sequences are calculated to construct the distance matrix between individual

pairs of taxa. There are two different algorithms in distance-based methods, the cluster-based and the optimality based. The cluster-based method algorithms build a phylogenetic tree based on a distance matrix starting from the most similar sequence pair; and the algorithms include neighbour-joining (NJ) and Unweighted Pair Group Method with Arithmetic mean (UPGMA). NJ sequentially identifies neighbour pairs that minimize the total length of the tree and NJ does not assume a molecular clock, while UPGMA does. UPGMA can be used to construct phylogenetic trees if the rates of evolution are approximately constant among the different lineages (Graur & Li, 2000), but NJ builds a tree where the evolutionary rates are free to differ in different lineages. The optimality-based method algorithms compare numerous different tree topologies and select the one which is believed to best fit between computed distances in the trees and desired evolutionary distances (often referred to as actual evolutionary distance). Examples of algorithms of optimality-based methods are Fitch-Margoliash and minimum evolution.

Maximum Parsimony Methods for Phylogenetic Prediction

Maximum parsimony predicts the evolutionary tree or trees that minimize the number of steps required to generate the observed variation in the sequence from common ancestral sequences (Mount, 2004). In this method, a multiple sequence alignment (msa) is needed to predict which sequence positions are likely to correspond and these positions will appear in vertical columns in the msa (Mount, 2004). For each aligned position, phylogenetic trees that require the smallest number of evolutionary changes to produce the observed changes from an ancestral sequence are identified. This analysis is continued for every position in the sequence alignment and those trees that produce the smallest number of changes overall for all sequence positions are identified. The maximum parsimony method is best suited for sequences that are quite similar. However, there are some problem with this methods: the most parsimonious tree may not be unique; difficult to make valid statistical statement if there are many steps in a tree; branches with particularly rapid rates of change tend to attract one another (especially when the sequences are short).

Maximum-Likelihood Methods for Phylogenetic Prediction

Maximum-likelihood (ML) methods are especially useful for phylogenetic prediction when there is considerable variation among the sequences in the multiple sequence alignment to be analysed (Mount, 2004). While parsimony methods seek phylogenetic solutions that minimize the amount of evolutionary change required to explain a data set, ML methods attempt to find solutions that have a maximum probability of being correct, given a particular evolutionary model (Swofford et al., 1996). ML methods start with a simple model, such as a model of rates of evolutionary change in nucleic acid or protein sequences and tree models that represent a pattern of evolutionary change, and then they adjust the model until there is a best fit to the observed data. Moreover, unlike parsimony, ML methods consider branch length when calculating the probability of a particular tree being correct. However, the ML methods are similar to the maximum parsimony methods in that the analysis is performed on each column of a multiple sequence alignment (Mount, 2004).

Bootstrapping

Bootstrapping essentially tests whether the whole dataset is supporting the phylogenetic tree. It is a computational method for estimating confidence values for the branching points of a tree; and it produces multiple ´´ new ´´ data sets by random re-sampling from the original dataset (Graur & Li, 2000). The support for the branching pattern is measured as the percentage of the trees that show the same branching pattern; the values are indicated on the internal branches defining the clades (Graur & Li, 2000).

Background of the Studies

Different individual viruses or virus combinations are responsible for disease in sugar beet and it is very difficult to identify them solely on the basis of symptoms. Moreover, some viruses such as BtMV, BWYV, BMYV, BChV, *Beet yellowing virus* (BYV, family *Closteroviridae*, genus *Closterovirus*) can be present in the same plant (Wintermantel, 2005) showing yellowing (symptoms) on leaves in normal growing temperature. Sugar beet is widely grown in Skåne, a region in the southern part of Sweden. However, so far no molecular identification has been carried out in Sweden for BtMV or any one of the beet-infecting poleroviruses (BChV, BMYV, BWYV).

Aim of the present study

The main aim of this study was to identify and characterize (by transmission studies and sequence analyses) viruses associated with mosaic and yellowing symptoms in sugar beet collected from the Skåne region in Sweden. The suspected viruses were BtMV and BMYV.

Materials & Methods:

Virus Sources

Four sugar beet plants displaying mosaic or yellowing symptoms were collected in the southern part (Skåne) of Sweden and were grown at the SLU greenhouse for using in this experiment. Additionally, another four leaves samples were also used in this experiment as positive control.

Planting of Sugar Beet, Spinach, Lettuce and Red Beet in the Greenhouse

As a potyvirus, BtMV can be transmitted mechanically. Therefore, to study the transmission mechanisms as well as host range of BtMV, several plant species including sugar beet (*Beta vulgaris* subsp. *vulgaris*), red beet (*Beta vulgaris*) cv Rubia, lettuce (*Lactuca sativa*) cv Sonette, spinach (*Spinacia oleracea*) cv Long Standing Bloomsdale were planted in the greenhouse at the Genetics Centre, SLU, Uppsala. Hybrid sugar beet seeds were kindly donated by Syngenta Seeds AB, Landskrona, Sweden and approximately 26 sugar beet plants (22 of them grown from seeds and 4 plants collected from Skåne) were used for mechanical transmission tests. Approximately 15 plants each of spinach, lettuce, and red beet were grown in the greenhouse. Seeds of these plants were sown in compost soil

and each seedling was transferred to a pot when they reached a height of 2-3 cm. The conditions of the greenhouse were maintained at a temperature of 25-30°C with a moisture of about 70%-80%. Moreover, extra light was given for plant assimilation and watering was done every day.

Mechanical Inoculation Test of *Beet Mosaic Virus* (BtMV)

Mechanical inoculation tests were done in young plants of sugar beet, spinach, lettuce and red beet at the Genetics Centre greenhouse. The surface of the young leaves was dusted with a small amount of carborundum to scratch the leaves during inoculation. Virus-infected leaves (from Skåne) were ground in a plastic bag. The leaves were homogenized in two different ways: in water or in 0.03 M HEPES (pH 7.0). Subsequently, the leaves were wiped with the virus buffer suspension and twice in a week the inoculated plants were inspected to check whether any symptoms (of BtMV infection) had developed.

Enzyme-linked Immunosorbent Assay (ELISA) to Detect BtMV

DAS-ELISA with polyclonal antibody (Neogen Europe Ltd – ADGEN Phytodiagnostics) was used to detect possible BtMV infection (Clark et al., 1976). The ELISA plate was coated with 100 µl per well of IgG antibody diluted 1:1000 in coating buffer, incubated for 2 hrs at 37°C and later washed 3 times for 3 min with washing buffer. The leaf sample (approximately 3.7 cm^2) was ground in 1 ml sample buffer and 100 µl of ground sample was added to the coated and washed wells. Healthy leaf samples were used as negative controls in two wells and in another two wells only antibody was added as buffer control. After over night incubation at 4°C and washing 3 times for 3 min, 100 µl per well of conjugate (secondary antibody, IgG-AP, diluted 1: 1000 in sample buffer) was added and incubated again for 2 hrs at 37°C. Again after 3 washing steps (each of 3 min with washing buffer), 100 µl of the substrate solution (in a concentration of 0.5 µg P-nitrophenyl phosphate/1 ml substrate buffer) was added to each microtiter well and incubated at room temperature over night (or 1-2 hours). The absorbance was measured with an ELISA-photometer Benchmark Microplate Reader (Bio-Rad Laboratories) at a wavelength of 405 nm. The results of the samples were compared with the positive and negative controls. In the ELISA test the samples were regarded as positive (infected by BtMV) if they turned yellow after adding substrate solution and had a double reading (in the ELISA-photometer) compared to a negative sample (healthy sample).

The following ELISA buffers were used:

Coating Buffer

Loewe Coating

pH 9.6; for 1000 ml dest. water

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g

Washing Buffer

pH 7.4; for 1000 ml dest. water

NaCl	8.00 g
KH_2PO_4	0.20 g
$Na_2HPO_4 \cdot 12H_2O$	1.15 g
KCl	0.20 g
Tween 20	0.50 g

Sample Buffer (Extraction Buffer) / Conjugate Buffer
pH 7.4; for 1000 ml dest. water

NaCl	8.00 g
KH_2PO_4	1.00 g
$Na_2HPO_4 \cdot 12H_2O$	14.50 g
PVP40000	20.00 g
Tween	1 ml

Substrate Buffer
pH 9.8; 1000 ml dest. water
(pH adjusted with conc. HCl)
Diethanolamine 97.00 ml
P-nitrophenyl phosphate was used as substrate
Conjugate Buffer = Sample buffer

RNA Extraction

Sugar beet leaves were homogenized in liquid nitrogen using a mortar and pestle. The powder (0.5 g) was transferred to an Eppendorf (1.5 ml) tube containing 1 ml TRIZOL reagent [380 ml phenol (pH 4.3, Sigma P4682), 118.6 g guanidine thiocyanate (Sigma G9277 or Fluka 50981), 76.12 g ammonium thiocyanate (0.4 M Fluka O0038), 33.4 ml 3 M sodium acetate pH 5.0 (0.1 M), 50 ml glycerol; water was added up to 1 litre] and samples were incubated at room temperature for 5 minutes. After adding 0.2 ml of chloroform, the tubes were shaken for 15 seconds, and incubated at room temperature for 3 min followed by centrifugation in the cold room (13 000 RPM) for 15 min. The aqueous phase (top phase) was transferred to a fresh tube and after adding 0.5 ml isopropanol the tubes were incubated at room temperature for 10 minutes to precipitate the RNA. After centrifugation (13 000 RPM) in the cold room (4°C), the supernatant was removed and the pellets (white visible) were washed with 1 ml of 75% ethanol followed by another 5 min centrifugation (4°C, 13000 RPM). The RNA pellets were left to air dry for 25 min and later dissolved in 20 µl of water. The samples were incubated at 60°C for 10 minutes to dissolve the RNA faster and the concentration was measured with a NanoDrop™ (Thermo Fisher Scientific, Inc.)

Reverse Transcription (RT) & Polymerase Chain Reaction (PCR)

Reverse Transcription (RT)

(for potyvirus BtMV and polerovirus/luteovirus)

RT was carried out using different reverse primers (Table 2) such as universal primers for the families *Potyviridae* or *Luteoviridae* and the BtMV-specific primer BM1.

One µl RNA sample and 1 µl (10 µM) reverse primer (Table 2) were mixed with 10 µl Milli-Q water, incubated at 70°C for 10 minutes and rapidly cooled on ice for 5 min. Then, 4 µl 5x reaction buffer (Fermentas), 2 µl dNTPs (10 mM)(Fermentas), 1 µl RiboLock ™ RNase Inhibitor (Fermentas) were added and incubated at 37°C for 5 minutes followed by adding 1 µl M-MLV Reverse Transcriptase RevertAid ™ (Fermentas). The reaction was incubated at 42°C for 1 hour (with Lu4 or M4T rev-primer) or 45 min (with BM1 rev-primer).

Table 2: Primers used for detection of *Beet mosaic virus* and luteoviruses by RT-PCR.

Primers target	For Reverse Transcription	For PCR amplification	References
Potyviridae-specific universal primers	M4T : 5′-GTT TTC CCA GTC ACG AC$(T)_{15}$-3′	Sprimer: 5′-GGX AAY AAY AGY GGX CAZ CC-3′, X= A,G,C or T; Y= T or C; Z= A or G	Chen et al., 2001
		M4: 5′-GTT TTC CCA GTC ACG AC-3′	
Beet mosaic virus-specific primers	BM1: 5′-CAGTTGCGAGTGTACGTAGT-3′	Reverse primer BM1 & Forward primer BM2: 5′-CATACATGCCTCGTTATGGC-3′	Glasa et al., 2003
		Reverse primer BM1 , & Forward primer BM3: 5′-GTGCCACAACAAGTTGATGC-3′	
Luteoviridae-specific universal primers	Lu4 : 5′-GTCTACCTATTTGG-3′	Upstream primer Lu1 : 5′-CCAGTGGTTRTGGTC-3′, R=G or A	Robertson et al., 1991
		Downstream non-degenerate primer, Lu4 : 5′-GTCTACCTATTTGG-3′	

Conditions for PCR Amplification

For Detection of Potyvirus and *Beet Mosaic Virus*

Different PCR amplifications were carried out by *Potyviridae*-specific universal primers (degenerate Sprimer; primer M4), and by the two BtMV-specific primer pairs BM1/BM2 and BM1/BM3 (reverse primer BM1, two forward primers BM2 and BM3) (Table 2).

