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Virome characterization of a European pear germplasm collection

Núria Fontdevila Pareta

Promotor: Prof. Sébastien Massart

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Virome characterization of a European pear germplasm collection

Núria FONTDEVILA PARETA

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Abstract

The work done during this doctoral thesis focuses on studying the virome of pome fruit trees and the characterization of novel viruses, particularly those discovered by high-throughput sequencing (HTS).

Viruses associated with economically significant pome fruit diseases on commercial cultivars have been thoroughly studied and characterized over the years. Nevertheless, beyond commercial cultivars, a significant genetic diversity is preserved in collections of genetic resources. Therefore, germplasms are essential for conserving plant genetic diversity and as a source of genetic material to introduce new traits for breeding and for direct use for food production. The focus was to assess the viral status of old historical cultivars maintained in these collections, which will also provide helpful information to plant breeders about possible resistant or tolerant fruit tree cultivars.

Advancements in HTS technologies and data analysis tools have made it easier to screen plant communities at a larger scale to study their virome. Moreover, an extra benefit or advantage of HTS is the discovery of new viruses at a faster rate. To manage this influx, during this thesis, a collective effort was undertaken to develop new guidelines in the form of a framework to help researchers prioritize their efforts after finding a new plant virus, or a viroid, and decide what the following steps to take, with a particular emphasis on a more fluid communication with the other stakeholders involved in the various steps, such as plant protection authorities, or National Plant Protection Organizations (NPPOs), and farmer associations are. Furthermore, the newly created framework includes input from various experts, providing a more transversal view and approach to virus characterization and integrating novel approaches and innovative data analysis tools.

In summary, the created framework is adapted to the current rate of virus discovery and provides an improved prioritization for filling knowledge and data gaps. It consists of four distinct steps adapted to include a multi-stakeholder feedback loop. The four steps include (i) detection test, confirmation of detection and genome sequence; (ii) contextual information gathering and notification to stakeholders; (iii) evaluation of the association between symptoms and virus presence; and, (iv) completion of data gaps to strengthen the risk evaluation process. The key elements include a more comprehensive prioritization and organization of the various steps to take into consideration the distinction and nuance between ecological and commercial interest of the identification of a novel virus, early data sharing among researchers and involved stakeholders for providing context to new virus findings (i.e., host range, diversity, and distribution), public database screening, and exploitation of genomic information to predict biological properties.

Then, the created framework was put to practice in this thesis to characterize a novel virus identified in pear trees during the extensive virome investigation to study the germplasm collection of apple and pear trees from the Walloon Agricultural Research

Center (CRA-W). Six apple trees and 128 pear trees were analyzed as pools using HTS techniques and/or tested individually for targeted viruses by RT-PCR. As mentioned before, during the virome survey, a novel velarivirus, tentatively named Pyrus virus A (PyVA), and four known viruses were identified, namely apple stem pitting virus (ASPV), apple chlorotic leaf spot virus (ACLSV), apple rubbery wood virus 1 (ARWV-1), and Citrus virus A (CiVA). The pear germplasm collection from Kozjanski Park (Slovenia) and a viral collection from Agroscope (Nyon, Switzerland) were also surveyed for the new pear virus and for three known viruses, namely CiVA, ARWV-1, and apple rubbery wood virus 2 (ARWV-2) to study their prevalence and geographic distribution.

This study successfully combined pooled HTS analyses and an alien control strategy to monitor sample cross-talk and maximize the number of germplasms tested, while also implementing targeted RT-PCR tests on individual samples for accurate detection. It reports and describes a new velarivirus discovered in pear trees and the first detections of CiVA in Belgium, Switzerland, and Slovenia, and ARWV-1 and -2 in Switzerland.

Résumé

Le travail effectué dans le cadre de cette thèse de doctorat porte sur l'étude du virome des arbres fruitiers à pépins et la caractérisation de nouveaux virus, en particulier ceux découverts par séquençage à haut débit (HTS).