For *Potyviridae*-specific universal primers (Sprimer & M4), every 50 µl PCR contained 2 µl template cDNA, 2 µl (10 µM) of each amplification primer, 0.5 µl (5 U/µl) Taq DNA polymerase (Invitrogen), 1 µl dNTPs (10 µM)(Fermentas), 5 µl 10XBuffer (Invitrogen), 1.5 µl $MgCl_2$ (50 mM)(Invitrogen) and 36 µl H_2O. The PCR was carried out with a PTC-100 programmable thermal controller (MJ Research Inc) or a Minicycler (MJ Research Inc) using the following program (Chen et al., 2001): initial denaturation at 94°C for 2 min; amplification for 30 cycles each of 30 sec at 94°C, 1 min at 47°C and 2 min at 72°C; and a final extension for 10 min at 72°C.

For BtMV-specific primers (BM1/BM2 & BM1/BM3), the amplification was carried out as above, and if 5 µl 10XBuffer (-$MgCl_2$)(Fermentas) was used then 3 µl $MgCl_2$ (50 mM)(Fermentas) was added with 34.5 µl H_2O. The PCR was carried out using the following program (Glasa et al., 2003): initial denaturation at 94°C for 5 min; amplification for 30 cycles each of 30 s at 94°C, 30 s at 58°C and 45 s at 72°C; and a final extension for 10 min at 72°C.

For Detection of Poleroviruses

Sometimes both BtMV and BMYV (polerovirus) can give similar mosaic symptoms. To check the possibility whether the suspected samples were infected with polerovirus instead of BtMV the upstream degenerate primer Lu1 and

downstream non-degenerate primer Lu4 (Table 2) were used to detect polerovirus/luteovirus as described by Robertson et al. (1991). The PCR amplification was carried out as described above and using the following program: initial denaturation at 94°C for 1 min; amplification for 40 cycles each of 30 s at 94°C, 1 min at 41°C and 2 min at 72°C; and a final extension for 10 min at 72°C.

Agarose Gel Electrophoresis

The PCR products were analysed on 1% agarose gel with 0.5xTBE and were stained with ethidium bromide (1 µg/ml). In the gel electrophoresis, 12 µl of samples and marker were run for identification of the band, and 30 µl of samples and marker were run for gel purification and cloning in the following way:

1.0 g agarose + 100 ml 0.5XTBE + 1 µl EtBr = 1% agarose gel

To identify the band

Samples:		Marker:	
	10 µl PCR product		1 µl λ *Eco*RI/*Hind*III (0.5 µg)
	2 µl 6XLoading buffer		2 µl 6XLoading buffer
	12 µl total		9 µl Milli-Q water
			12 µl total

To purify the PCR product and for cloning

Samples:		Marker:	
	25 µl PCR product		2.5 µl λ *Eco*RI/*Hind*III (0.5 µg)
	5 µl 6XLoading buffer		5 µl 6XLoading buffer
	30 µl total		22.5 µl Milli-Q water
			30 µl total

Gel Extraction and Purification

Purification of DNA fragments was done using the QIAquick Gel Extraction Micro Centrifuge and Vacuum Protocol according to the manufacturer's instructions. Briefly, the DNA band was excised under UV light from EtBr-stained agarose gel with a clean, sharp scalpel and transferred to an Eppendorf tube. Then, 3 volumes of Buffer QG was added to 1 volume of gel. For dissolving the gel completely, the tube was incubated at 50°C for 10 min and vortexed every 2-3 min during the incubation. When the gel slice was dissolved completely and the colour of the mixture turned into yellow, 1 gel volume of isopropanol was added to the sample and mixed. A QIAquick spin column was placed in the provided 2 ml collection tube and the sample was added to the QIAquick column. After 1 min centrifugation the flow-through was discarded. Buffer QG (0.5 ml) was added to the QIAquick column and the solution was centrifuged for 1 min and the flow-through was discarded. Buffer PE (0.75 ml) was added to the column, centrifuged for 1 min and the flow-through was discarded, followed by another 1 min centrifugation. The QIAquick column was placed into a clean 1.5 ml micro centrifuge tube, and 50 µl of Buffer EB (10 mM Tris HCl, pH 8.5) was added to the centre of the QIAquick membrane, followed by centrifugation for 1 min. Additional 30 µl elution buffer was added to the centre of the QIAquick

membrane. The column was let to stand for 1 min and again centrifuged for 1 min.

Cloning PCR Products with pJET1.2/blunt Cloning Vector

The purified PCR fragments were cloned using CloneJET™ PCR Cloning kit (Fermentas) and Subcloning Efficiency DH5α Competent Cells (Invitrogen). The following steps were carried out for cloning and analyses:

1. Culture Media and Agar Plates

Every 1 litre of LB-Agar media had the following components:

Tryptone	10 g
Yeast X (Bacto Yeast extract)	5 g
NaCl	10 g
pH 7.0 with 1 *N* NaOH	
Agar (Bacto Agar)	15 g

The components above were mixed with destilled water (up to 1 litre), autoclaved and cooled. Then, 1 ml ampicillin (100 μg/ml) was added to 1 litre of LB agar. Another 1 litre culture medium was prepared as described above but no agar was added to the medium.

2. Ligation of PCR Fragments into pJET1.2/blunt Cloning Vector

The CloneJET™ PCR Cloning kit (Fermentas) has the cloning vector pJET1.2/blunt containing a lethal gene, which is disrupted by ligation of a DNA insert into the cloning site. Therefore only cells with recombinant plasmids were able to propagate, eliminating the need for expensive blue/white screening. The vector also contains an expanded multiple cloning site, as well as a T7 promoter for *in vitro* transcription (Figure 5 & Table 3). The pJET1.2/blunt is a linearized cloning vector, which accepts insert from 6 bp to 10 kb. The 5′-ends of the vector cloning site contain phosphoryl groups; therefore, phosphorylation of the PCR primers was not required.

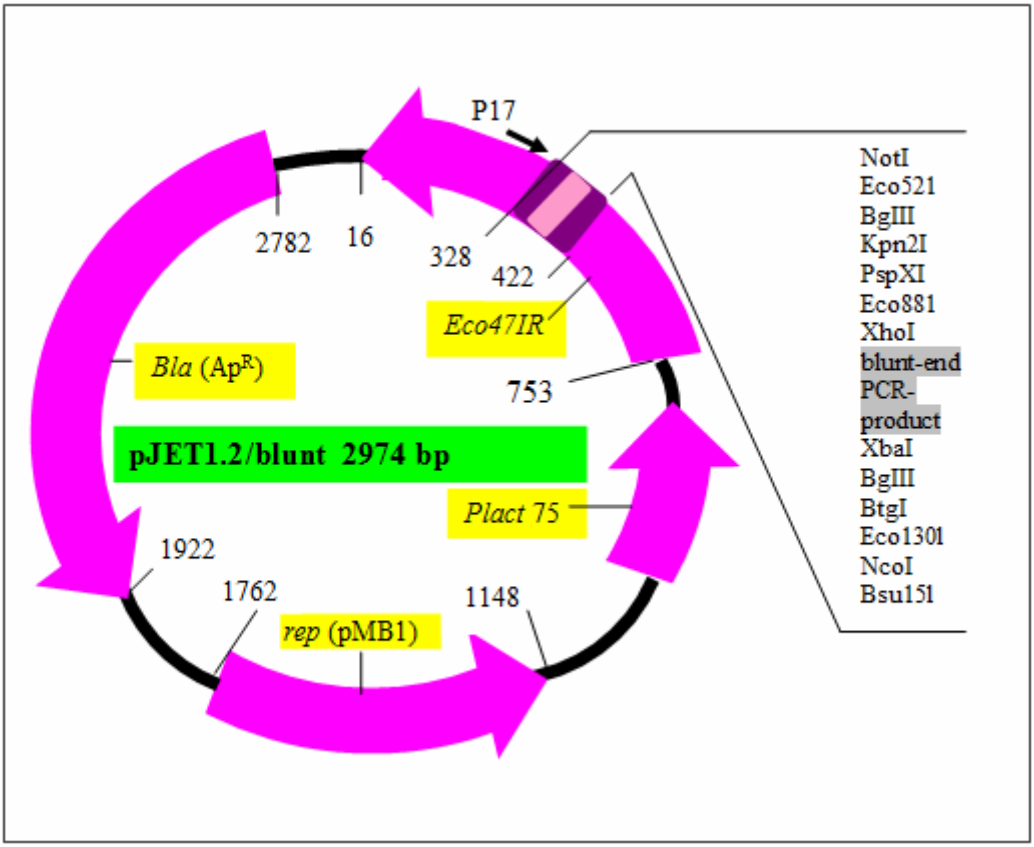


Figure 5: pJET1.2/blunt Vector Map
(Source: http://fermentas.kd-services.de/fermentas-shop/product_info.php?info=p743)

Since PCR products generated by *DreamTaq/Taq* DNA polymerase have 3´-dA overhangs, the ligation was done using the sticky-end cloning protocol of the CloneJET™ PCR Cloning kit. Prior to ligation a blunting procedure was done in the following way:

2X reaction buffer	10 µl
PCR product	2 µl
Water, nuclease-free	5 µl
DNA blunting enzyme	1 µl
	Total 18 µl

The reaction mixture was vortexed briefly, centrifuged for 3-5 s, and incubated at 70°C for 5 min and chilled briefly on ice.

Table 3: Genetic Elements of pJET1.2/blunt cloning vector

Element	Function	Position (bp)
rep (pMB1)	Replicon (rep) from the pMBI plasmid responsible for the replication of pJET1.2	1762-1148
Replication start	Initiation of replication	1162 ±1
bla (Ap^R)	β-lactamase gene conferring resistance to ampicillin. Used for selection and maintenance of recombinant *E.coli* cells	2782-1922
eco47IR	Lethal gene *eco47IR* enables positive selection of recombinant plasmid	753-16
P_{lacUV5}	Modified P_{lac} promoter for expression of the *eco47IR* gene at a level sufficient to allow for positive selection	892 -769
T7 promoter	T7 RNA polymerase promoter for *in vitro* transcription of the cloned insert	305-324
Multiple cloning site (MCS)	Mapping, screening and excision of the cloned insert	422-328
Insertion site	Blunt DNA ends for ligation with insert	371-372
Primer binding sites:		
pJET1.2 forward sequencing primer	Sequencing of insert, colony PCR	310-332
pJET1.2 reverse sequencing primer	Sequencing of insert, colony PCR	428-405

Primer sequences

pJET1.2 forward sequencing primer, 23-mer	5'-CGACTCACTATAGGGAGAGCGGC-3'
pJET1.2 reverse sequencing primer, 24-mer	5'-AAGAACATCGATTTTCCATGGCAG-3'

(Source: http://fermentas.kd-services.de/fermentas-shop/product_info.php?info=p743)

To make a ligation reaction of 20 µl, 1 µl each of pJET1.2/blunt cloning vector (50 ng/µl) and T4 DNA ligase (5 U/µl) were added to the blunting reaction. The ligation mixture was vortexed briefly, centrifuged for 3-5 s and incubated at 14°C overnight and used directly for bacterial transformation.

3. Transformation Using Subcloning Efficiency™ DH5α™ Competent Cells

Fifty µl Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen) were gently mixed with 5 µl ligation mixture (from previous day) in a 2 ml Eppendorf tube and kept on ice for 30 min followed by a 20 s heat-shock in a water bath at 37°C and 2 min incubation on ice. At room temperature, 900 µl of liquid LB medium were added and the bacteria were incubated for 1h 30 min at 37°C with shaking to allow transformed bacteria to recover and express the antibiotics resistance gene. Then, 100 µl of each transformation reaction were plated and spread with glass rod (dipped in ethanol, burned and then used for gentle spread)

on LB-agar plates containing the antibiotic ampicillin. The plates were incubated at 37°C overnight. Since we used pJET1.2/blunt cloning vector, no X-gal or blue/white screening was needed (CloneJET™ PCR Cloning kit protocol No K1231/K1232, Fermentas Life Sciences).

4. Overnight Culture

The selected colonies were taken from the LB-agar plates with a platinum stick. The colonies were added to 4 ml liquid LB (with ampicillin) and incubated again overnight at 37°C.

5. Plasmid Purification

Plasmid DNA from overnight cultures was purified prior to sequencing using the GeneJet Plasmid Miniprep Kit (Fermentas). For this purpose, the samples were added to 2 ml tubes and centrifuged (11000 RPM) for 1 min. The white pellet was kept after discarding the upper fluid, and 250 µl of Resuspension Solution was added. The pellet was resuspended by shaking. The Lysis Solution (250 µl) and Neutralization Solution (350 µl) were added, and mixed thoroughly by inverting the tube 4-6 times until the solution became viscous and slightly clear. After 5 min of centrifugation, the supernatant was loaded to the supplied GeneJET spin column. Another 1 min centrifugation was done and after discarding the flow-through the column was put back into the same collection tube. Then, 500 µl Wash Solution (diluted with ethanol prior to first use) was added to the column, centrifuged for 1 min, the flow-through was discarded, the column was placed back into the same collection tube and the whole procedure was repeated once again. After an additional 1 min centrifugation the spin column was placed into a clean 1.5 ml Eppendorf tube, and 50 µl Elution Buffer (buffer EB) was added to the centre of the spin column. After incubation at room temperature for 2 min (with open cap) the tube was centrifuged for 2 min (11000 RPM), the column was discarded and the purified plasmid DNA was stored at -20 °C.

6. Insert Excision & Gel Electrophoresis

The insert was cut out from the plasmid DNA using *Bgl*II restriction Reaction Mix:

10X Buffer O with BSA	1 µl (Fermentas)
Plasmid (purified)	2 µl
*Bgl*II (10 U/µl)	0.5 µl
Milli-Q water	6.5 µl
	Total 10 µl

The reaction mix was incubated at 37°C for 1 hour.

Digested DNA was separated by agarose gel electrophoresis and the fragments were visualised by ethidium bromide staining and UV irradiation according to the procedure described before.
For agarose gel electrophoresis, 12 µl of reaction mix or marker were used:

Samples:		Marker:	
10 µl	Reaction mix from step 6	1 µl λ *Eco*RI/*Hind*III (0.5 µg)	
2 µl	6XLoading buffer	2 µl	6XLoading buffer
12 µl	total	9 µl	Milli-Q water
		12 µl	total

Sequencing & Phylogenetic Analyses

Sequencing and Alignment: The clones were sequenced (Macrogen Inc., Seoul, South Korea) in both directions and the obtained sequences were edited (removing the sequences of primers and vectors) in the BioEdit (Hall, 1999) sequence editor and then used for a search in GenBank with BLASTn (Altschul et al., 1990) to identify the sequences. All virus sequences obtained in this study and their closest matches from GenBank were aligned using the progressive alignment programme ClustalW (Thompson et al., 1994). Nucleotide sequences were translated using the Translate tool at the Expasy homepage (http://www.expasy.org/). Search of GenBank with BLASTp and BLASTx were done to identify homologous amino acid sequences.

The Best Evolutionary Model Selection & Phylogenetic Analyses

Phylogenetic and molecular evolutionary analyses were carried out using nucleotide sequences and the programmes MEGA version 4 (Tamura et al., 2007), PAUP* 4.0 (Swofford, 2002) and TreeView (Page, 1996).

PAUP*4.0 supported software Mrmodeltest2.3 (Nylander, 2004) was used to select the best evolutionary model. The NEXUS data files (from alignment) and *mrmodelblock* were executed in the PAUP*4.0 command prompt to obtain *mrmodel.score*, which gave an *outfile* while running *Mrmodeltest2.3* in the Windows command prompt. The *outfile* contained suggested best models from Hierarchical Likelihood Ratio Tests (hlRT) and Akaike Information Criterion (AIC) analysis. *Mrmodeltest2.3* gave a PAUP*4.0 block, which was added to the NEXUS data files to execute the suggested best evolutionary model files in PAUP*4.0. Different optimality set criteria (maximum likelihood, minimum evolutionary distance and maximum parsimony) were used to obtain different phylogenetic trees. The final tree was generated in PAUP*4.0 using the distance-based tree-building method Neighbour-joining. The robustness of the internal branches of the tree was estimated by bootstrap analysis using 10000 replications. Trees were viewed in the TreeView software.

Results

Mechanical Inoculation Tests

In this transmission test, red beet, lettuce and spinach were used since it was known that BtMV can also naturally infect those plants similar as sugar beet (Hasan, 2004; Xiang et al., 2007; Wang et al., 2008). However, no symptoms were visible from any of the plants after transmission by mechanical inoculation (Table 4).

Table 4: Results of mechanical inoculation/transmission test with suspected *Beet mosaic virus* from Skåne.

Test plants	Varieties/ Cultivars	Total number of plants	Repetitions	Symptoms 2-3 weeks after inoculation (Yes/No)
Sugar beet (*Beta vulgaris*)	-	26	4	No
Red beet (*Beta vulgaris*)	Rubia	15	3	No
Spinach (*Spinacia oleracea*)	Long Standing Bloomsdale	15	3	No
Lettuce (*Lactuca sativa*)	Sonette	15	3	No

Therefore, the results show that the initial plants most likely were not infected by a mechanically transmissible virus, such as BtMV.

Serological Test (ELISA) for *Beet Mosaic Virus*

Since several mechanical inoculation/transmission tests (to detect BtMV) were conducted on sugar beet, it was also necessary to carry out ELISA tests (as follow up experiment) on the different growth stages of the same sugar beet plants after inoculation from the virus source. Therefore, the 1st and 2nd ELISA tests were conducted approximately at four weeks, 3rd ELISA test at five weeks and 4th ELISA test at eight weeks after inoculation (Table 5 and 6). In the fourth ELISA test five additional sugar beet plant samples (Inoculated Plant A, B, C, D and E in Table 6) were also used.

Table 5: 1st, 2nd & 3rd ELISA plate reports at 405 nm for detection of *Beet mosaic virus*.

Name (Buffer/Control/plant)		**1st ELISA test**	**2nd ELISA test**	**3rd ELISA test**
Buffer with antibody	1st Sample *	0.065	0.160	0.292
	2nd Sample	0.058	0.172	0.243
Negative Control	1st Sample	0.104	0.220	0.269
	2nd Sample	0.103	0.220	0.323
Positive Control – ELISA kits	1st Sample	0.292	0.327	0.493
	2nd Sample	0.325	0.294	0.560
Source plant - Skåne 0	1st Sample	-	0.232	0.412
	2nd Sample	-	0.255	0.487
Inoculated Plant 1	1st Sample	0.090	0.184	0.344
	2nd Sample	0.092	0.190	0.321
Inoculated Plant 2	1st Sample	0.086	0.214	0.274
	2nd Sample	0.079	0.195	0.296
Inoculated Plant 3	1st Sample	0.669	0.196	0.293
	2nd Sample	0.089	0.200	0.363
Inoculated Plant 4	1st Sample	0.089	0.201	0.350
	2nd Sample	0.096	0.203	0.339

* 1st sample and 2nd sample mean that in every ELISA test two samples (plant sap) were taken from each plant (leaf).

Table 6: 4th ELISA plate report at 405 nm for detection of *Beet mosaic virus*.

Name (Buffer/Control/plant)		4th ELISA test
Buffer with antibody	1st Sample *	0.117
	2nd Sample	0.150
Negative Control	1st Sample	0.366
	2nd Sample	0.466
Source plant - Skåne 1	1st Sample	0.472
	2nd Sample	0.486
Positive Control	Supplied with ELISA Kits	0.862
	Source plant - Skåne 2	0.380
Inoculated Plant 1	1st Sample	0.194
	2nd Sample	0.253
Inoculated Plant 2	1st Sample	0.286
Inoculated Plant 3	1st Sample	0.288
Inoculated Plant 4	1st Sample	0.415
Inoculated Plant A	1st Sample	0.481
Inoculated Plant B	1st Sample	0.174
	2nd Sample	0.217
Inoculated Plant C	1st Sample	0.301
	2nd Sample	0.566
Inoculated Plant D	1st Sample	0.471
	2nd Sample	0.524
Inoculated Plant E	1st Sample	0.202
	2nd Sample	0.312

* 1st sample and 2nd sample mean that in ELISA test two samples (plant sap) were taken from each plant (leaf).

However, ELISA analysis for BtMV did not show any clear differences between the inoculated plants and healthy control plants (except an experimental error, the 1st sample of the inoculated plant 3 in the 1st ELISA test) (Table 5). The quality of the antibody used in the ELISA might not be good enough since in the 2nd and 3rd ELISA tests the positive control supplied with the ELISA kit did not give double reading compared to the negative control (Table 5).

In the 4th ELISA test (Table 6), only the positive control sample supplied with the ELISA kits gave a clear positive result (plate reading 0.862 compared to 0.366 and 0.466 in the negative control), which demonstrates the authenticity of the ELISA test. The two suspected positive samples collected from Southern Sweden, Skåne 1 and Skåne 2, did not give any positive results because their ELISA plate readings were not double that of the negative control (Table 6).

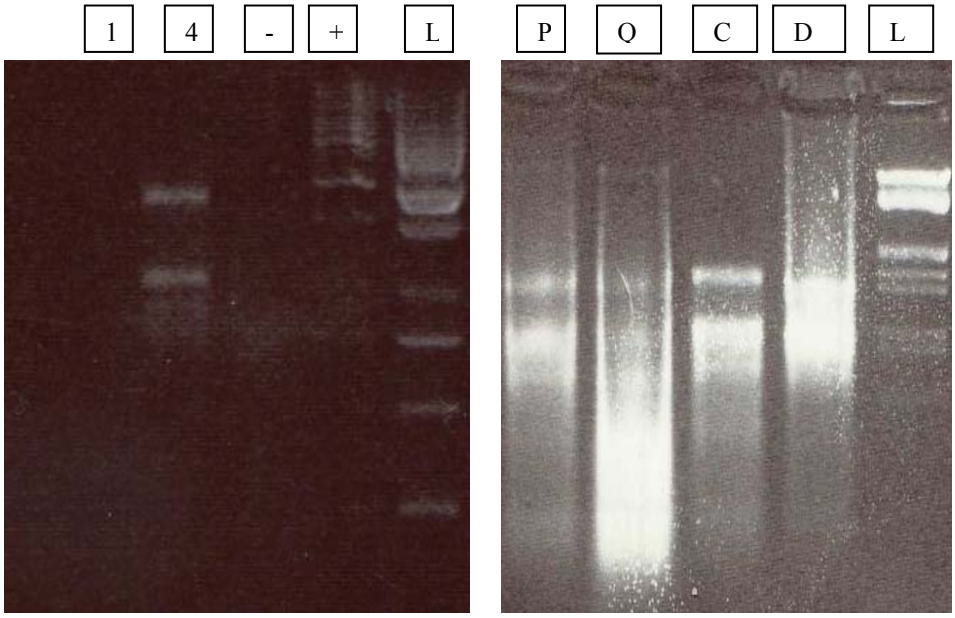
Therefore, it was confirmed from the ELISA tests that none of the mechanically inoculated plants or the suspected positive samples collected from the southern part of Sweden were infected with BtMV (Table 5 and 6).

RNA Extraction

RNA was extracted from eight sugar beet plants and almost all of the extracts contained the expected nucleic acid concentration (Table 7) except for the Inoculated Plant sample C, which showed comparatively lower concentration in the NanoDrop analysis.

Table 7: Average RNA concentration (ng/µl) measured with NanoDrop

Dilution	Sample	Concentration of RNA (ng/µl)	Sample	Concentration of RNA (ng/µl)
Measured with 1:10 Dilution	Skåne 0	291.90	Negative Control	294.05
	Inoculated Plant 1	161.20	Inoculated Plant 4	87.77
measured without dilution	Skåne 3	2643.2	Skåne 1	2491.82
	Inoculated Plant C	485.95	Inoculated Plant D	1497.35



a **b**

Figure 6: Gel from total RNA extract of sugar beet plants a) from left: 1 & 4 Inoculated plants samples at SLU greenhouse, negative sample (-), sample Skåne 0 (+), L Marker (λ *Eco*RI/*Hind*III). b) from left: P, sample Skåne 2; Q, sample Skåne 1; C & D, Inoculated plant samples at SLU greenhouse; L Marker (λ *Eco*RI/*Hind*III).