Les virus associés aux maladies des fruits à pépins d'importance économique sur les cultivars commerciaux ont été étudiés et caractérisés de manière approfondie au fil des ans. Néanmoins, au-delà des cultivars commerciaux, une diversité génétique importante est préservée dans les collections de ressources génétiques. Les germoplasmes sont d'importantes sources de matériel génétique pouvant permettre l'introduction de nouvelles caractéristiques pour la sélection et l'amélioration variétale. L'objectif principal de cette thèse est d'évaluer le statut viral des cultivars historiques/anciens conservés dans ces collections, ce qui fournira également des informations utiles aux sélectionneurs de plantes sur d'éventuels cultivars d'arbres fruitiers résistants ou tolérants.

Les progrès des technologies HTS et des outils d'analyse des données ont facilité le criblage des communautés végétales à grande échelle afin d'étudier leurs viromes et accéléré la découverte de nouveaux virus. Pour gérer cet afflux, un effort collectif a été entrepris au cours de cette thèse afin élaborer de nouvelles lignes directrices destinées à aider les chercheurs à hiérarchiser leurs efforts après la découverte d'un nouveau virus végétal, ou d'un viroïde, et à décider des prochaines étapes à suivre, en mettant particulièrement l'accent sur une communication plus fluide avec les autres parties prenantes impliquées dans les différentes étapes, telles que les autorités chargées de la protection des végétaux, ou les organisations nationales de protection des végétaux (NPPO), ou les associations d'agriculteurs. Le framework nouvellement créé comprend des contributions d'experts de divers horizons, ce qui permet d'avoir une vision et une approche plus transversales de la caractérisation des virus et d'intégrer de nouvelles approches et des outils d'analyse de données novateurs.

Le framework proposé est adapté au rythme actuel de découverte des virus et permet de mieux hiérarchiser les priorités pour combler les lacunes en matière de connaissances et de données manquantes. Il se compose de quatre étapes distinctes adaptées pour inclure un retour d'expérience multipartite. Les éléments clés comprennent une hiérarchisation et une organisation complètes des différentes étapes, un partage précoce des données entre les chercheurs et les autres parties prenantes afin de fournir un contexte aux nouvelles découvertes de virus (comme, la gamme d'hôtes, la diversité et la distribution), une sélection des bases de données publiques ainsi que l'exploitation des informations génomiques pour prédire les propriétés biologiques.

Par la suite, le framework crée a été mis en pratique dans cette thèse pour caractériser un nouveau virus identifié chez le poirier au cours de la vaste enquête sur le virome de la collection de germoplasme de pommiers et de poiriers du Centre wallon de recherche agronomique (CRA-W). Au total, six pommiers et 128 poiriers ont été analysés en groupes à l'aide de techniques HTS et/ou testés individuellement

pour des virus ciblés par RT-PCR. Comme mentionné précédemment, un nouveau velarivirus, provisoirement nommé Pyrus virus A (PyVA), et quatre virus connus ont été identifiés au cours de l'étude du virome, à savoir l'apple stem pitting virus (ASPV), l'apple chlorotic leaf spot virus (ACLSV), l'apple rubbery wood virus 1 (ARWV-1), et le Citrus virus A (CiVA). La collection de germoplasme de poirier de Kozjanski Park (Slovénie) et une collection virale d'Agroscope (Nyon, Suisse) ont également été étudiées pour le nouveau virus du poirier et pour trois virus connus, à savoir CiVA, ARWV-1, et apple rubbery wood virus 2 (ARWV-2), afin d'étudier leur prévalence et leur distribution géographique.

Cette étude a combiné avec succès des analyses HTS groupées et une stratégie utilisant des contrôle alien pour surveiller les potentiels échanges entre les échantillons et maximiser le nombre de germoplasmes testés, tout en mettant en œuvre des tests RT-PCR ciblés sur des échantillons individuels pour une détection plus précise. Cette thèse rapporte et décrit un nouveau velarivirus découvert chez les poiriers et les premières détections de CiVA en Belgique, en Suisse et en Slovénie, et d'ARWV-1 et -2 en Suisse.