Sometimes the NanoDrop or other spectrophotometric measurements can give false results. Therefore, samples were also run on a gel to verify the RNA concentration and to check if the RNA was intact. In this case, except for inoculated plant sample 1 and the negative sample (-), RNA was visible for all samples (Figure 6). It seems that an experimental error might have occurred either

during measurement of concentration or loading samples on the gel, since according to the concentration measurements, the RNA yield of sample 1 and negative sample (-) were acceptable, but there was almost nothing visible in the gel picture (Table 7 and Figure 6).

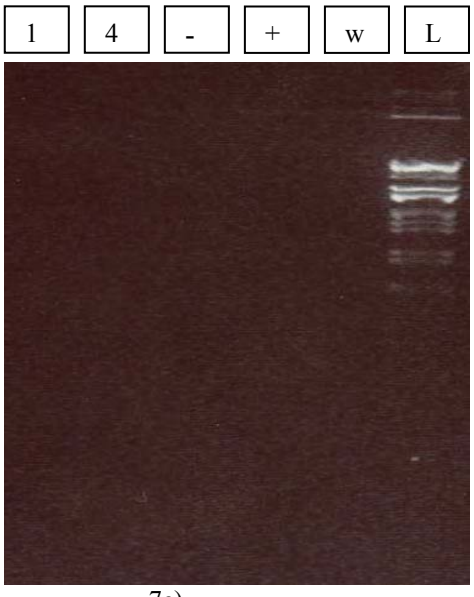
RT-PCR Amplification

Detection of Potyvirus and *Beet Mosaic Virus*

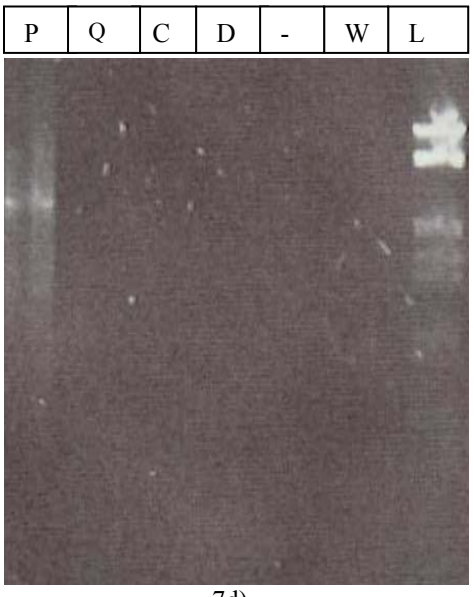
To check whether the symptomatic samples collected from Southern Sweden (sample Skåne 0, Skåne 1 & Skåne 3) and inoculated plants grown in the SLU greenhouse (Plant sample 1 and 4) were really infected by any potyvirus, several PCR amplifications were conducted with *Potyviridae*-specific primers (S-primer/primer M4, Table 2), but none of them gave the expected band of 1.7 kb (Figure 7).

7a)

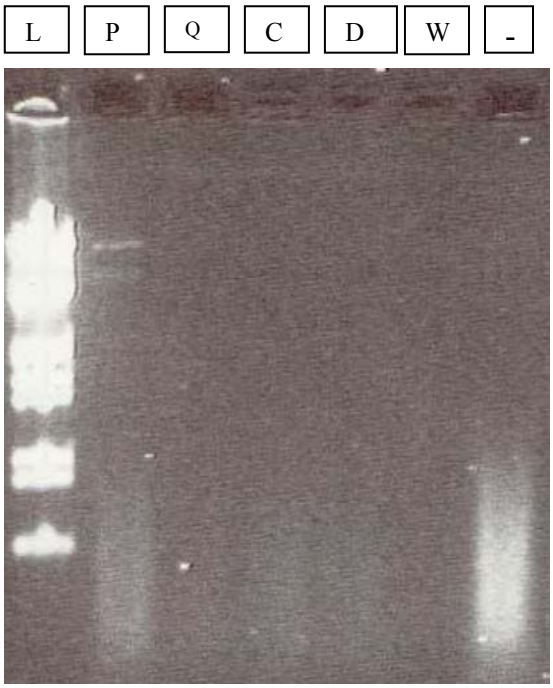
7b)



7c)



7d)



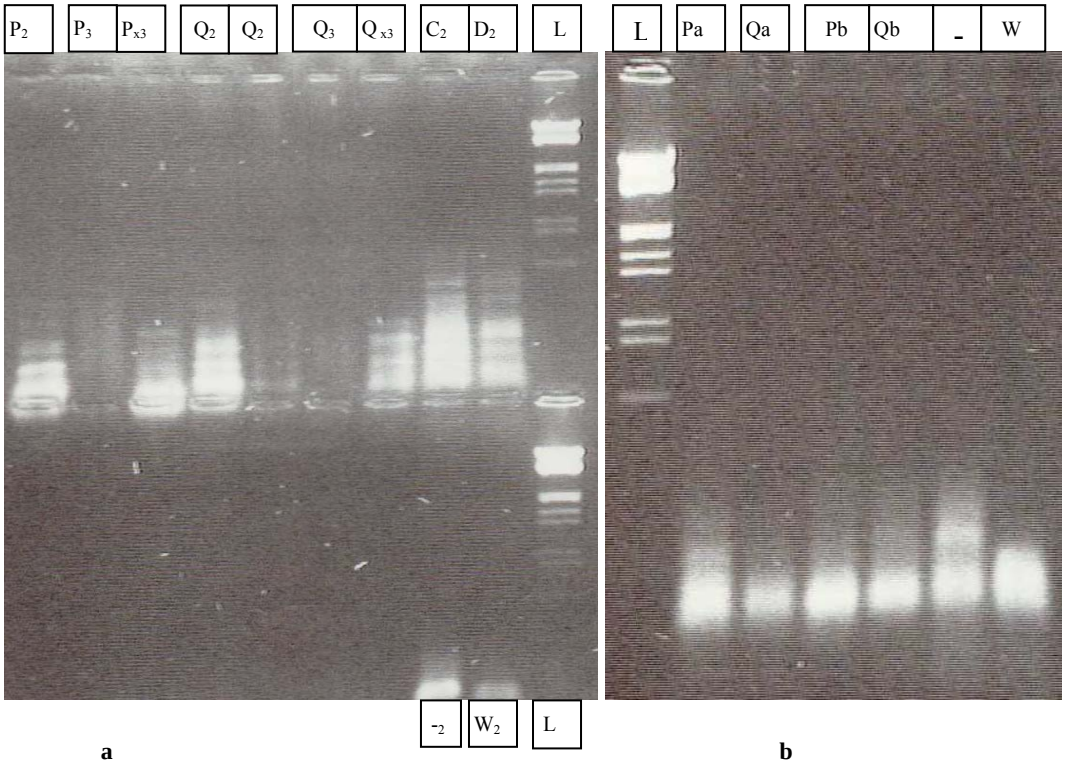
7e)

Figure 7: RT-PCR analysis of sugar beet samples using universal potyvirus primers.

a), b) and c):
1 & 4, inoculated plant samples; (-), sample without inoculation; (+), sample Skåne 0; W, water; L, Marker λ *Eco*RI+*Hind*III;

d) and e):
P, sample Skåne 3; Q, sample Skåne 2;
C and D, inoculated plant samples; (-), sample without inoculation; L, Marker λ *Eco*RI/*Hind*III.

The expected 1.7 kb band for potyvirus infection was not found.



a b

Figure 8: RT-PCR with *Beet mosaic virus*-specific primers (BM1/BM2 and BM1/BM3). Samples P_2, Q_2, C_2, D_2, W_2, $(-)_2$, Pa, Qa, W and (-) were run with BM1/BM2 and the expected product size is 244 bp. Samples P_3, P_{x3}, Q_3, Qx_3, P_b and Q_b were run with BM1/BM3 and the expected product size is 755 bp. Samples C_2 and D_2 were inoculated plant sample; (-) & $(-)_2$ samples without inoculation or negative sample; P_2, P_3, P_{x3}, Pa and Pb several samples of the plant Skåne 3; Q_2, Q_3, Qx_3, Qa, and Qb several samples of the plant Skåne 1; W & W_2= water, L = Marker λ *Eco*RI/*Hind*III. No PCR amplification gave a positive result.

Similarly, PCR amplification with BtMV-specific primers (BM1/BM2 & BM1/BM3) did not give the expected bands (244 bp for BM1/BM3 and 755 bp for BM1/BM3) for any of the samples collected from southern Sweden (P_2, P_3, P_{x3}, Q_2, Q_3, Qx_3 in Figure 8a and Pa, Qa, Pb, Qb in Figure 8b) or inoculated plant samples (C_2, D_2 in Figure 8a) grown in the SLU greenhouse. Therefore, from the PCR tests we could conclude that neither the samples collected from southern Sweden nor the plants grown at the SLU greenhouse inoculated with extract from the collected samples were infected with BtMV or any potyvirus.

Detection of Polero/Luteovirus & Purification of PCR fragment

After running PCR using *Luteoviridae*-specific universal primers (Lu1 & Lu4) the expected band of ca 500 bp was obtained for the samples from Skåne (P & Q in Figure 9). That gave a clear indication that the symptomatic samples were infected by a member of the family *Luteoviridae* (and not by BtMV from the family *Potyviridae* as primarily assumed). Moreover, since polero/luteovirus cannot be transmitted mechanically, it was expected that the mechanically inoculated plants (C & D in Figure 9) grown in the SLU greenhouse would be negative in the PCR test (Figure 9).

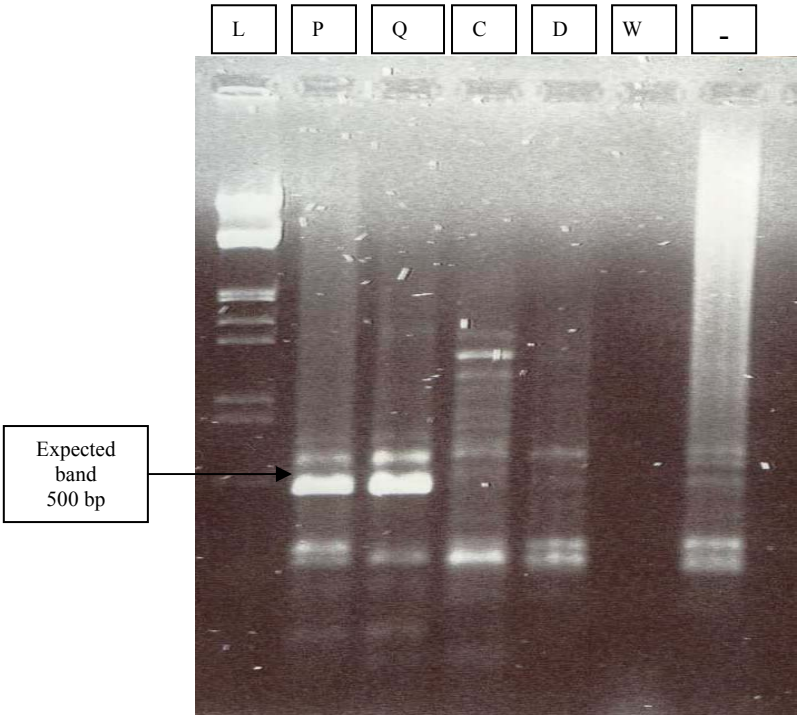


Figure 9: PCR of *CP* gene of polero/luteovirus detected with *Luteoviridae*-specific primers Lu1/Lu4. Lane L is DNA size marker (λ *Eco*RI/*Hind*III). Lanes P (Skåne 3), Q (Skåne 1) are samples collected from southern Sweden; Lanes C, D are inoculated plant samples from SLU greenhouse; W, water; (-) sample without inoculation or negative sample. The expected DNA fragment of 500 bp represents a part of polero/luteovirus *CP* gene.

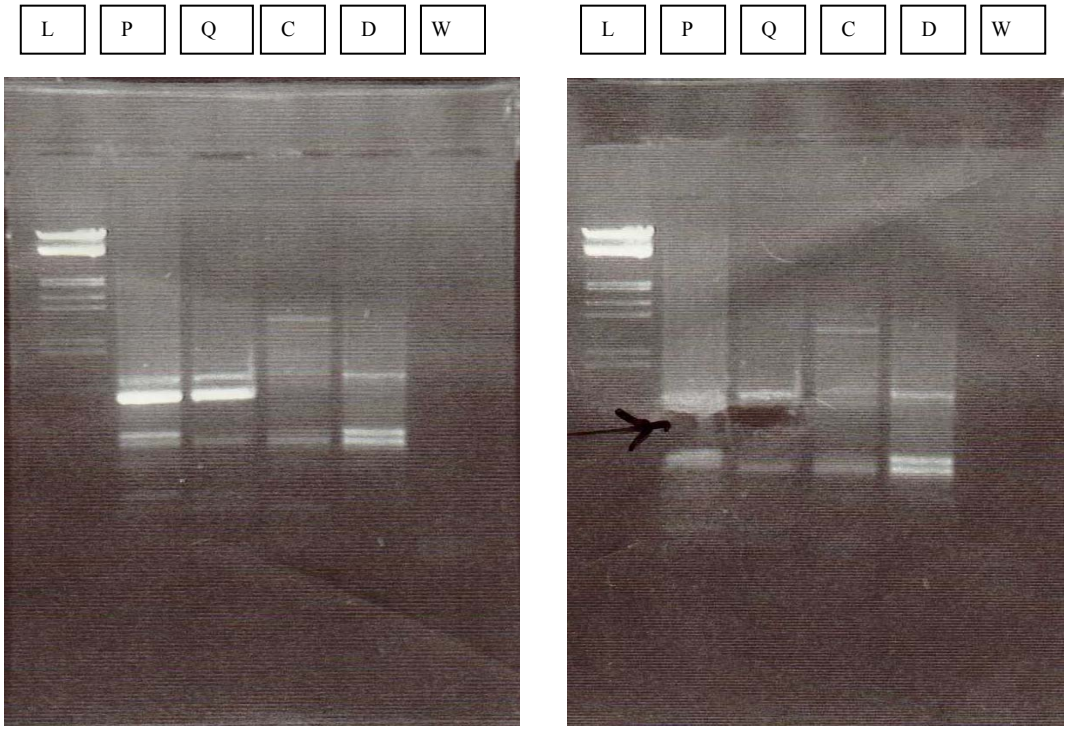


Figure 10. Purification of PCR product for polero/luteovirus *CP* gene. Cutting *CP* DNA band from the gel. a) before cutting b) after cutting. Lane L is DNA size marker (λ *Eco*RI/*Hind*III). Lane P (Skåne 3), Q (Skåne 1) are samples collected from southern Sweden; Lanes C, D are inoculated plant samples from SLU greenhouse; W, water.

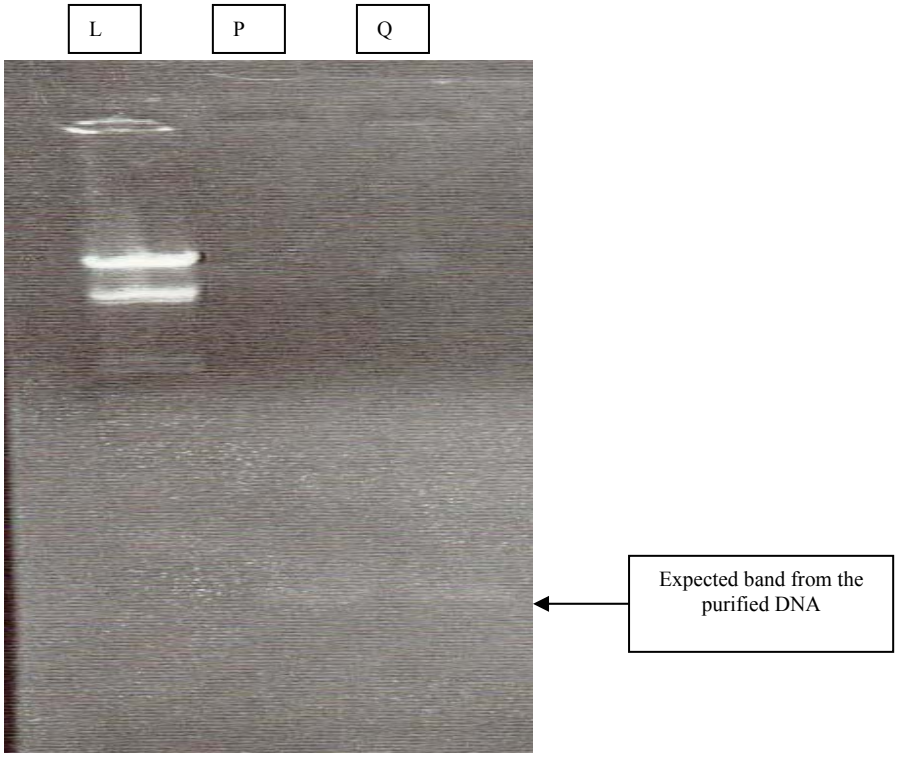


Figure 11: Gel electrophoresis of purified PCR products. L= Marker λ *Eco*RI/*Hind*III; Lanes P = Skåne 3 and Q = Skåne 1.

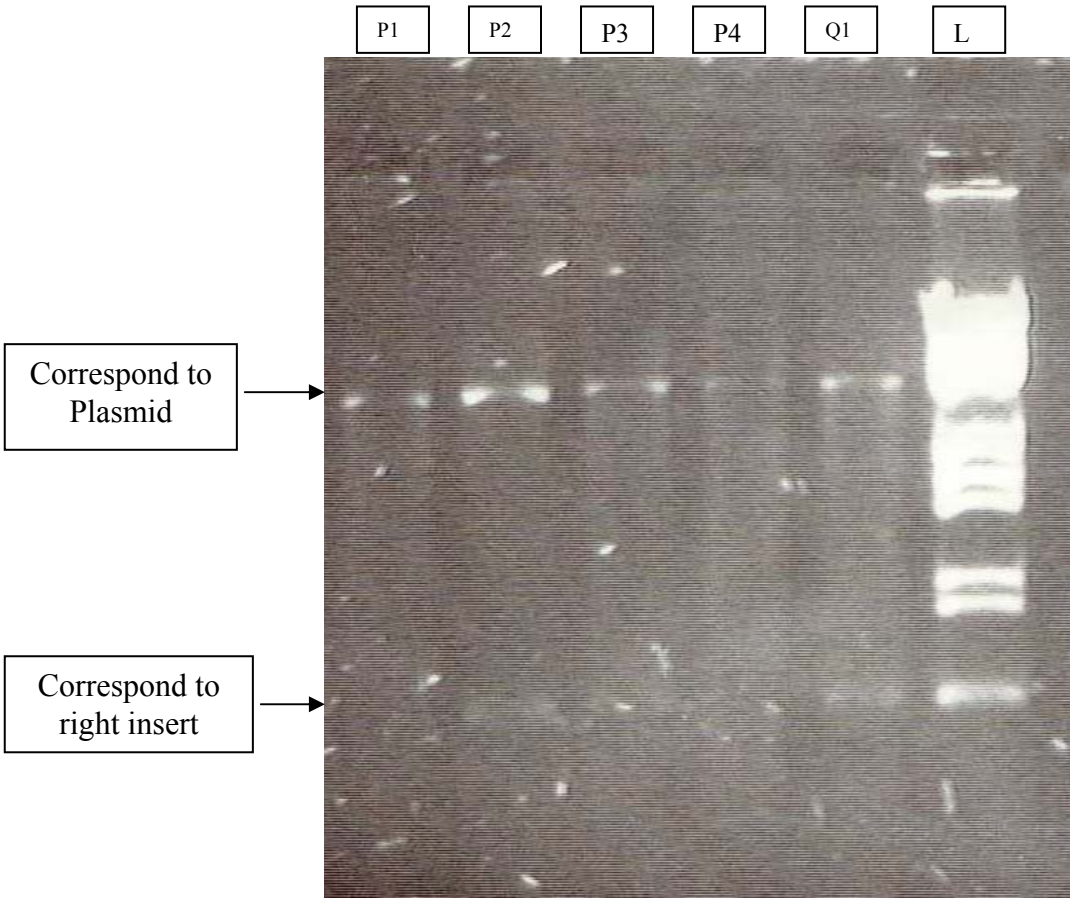


Figure 12: Restriction enzyme (*Bgl*II) digestion of plasmids to see if they contained the PCR product of the *CP* gene. Lanes P1, P2, P3 and P4 are clones from isolate P (Skåne 3) and Q1 is a clone from isolate Q (Skåne 1); lane L is marker (λ *Eco*RI/*Hind*III). The upper band corresponds to plasmid vector and the lower band corresponds to the right insert. Only clones P3, P4 and Q1 were sent for sequencing.

The PCR products for the polero/luteovirus *CP* gene were excised from the gel (Figure 10) and purified. The DNA concentration of the purified bands, measured in the NanoDrop, were 3.72 ng/µl for sample P (Skåne 3) and 3.88 ng/µl for sample Q (Skåne 1). Gel electrophoresis showed that sufficient amounts of DNA had been obtained for two samples (P & Q in Figure 11), which were subsequently used for cloning.

Restriction enzyme (*Bgl*II) digestions confirmed that the plasmids contained the right insert corresponding to the *CP* gene (Figure 12).

Table 8: DNA concentration (ng/µl) of purified plasmids DNA.

Sample Name	DNA Concentration (ng/ µl)	Sequenced (Yes/No)
P1	26.59	No
P2	29.53	No
P3	43.31	Yes
P4	43.61	Yes
Q1	46.28	Yes

The yields from the plasmid DNA extractions were low. Therefore, only three clones (P3, P4 & Q1), which have comparatively higher DNA concentration (Table 8), were sent for sequencing.

Sequence and Phylogenetic Analyses

Analysis of amplification products (*CP* gene) from the virus isolates (sample P3 was renamed SE1 and sample Q1 SE2) collected from Skåne revealed

Box 1: DNA sequences (nt = 505) of two clones (SE1 and SE2) in both directions.

>SE1-R (5´-3´)
CAAACCTCTCGGACAACACAACGCCGACCTAGACGACGACGAAGAGGTAACAA
CCGGACAAGAAGAACTGTTTCTACCAGAGGAACAGGTTCGAGCGAGACATTCGT
TTTCTCAAAAGACAATCTCGCGGGAAGTTCCAGCGGAGCAATCACGTTCGGGCC
GAGTCTATCAGACTGCCCGGCATTCGCTGATGGAATGCTCAAGGCCTACCATGA
GTATAAAATCTCGATGGTCATCTTGGAGTTCGTCTCCGAGGCCTCTTCCCAAAAT
TCCGGTTCCATCGCTTACGAGCTGGACCCACACTGTAAACTCAGTGCCCTTTCAT
CAACCATTAACAAGTTCGGGATCACAAAGCCCGGCAGGAGGGCATTTACAGCGT
CTTACATCAACGGGACGGACTGGCATGACGTTGCCAAGGACCAATTCAGGATCC
TCTACAAAGGCAATGGTTCCTCATCGATAGCTGGTTCTTTTAGAATCACCATGAA
ATGCCAGTTCCACAATC

>SE2-F (5´-3´)
CAAACCTCTCGGACAACACAACGCCGACCTAGACGACGACGAAGAGGTAACAA
CCGGACAAGAAGAACTGTTTCTACCAGAGGAACAGGTTCGAGCGAGACATTCGT
TTTCTCAAAAGACAATCTCGCGGGAAGTTCCAGCGGAGCAATCACGTTCGGGCC
GAGTCTATCAGACTGCCCGGCATTCGCTGATGGAATGCTCAAGGCCTACCATGA
GTATAAAATCTCGATGGTCATCTTGGAGTTCGTCTCCGAGGCCTCTTCCCAAAAT
TCCGGTTCCATCGCTTACGAGCTGGACCCACACTGTAAACTCAGTGCCCTTTCAT
CAACCATTAACAAGTTCGGGATCACAAAGCCCGGCAGGAGGGCATTTACAGCGT
CTTACATCAACGGGACGGACTGGCATGACGTTGCCAAGGACCAATTCAGGATCC
TCTACAAAGGCAATGGTTCCTCATCGATAGCTGGTTCTTTTAGAATCACCATGAA
ATGCCAGTTCCACAATC