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List of acronyms

aa	Amino acid
Acc. nº	Accession number
ACLSV	Apple chlorotic leaf spot virus
ApMV	Apple mosaic virus
ARWV-1	Apple rubbery wood virus 1 (Rubodvirus mali)
ARWV-2	Apple rubbery wood virus 2 (Rubodvirus prosserense)
ASGV	Apple stem grooving virus
ASPV	Apple stem pitting virus
BLAST	Basic Local Alignment Search Tool
CE	Common Era
CiVA	Citrus virus A (Coguvirus eburi)
cm	Centimeters
CRA-W	Centre Wallon de Recherches Agronomiques
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dsRNA	Double-stranded RNA
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunoassay
EPPO	European and Mediterranean Plant Protection Organization
EU	European Union
HDA	Helicase-dependent amplification
HTS	High Throughput Sequencing
ICTV	International Committee on Taxonomy of Viruses
IPM	Integrated Pest Management
IPPC	International Plant Protection Convention
ISPM	International Standards for Phytosanitary Measures
LAMP	Loop-Mediated Isothermal Amplification
MdoVA	Malus domestica virus A
Mt	Megatonne (10^6 tonnes)
NCBI	The National Center for Biotechnology Information
NPPO	National Plant Protection Organization
nt	Nucleotide
ORF	Open Reading Frame

PhCMoV	Physostegia chlorotic mottle virus
PCR	Polymerase Chain Reaction
PPV	Plum pox virus
PRA	Pest Risk Analysis
PvEV-1	Phaseolus vulgaris alphaendornavirus 1
PvEV-2	Phaseolus vulgaris alphaendornavirus 2
PvEV-3	Phaseolus vulgaris alphaendornavirus 3
PyVA	Pyrus virus A
RNA	Ribonucleic acid
RNQP	Regulated Non-Quarantine Pest
RPPO	Regional Plant Protection Organization
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SBL	Sequencing by ligation
SBS	Sequencing by synthesis
siRNA	Small interfering RNA
SMRT	Single-molecule real-time
SNP	Single Nucleotide Polymorphism
SRA	Sequence Read Archive
TMV	Tobacco mosaic virus
VANA	Virion-associated nucleic acid
(–)ssRNA	(Negative-sense) Single-stranded RNA
(+)ssRNA	(Positive-sense) Single-stranded RNA

Chapter 1

General introduction

1. Pome fruit trees

1.1. Global overview

Over the centuries, fruit trees have been domesticated and improved by breeding and interspecific hybridization, thus producing a high genetic variability within species and complicating their phylogeny description (Potter et al., 2007; Shulaev et al., 2008). Pome and stone fruit are part of the Rosaceae family, consisting of approximately 3,000 species, including many essential fruits, nuts, and ornamental and woody crops (Judd et al., 2002; Mabberley, 1997). For example, the Spiraeoideae subfamily contains the genus *Prunus* (tribe Amygdaleae). It includes stone fruit species such as peach (*Prunus persica*), plum (*Prunus domestica*), and sweet cherry (*Prunus avium*), as well as the subtribe Pyrinae, which englobes the pome fruits apple (*Malus* spp.) and pear (*Pyrus* spp.), which are two of the most widely grown fruit trees (Potter et al., 2007). The critical difference between pome and stone fruits is the structure of their fruit, which can have a wide range of phenotypic variation. Pome fruits get their name from their fruit structure, derived from several fused carpels surrounded by the hypanthium, the fruit's flesh. The hypanthium surrounds the inner pericarp, which contains the seeds and is often called the core. In comparison, stone fruits comprise a thin skin or outer wall called the exocarp, a fleshy portion or mesocarp, and a woody stone known as the endocarp that protects the seed (Webster and Palmer, 2017).