>SE1-F (3´-5´)
GATTGTGGAACTGGCATTTCATGGTGATTCTAAAAGAACCAGCTATCGATGAGG
AACCATTGCCTTTGTAGAGGATCCTGAATTGGTCCTTGGCAACGTCATGCCAGTC
CGTCCCGTTGATGTAAGACGCTGTAAATGCCCTCCTGCCGGGCTTTGTGATCCCG
AACTTGTTAATGGTTGATGAAAGGGCACTGAGTTTACAGTGTGGGTCCAGCTCGT
AAGCGATGGAACCGGAATTTTGGGAAGAGGCCTCGGAGACGAACTCCAAGATG
ACCATCGAGATTTTATACTCATGGTAGGCCTTGAGCATTCCATCAGCGAATGCCG
GGCAGTCTGATAGACTCGGCCCGAACGTGATTGCTCCGCTGGAACTTCCCGCGA
GATTGTCTTTTGAGAAAACGAATGTCTCGCTCGAACCTGTTCCTCTGGTAGAAAC
AGTTCTTCTTGTCCGGTTGTTACCTCTTCGTCGTCGTCTAGGTCGGCGTTGTGTTG
TCCGAGAGGTTTG

>SE2-R (3´-5´)
GATTGTGGAACTGGCATTTCATGGTGATTCTAAAAGAACCAGCTATCGATGAGG
AACCATTGCCTTTGTAGAGGATCCTGAATTGGTCCTTGGCAACGTCATGCCAGTC
CGTCCCGTTGATGTAAGACGCTGTAAATGCCCTCCTGCCGGGCTTTGTGATCCCG
AACTTGTTAATGGTTGATGAAAGGGCACTGAGTTTACAGTGTGGGTCCAGCTCGT
AAGCGATGGAACCGGAATTTTGGGAAGAGGCCTCGGAGACGAACTCCAAGATG
ACCATCGAGATTTTATACTCATGGTAGGCCTTGAGCATTCCATCAGCGAATGCCG
GGCAGTCTGATAGACTCGGCCCGAACGTGATTGCTCCGCTGGAACTTCCCGCGA
GATTGTCTTTTGAGAAAACGAATGTCTCGCTCGAACCTGTTCCTCTGGTAGAAAC
AGTTCTTCTTGTCCGGTTGTTACCTCTTCGTCGTCGTCTAGGTCGGCGTTGTGTTG
TCCGAGAGGTTTG

a sequence of 505 nucleotides (nts) (excluding the primer and vector sequences) (Box 1). The forward and reverse sequences of the two clones were completely identical (Box 1), showing that the sequences are correct. Also the sequences of SE1 and SE2 were 100% identical (Box 1).

Comparison of SE1 and SE2 to the sequences in GenBank using BLASTn (Altschul et al., 1990) showed 99% nucleotide identity (Table 9) and 100% amino acid identity (using BLASTp & BLASTx, data not shown) with the CP of BMYV isolates. The sequence of isolate SE1 was submitted to GenBank and the accession number FN827048 was obtained.

Table 9. Polerovirus *CP* sequences (nt) used in this study: BMYV-*Beet mild yellowing virus.*

Virus isolates	**Sequence (nt) identity (%) with SE1/SE2 (Accession No. FN827048)**	**Geographic location**	**Accession No.**
Beet mild yellowing virus			
BMYV-1	99	Suffolk, UK	AF167474
BMYV-N9	99	France	EU022498.1
BMYV-N18	99	France	EU022499.1
BMYV-N24	99	France	EU022500.1
BMYV-Th	99	France	EU022501.1
BMYV-G44	99	France	EU022502.1
BMYV-56r	99	Suffolk, UK	AF167482
BMYV-Broom's Barn	99	UK	EF107543.1
BMYV-N32	99	France	EU148510.1
BMYV-2ITB	98	Eure et Loire, France	X83110.1
BMYV-N27	98	France	EU148509.1
BMYV-J	98	Haut-Rhin, France	AF167480
BMYV-L	98	Haut-Rhin, France	AF167481
BMYV-N20	98	France	EU022496.1
BMYV-26	96	Poland	EU022504.1
BMYV-M5	96	Poland	EU022505.1
BMYV-Md	96	Poland	EU022507.1
BMYV- O37	96	France	EU022497.1
BMYV-D13	96	Poland	EU022503.1
BMYV-M8	96	Poland	EU022506.1
BMYV-19K	96	Poland	EU148508.1
BMYV-IPP	96	Germany	DQ132996.1
BMYV-Iran	96	Iran	AF167479

Table 10. Polerovirus *CP* sequences (nt) used in this study. Other poleroviruses: BChV- *Beet chlorosis virus*, BWYV-*Beet western yellows virus*, TuYV-*Turnip yellows virus*.

Virus isolates	Sequence (nt) identity (%) with SE1/SE2 (Accession No. FN827048)	Geographic location	Accession No.
Beet western yellows virus			
BWYV-28b	93	Iran	L39984.1
BWYV-fev	92	Haut-Rhin, France	AF167478
BWYV-Col	94	Haut-Rhin, France	AF167477
Beet chlorosis virus			
BChV-2a	93	Suffolk, UK	AF167475
BChV-Colorado	93	USA	AF167483
BChV-M26	93	France	EU022510.1
BChV-CR	92	California, USA	AF352025.1
Turnip yellows virus			
TuYV-GB	92	UK	AF167486
TuYV-BN5	92	Germany	AF167484

Phylogenetic analyses were carried using the 505 nt (*CP*) sequence of these Swedish isolates (SE1 and SE2). Two different evolutionary best fit models by *Mrmodeltest2*, (K80+G) and (K80+I) were found from Hierarchical Likelihood Ration Tests (hLRTs) and Akaike Information Criterion (AIC)(Table 11).

Table 11. Best-fit model from the *Mrmodeltest2*.

No. of taxa (ntax) and nucleotides (nchar) used in the alignment	Best-fit model & likelihood setting in PAUP* *by Mrmodeltest2*	
	HIERARCHICAL LIKELIHOOD RATIO TESTS (hLRTs) Model (K80+G)	AKAIKE INFORMATION CRITERION (AIC) Model (K80+I)
ntax=34 nchar=505	Lset Base=equal Nst=2 TRatio=5.1864 Rates=gamma Shape=0.0968 Pinvar=0;	Lset Base=equal Nst=2 TRatio=5.1680 Rates=equal Pinvar=0.7266;

Neighbour-joining (Saitou & Nei, 1987) tree search after 10000 bootstrap replicates with bootstrap value (Felsenstein, 1985) of higher than 50%, showed that both of the isolates from Sweden (SE1 and SE2) are very closely related to the British isolate BMYV-Broom's Barn (Stevens & Vigano, 2007) (Figure 12, 13 & 14). There were no significant differences found when using different methods (maximum parsimony, maximum likelihood and minimum evolutionary distances) or models (Table 11 and Figure 12, 13 & 14).

Phylogenetic trees from the alignment with CP-encoding genes showed that the BMYV isolates could be divided into three clades: a) BMYV isolates 1, SE1, SE2, N32, 56r, N24, Th, N9, N18 and G44; b) BMYV isolates N27, N20, 2ITB, L and J; c) BMYV isolates O37, M8, D13, Iran and IPP; d) BMYV isolates Md, 19k, 26 & M5. Another clades was formed by the other poleroviruses: BChV-2a,

TuYV-BN5, BWYV-fev, TuYV-GB, BChV-Colorado, BChV-CR and BWYV-28b (Figure 12, 13 & 14). Some of the BMYV isolates grouped according to the in geographic origin (e.g., Poland or France), but in other cases there was no grouping according to geographical origin.

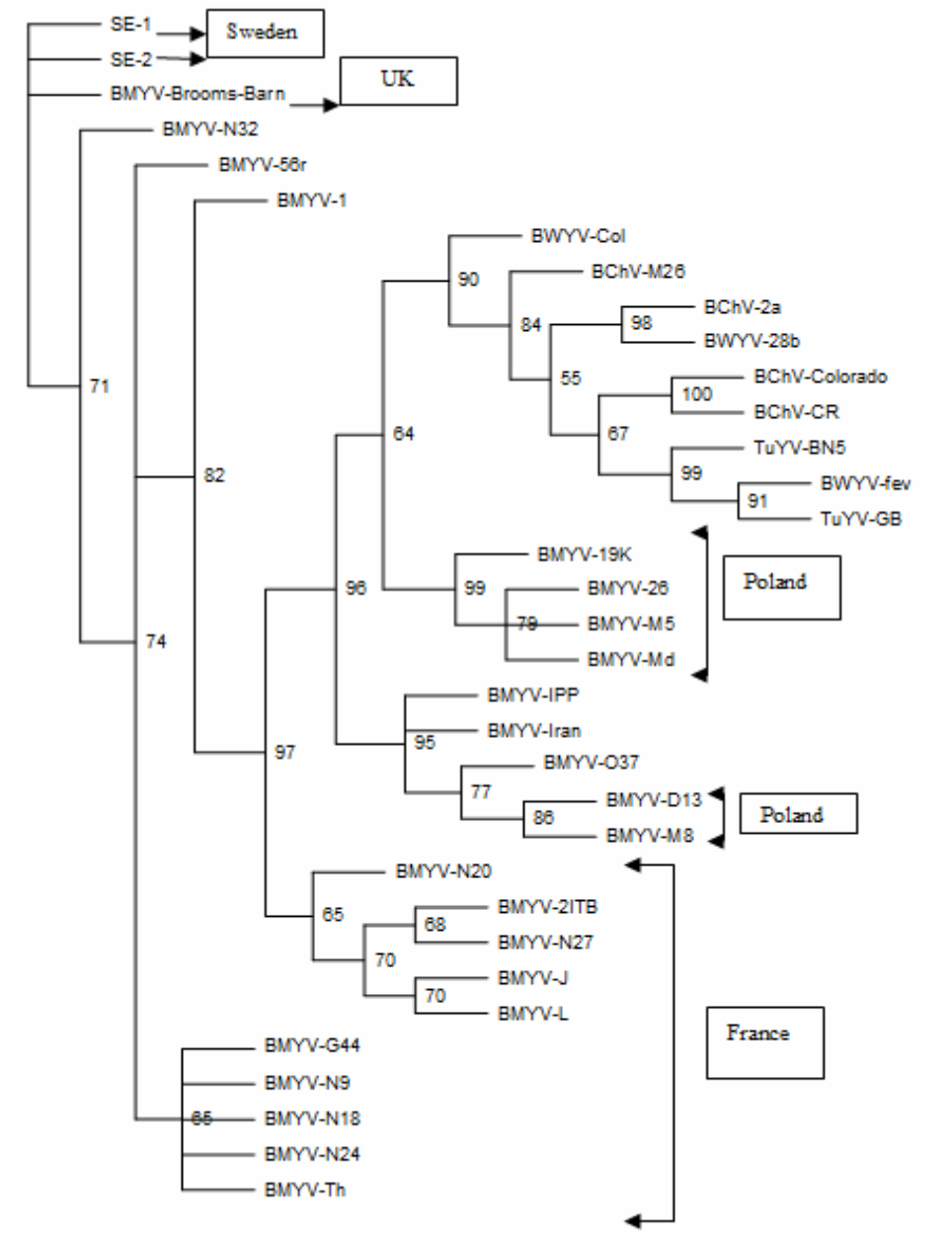


Figure 12: Bootstrap neighbour-joining tree from Hierarchical Likelihood Ration Tests (hLRTs) with (K80+G) best fit model selected by Mr.modeltest2.3 (Nylander, 2004). Bootstrapped phylogenetic tree constructed from the 505 nt sequence of the *CP* gene of *Beet mild yellowing virus* isolates (SE1 & SE2) from southern part of Sweden showing relationships with other poleroviruses. Only bootstrap values higher than 50% are presented after 10000 replicates. The accession numbers and geographic locations of the isolates are shown in Table 9 and 10. The phylogenetic analyses were conducted in PAUP* 4.0 (Swofford, 2002).

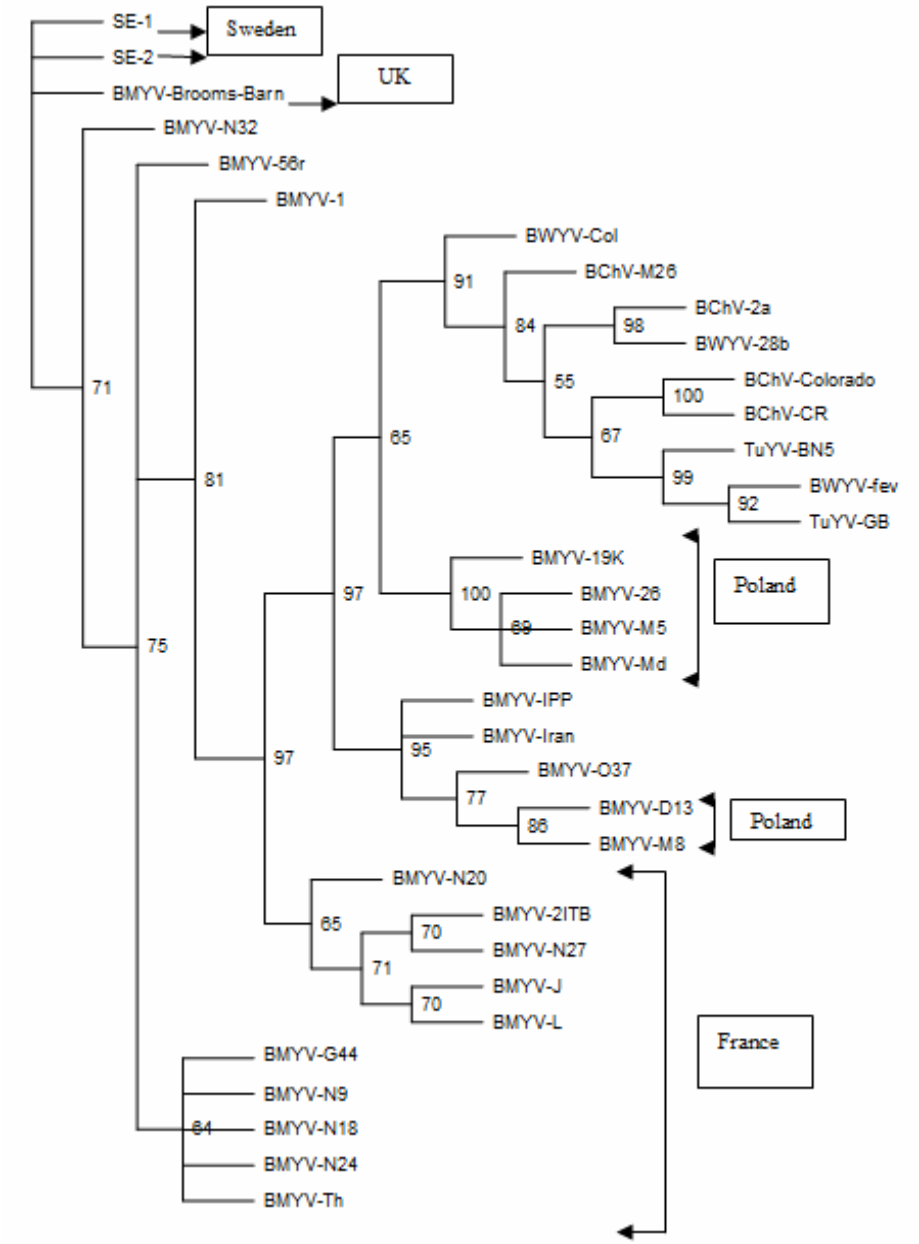


Figure 13: Bootstrap neighbour-joining tree from Akaike Information Criterion (AIC) with (K80+I) best fit model selected by Mr.modeltest2.3 (Nylander, 2004). Bootstrapped phylogenetic tree constructed from the 505 nt sequence of the *CP* gene of *Beet mild yellowing virus* isolates (SE1 & SE2) from southern part of Sweden showing relationships with other poleroviruses. Only bootstrap values higher than 50% are presented after 10000 replicates. The accessions numbers and geographic locations of isolates are shown in Table 9 and 10. The phylogenetic analyses were conducted in PAUP* 4.0 (Swofford, 2002).

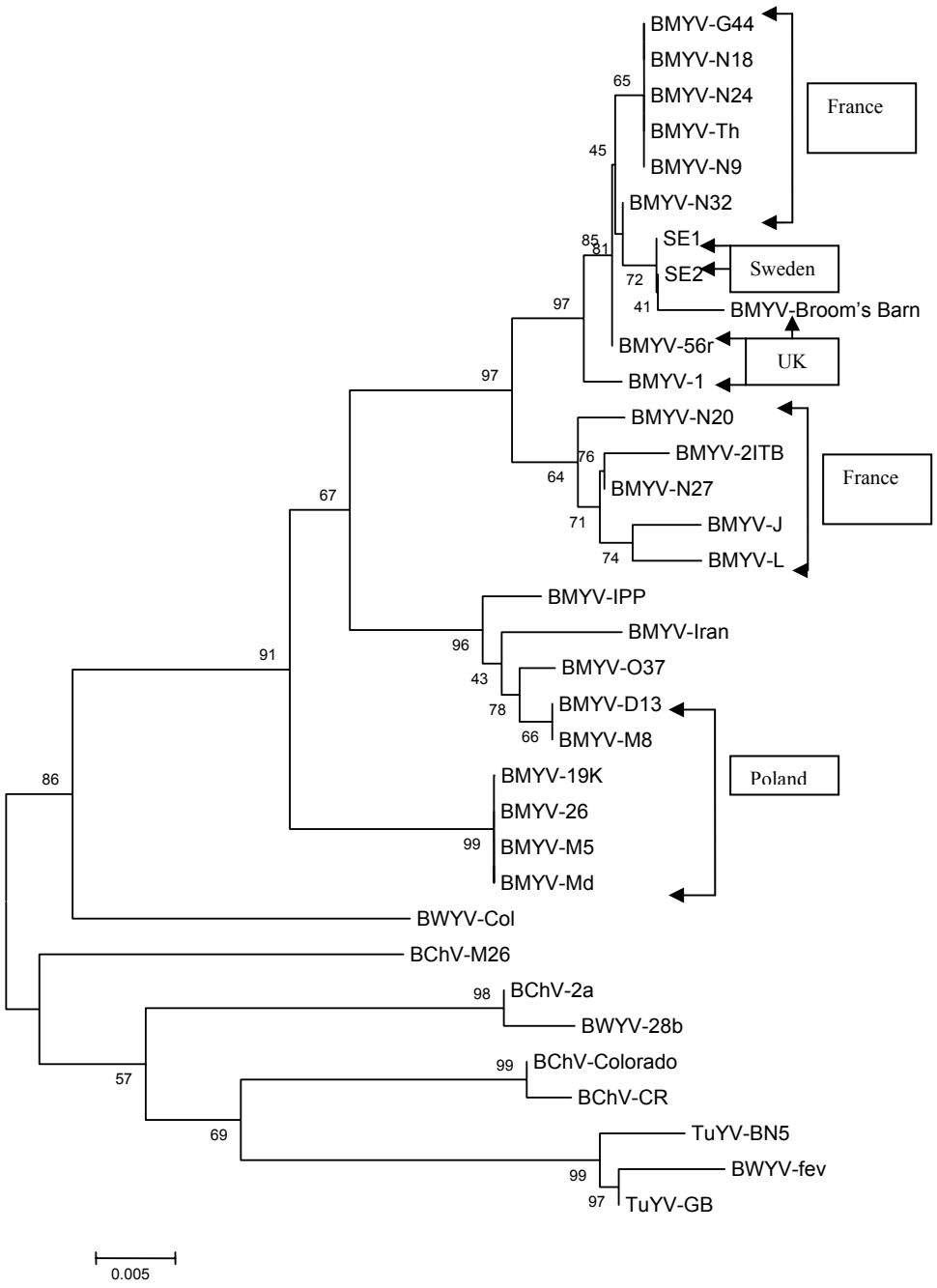
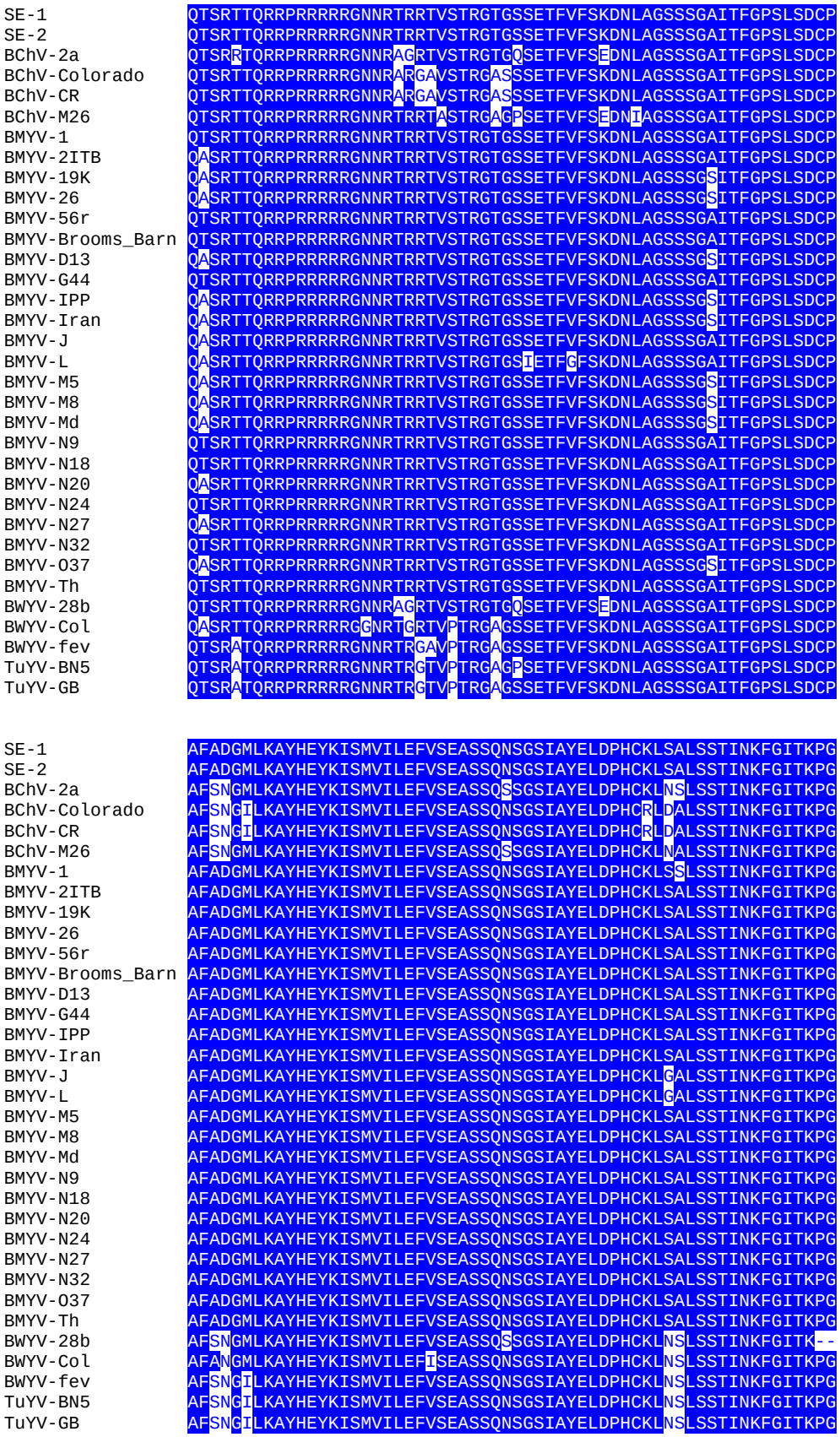


Figure 14: Bootstrap phylogenetic tree constructed from the 505 nt sequence of the *CP* gene of *Beet mild yellowing virus* isolates (SE1 & SE2) from southern part of Sweden showing relationship with other poleroviruses. Only bootstrap values higher than 50% are presented after 10000 replicates. The evolutionary history was inferred using the Neighbour-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The accessions numbers and geographic locations of isolates are shown in Table 9 and 10. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).