Pome fruit trees are usually grown and cultivated in areas with temperate climates and well-defined annual seasons, with apples and pears being the most produced fruits, with 93 Mt and 25 Mt produced of each worldwide in 2021, respectively. In 2021, mainland China, Turkey, The United States of America, Poland, and India were the five leading apple producers. That same year, Belgium was the world's sixth most significant producer of pears and the first in Europe, with the second and third being The Netherlands and Spain, respectively. The major pome fruit species cultivated are deciduous, meaning trees lose their leaves in autumn. The different phases of the annual cycle of deciduous fruit trees are triggered by plant responses to climate (Kurokura et al., 2013). One of these phases is winter dormancy, used by the tree to survive winter conditions and avoid being damaged by the cold weather. Then, to break dormancy, a period of winter chilling with sufficient exposure to cold temperatures, known as vernalization, is needed, although it varies from cultivar to cultivar (Darbyshire et al., 2011; Guo et al., 2014). Cultivation of pome fruit trees has traditionally been restricted to areas in temperate climates with the required conditions for the annual cycle of the fruit trees. Thus, temperate fruit trees grown in tropical or subtropical regions may not have a sufficient winter chill (Pio et al., 2018). Several models and attempts have been made to study and understand the requirements for different plant species and cultivars (Lloret et al., 2022). The high diversity of cultivars within pome fruit trees results from most of the species being heterozygous; hence, each tree's potential offspring is highly variable (Miller and Gross, 2011). This

would have been advantageous to fruit trees, allowing at least a fraction of the future offspring to survive in different environments and conditions. However, this high variability would have also created a high fruit diversity and uncertainty on the genotypic and phenotypic characteristics of the offspring. To target specific traits and improve the phenotype, humans domesticated fruit trees through hybridization, selecting specific phenotypes, and clonal propagation (Singh et al., 2021).

For wild species, fruit trees are propagated via pollen and seed dispersal. Regardless of their domestication, other forms of propagation arose to allow plant breeders to reproduce the desired characteristics of the cultivar. Nowadays, both rootstocks and scions are obtained using vegetative or clonal propagation, albeit rootstocks are also propagated via division (i.e., layering or marcotting) and cuttings (i.e., micropropagation or hardwood) (Webster, 1995). Most pome and stone fruits are propagated through budding or grafting of the scion onto a rootstock, which allows growers to reproduce the desired characteristics of the cultivar. The type of rootstock selected will affect the growth and survival of the scion (i.e., resistance to soilborne pathogens). Rootstocks directly influence the characteristics of the scion, the fruit set and the productivity, and fruit characteristics. The correct cultivar, combined with the correct rootstock, is the base of a thriving orchard, and it is essential to prevent incompatibility between rootstock and scion, which can lead to bead and necrosis of the scion. This incompatibility can have a genetic or a viral origin, so their health status is critical to guarantee the tree's survival. Other consequences of pathogen infections could be a reduction in tree growth or fruit quality. Usually, in apple cultivation, the rootstocks used are the same as the scion, although other pome fruit trees may use different species for the rootstock and the scion. For example, quince is often used as a rootstock for pear trees. Biotic and abiotic factors will highly influence the choice of rootstock and will change depending on the orchard site and the management strategy.

1.2. Major diseases and pests

Pome fruit trees are affected by infectious diseases caused by fungi, bacteria, viruses, phytoplasmas, viroids, virus-like agents, and nematodes; non-infectious disorders caused by environmental, genetic, physiological, and nutritional factors; and arthropod pests that can hurt orchard productivity and fruit quality. Viral diseases are described in section 2.2. Some of pome fruit trees' most notable diseases and pathogens include apple scab, fire blight, brown rot, pests such as codling moths and aphids, and postharvest decay.