SE-1 QTSRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGAITFGPSLSDCP
SE-2 QTSRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGAITFGPSLSDCP
BChV-2a QTSRRTQRRPRRRRRGNNRAGRTVSTRGTGQSETFVFSEDNLAGSSSGAITFGPSLSDCP
BChV-Colorado QTSRTTQRRPRRRRRGNNRARGAVSTRGASSSETFVFSKDNLAGSSSGAITFGPSLSDCP
BChV-CR QTSRTTQRRPRRRRRGNNRARGAVSTRGASSSETFVFSKDNLAGSSSGAITFGPSLSDCP
BChV-M26 QTSRTTQRRPRRRRRGNNRTRRTASTRGAGPSETFVFSEDNIAGSSSGAITFGPSLSDCP
BMYV-1 QTSRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGAITFGPSLSDCP
BMYV-2ITB QASRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGAITFGPSLSDCP
BMYV-19K QASRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGSITFGPSLSDCP
BMYV-26 QASRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGSITFGPSLSDCP
BMYV-56r QTSRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGAITFGPSLSDCP
BMYV-Brooms_Barn QTSRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGAITFGPSLSDCP
BMYV-D13 QASRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGSITFGPSLSDCP
BMYV-G44 QTSRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGAITFGPSLSDCP
BMYV-IPP QASRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGSITFGPSLSDCP
BMYV-Iran QASRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGSITFGPSLSDCP
BMYV-J QASRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGAITFGPSLSDCP
BMYV-L QASRTTQRRPRRRRRGNNRTRRTVSTRGTGSIETFGFSKDNLAGSSSGAITFGPSLSDCP
BMYV-M5 QASRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGSITFGPSLSDCP
BMYV-M8 QASRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGSITFGPSLSDCP
BMYV-Md QASRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGSITFGPSLSDCP
BMYV-N9 QTSRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGAITFGPSLSDCP
BMYV-N18 QTSRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGAITFGPSLSDCP
BMYV-N20 QASRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGAITFGPSLSDCP
BMYV-N24 QTSRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGAITFGPSLSDCP
BMYV-N27 QASRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGAITFGPSLSDCP
BMYV-N32 QTSRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGAITFGPSLSDCP
BMYV-O37 QASRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGSITFGPSLSDCP
BMYV-Th QTSRTTQRRPRRRRRGNNRTRRTVSTRGTGSSETFVFSKDNLAGSSSGAITFGPSLSDCP
BWYV-28b QTSRTTQRRPRRRRRGNNRAGRTVSTRGTGQSETFVFSEDNLAGSSSGAITFGPSLSDCP
BWYV-Col QASRTTQRRPRRRRRGGNRTGRTVPTRGAGSSETFVFSKDNLAGSSSGAITFGPSLSDCP
BWYV-fev QTSRATQRRPRRRRRGNNRTRGAVPTRGAGSSETFVFSKDNLAGSSSGAITFGPSLSDCP
TuYV-BN5 QTSRATQRRPRRRRRGNNRTRGTVPTRGAGPSETFVFSKDNLAGSSSGAITFGPSLSDCP
TuYV-GB QTSRATQRRPRRRRRGNNRTRGTVPTRGAGSSETFVFSKDNLAGSSSGAITFGPSLSDCP
SE-1 AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
SE-2 AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
BChV-2a AFSNGMLKAYHEYKISMVILEFVSEASSQSSGSIAYELDPHCKLNSLSSTINKFGITKPG
BChV-Colorado AFSNGILKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCRLDALSSTINKFGITKPG
BChV-CR AFSNGILKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCRLDALSSTINKFGITKPG
BChV-M26 AFSNGMLKAYHEYKISMVILEFVSEASSQSSGSIAYELDPHCKLNALSSTINKFGITKPG
BMYV-1 AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSSLSSTINKFGITKPG
BMYV-2ITB AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
BMYV-19K AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
BMYV-26 AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
BMYV-56r AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
BMYV-Brooms_Barn AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
BMYV-D13 AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
BMYV-G44 AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
BMYV-IPP AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
BMYV-Iran AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
BMYV-J AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLGALSSTINKFGITKPG
BMYV-L AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLGALSSTINKFGITKPG
BMYV-M5 AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
BMYV-M8 AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
BMYV-Md AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
BMYV-N9 AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
BMYV-N18 AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
BMYV-N20 AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
BMYV-N24 AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
BMYV-N27 AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
BMYV-N32 AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
BMYV-O37 AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
BMYV-Th AFADGMLKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLSALSSTINKFGITKPG
BWYV-28b AFSNGMLKAYHEYKISMVILEFVSEASSQSSGSIAYELDPHCKLNSLSSTINKFGITK--
BWYV-Col AFANGMLKAYHEYKISMVILEFISEASSQNSGSIAYELDPHCKLNSLSSTINKFGITKPG
BWYV-fev AFSNGILKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLNSLSSTINKFGITKPG
TuYV-BN5 AFSNGILKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLNSLSSTINKFGITKPG
TuYV-GB AFSNGILKAYHEYKISMVILEFVSEASSQNSGSIAYELDPHCKLNSLSSTINKFGITKPG
SE-1 RRAFTASYINGTDWHDVAKDQFRILYKGNGSSSIAGSFRITMKCQFHN
SE-2 RRAFTASYINGTDWHDVAKDQFRILYKGNGSSSIAGSFRITMKCQFHN
BChV-2a RRAFTASYINGTEWHDVAKDQFRILYKGNGSSSIA----------
BChV-Colorado RRAFTASYINGTDWHDVAKDQFRILYKGNGSSSIA----------
BChV-CR RRAFTASYINGTDWHDVAKDQFRILYKGNGSSSIAGSFRITIKCQFH-
BChV-M26 RRTFTASYINGTEWHDVAKDQFRILYKGNGSSSIAGSFRITIKCQFH-

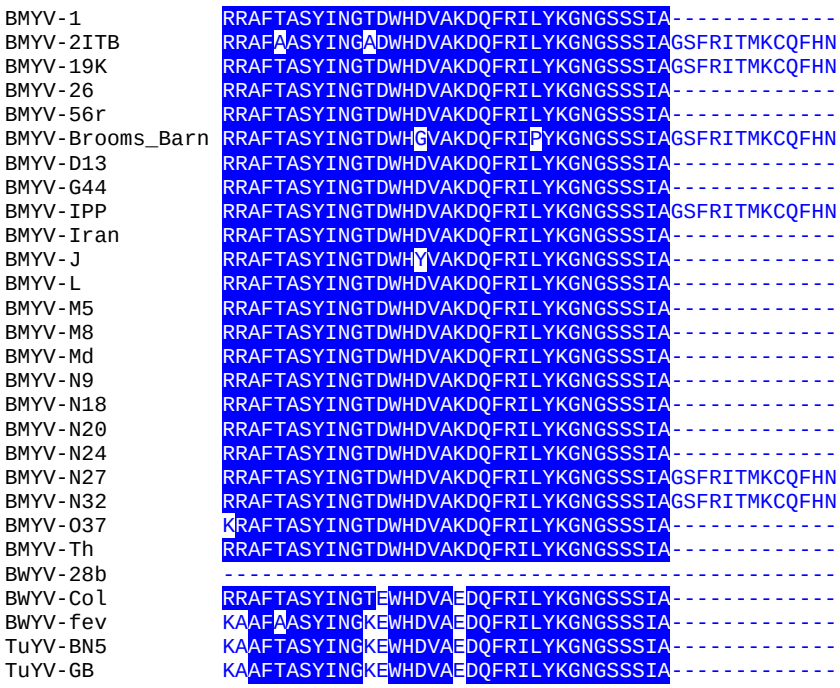


Figure 15. Alignment of deduced amino acid sequences of the CP of poleroviruses. The alignment was done using ClustalW algorithm in MEGA 4.0.2. Shading of the amino acids was performed with the Boxshade program (http://bioweb.pasteur.fr/seqanal/interfaces/boxshade.html). Identical amino acids (conserved sites) are shaded in blue.

The deduced amino acid sequences of the two Swedish BMYV isolates (SE1 and SE2) and other poleroviruses isolates used (Table 9 and 10) in this study were aligned (Figure 15). It was highly conserved within BMYV, but less conserved between more distantly related poleroviruses (Figure 15); 133 positions were completely conserved among 168 positions and two variable sites (G↔D & P↔L) were found between the Swedish isolates of BMYV and BMYV-Broom's Barn (Figure 15).

Discussion

Testing for *Beet Mosaic Virus*

The main aim of the study was to identify and characterise the virus isolates from symptomatic sugar beet from a region (Skåne) of southern Sweden that were preliminary suspected to be BtMV. The transmission tests with different hosts (e.g., red beet, spinach, lettuce) were done as part of these studies. However, using mechanical transmission, ELISA and PCR it was proven that neither BtMV nor any other potyvirus was present in the collected samples.

Sometimes plants can handle virus infections and infection by BtMV may not give clear symptoms in the summer, usually in Sweden the summer temperature is between 20-25 °C. Therefore, a follow-up with serological and molecular diagnostic experiments was necessary to confirm the results of the transmission tests.

ELISA is usually a very reliable method, although for some viruses it can be difficult to obtain a good antiserum. In previous studies (Dekker et al., 1989) with

TMV it was also shown that there are limitations of different ELISA procedures for localizing epitopes in viral CP subunits. However, in this study the 4th ELISA tests gave strong evidence for the absence of BtMV in the collected samples (Table 6). Similarly, the RT-PCR tests with universal potyvirus primers or specific BtMV primers gave additional supportive evidence for the absence of BtMV in the samples.

Detection and Sequence Analyses of *Beet Mild Yellowing Virus*

It was assumed at the beginning of the experiment that there might be a mixed infection of BtMV and a polerovirus in the collected sample. However, the analyses support the idea of polerovirus infection only.

The primers (Lu1/Lu4) used in this experiment to detect BMYV were specific for all members of the family *Luteoviridae*. They were derived from conserved sequences of BYDV-PAV (genus: *Luteovirus*), BWYV (genus: *Polerovirus*) and PLRV (genus: *Polerovirus*) (Robertson et al., 1991). Therefore, it was not possible to detect from RT-PCR amplification with these primers (Lu1/Lu4) whether the plants were infected by several polerovirus species (mixed infection) or solely with BMYV prior to sequencing. Previous phylogenetic analyses of beet poleroviruses have shown that sequences corresponding to the CP were closely related whereas those corresponding to P0 are highly divergent (Hauser et al., 2000). In our case, it was also observed in the phylogenetic trees (Figure 12, 13 and 14) that isolates from different beet polerovirus species grouped together. However, the viruses could be distinguished by multiplex RT-PCR using primers specially designed to the 5´ end of their respective genome (Stevens et al., 2005).

In the phylogenetic tree (Figure 12, 13 and 14), no strict grouping was observed according to geographic origin of the isolates. For some other viruses, there is a geographic grouping of isolates and this is important information regarding the evolutionary history of the virus. However, BMYV is transmitted in a persistent way by aphids and they can fly very long distances with the virus. There is a hypothesis that England´s southeastern coastal areas are the main origin of European leaf yellowing viruses (including BMYV) of sugar beet and from there viruses have spread by aphids; primarily to Holland, Belgium and northern France, and gradually southern part of Sweden (Nilsson & Larsson, 1990). It has been found that favourable conditions for aphid multiplication in Europe and strong southerly winds, which help aphids to travel long distances, are the most important factors for high incidence of BMYV (or other beet yellowing viruses) in Sweden (Wiktelius, 1977; Nilsson & Larsson, 1990).

Conclusions

To our knowledge, this is the first molecular identification and sequence information for BMYV from Sweden. However, more experiments and samples are needed to understand the host range and infection pattern. Transmission experiments with aphids should be carried out after failure to detect BtMV in the samples. More samples should be collected from different geographical locations in Skåne and times to see whether there is any variation in infection pattern and genome sequences. It would be interesting to find out if there are other viruses in these samples as well, or if it is BMYV that is responsible for the symptoms. It

could be tested by raising monoclonal antibodies (e.g., for distinguishing BWYV and BMYV) for ELISA, species-specific primers in multiplex RT-PCR. However, from this study once again, it was proven that solely symptomatology or serologicals tests can not always be reliable detection methods, and may have to be complemented with RT-PCR and sequencing.

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