Apple scab, or black spot, is a disease that mainly affects apple trees, although it could also affect other fruit trees such as pear. The causal agent of apple scab in apple trees is predominantly *Venturia inaequalis*. Apple scab can affect leaves, fruit and twigs. The first symptoms appear as olive-colored, velvety spots on the lower surface of sepals and leaves, which then turn grey. These lesions become metallic black and start appearing on the leaves' upper surface. Severely diseased leaves can eventually

desiccate and fall out. Furthermore, during fruit development and maturity, circular scab lesions may appear which, with time, may darken and turn scabby and crack (Chane and Boyraz, 2017).

Fire blight is a disease of apples, pears, and other rosaceous plants that can cause symptoms on various plant tissues, including flowers, leaves, petioles, fruits, young shoots, scaffold limbs, and rootstocks. The causal agent of this bacterial disease is the enterobacterium *Erwinia amylovora*. (Pusey, 2000). Symptoms of fire blight are characterized by necrosis and browning or blackening of dead plant tissue, which gives the appearance of the tissue being burned by fire (Zeng et al., 2021). Then, the disease can pass from infected flowers and shoots to larger branches, the trunk, and the rootstock (Pedroncelli and Puopolo, 2023).

Brown rot is a fungal disease of pome fruit trees caused by species of the genus *Monilinia* that produce severe blossom and twig blight, which can result in significant pre- and post-harvest losses. (Casals et al., 2022). Initial symptoms appear on the blossoms and can quickly propagate to flowers and stems. Once fruit develops and approaches maturity, small, circular, brown spots can appear and rapidly spread. From these spots, tufts of conidia can break through the skin of the infected area onto the fruit surface (Martini and Mari, 2014).

The ***codling moth*** (Cydia pomonella L.) is a significant pest infecting pome fruits, especially apples. Yield losses are due to damage caused by invading larvae in an infested fruit. Invading larvae first enter just under the skin of the fruit by forming a small cavity and keep feeding on the fruit until arriving at its core, thus damaging seeds and pulp. Infected fruits have a distinctive, red-colored hole at the point of entry, which may be covered in frass (Pajač et al., 2011). The pest overwinters in protected locations, such as bark or plant debris on the orchard floor, in its pupae form. With the weather changing in spring, females lay eggs on leaves and fruits, which, once hatched, larvae enter the fruits, leaving behind the characteristic reddish hole and causing internal damage by feeding on seeds and pulp. Once the larvae mature, they exit the fruit to pupate, thus creating silken cocoons in protected and sheltered places. The following generation of adults perpetuates the cycle, completing one or two cycles per year depending on weather conditions (Moinina et al., 2019; Pajač et al., 2011).

Aphids can harm fruit quality by reducing the fruit size, deforming its shape, or causing a premature fall of the fruit. Aside from the effect on fruits, aphids can also impact the tree by deforming its organs, producing chlorosis, and inducing the development of sooty mold due to honeydew and leaf fall. Furthermore, aphids are vectors of viruses such as plum pox virus (PPV), which produces Sharka disease (Sorensen, 2009).

Apple proliferation disease is a severe graft-transmissible and vector-borne disease that significantly affects apple trees, leading to substantial yield losses and fruit quality degradation. The disease is characterized by a range of symptoms, including

witches' broom growth, where multiple shoots emerge from a single node, and an overall bushy appearance of the tree. Infected trees often exhibit reduced vigor, smaller and deformed fruits, and early leaf reddening in the late summer. The causal agent, '*Candidatus* Phytoplasma mali', is transmitted by insect vectors such as leafhoppers and psyllids and through infected grafting material (CABI, 2021).

Pear decline is a debilitating disease caused by a phytoplasma, specifically '*Candidatus* Phytoplasma pyri', which results in significant health deterioration and productivity loss in pear trees. The disease manifests through a variety of symptoms, including stunted growth, leaf scorch, premature leaf drop, and a general decline in tree vigor. Severely affected trees may display reddish-brown discoloration of leaves and brittle, necrotic phloem tissue. The impact of this disease is significant, often leading to reduced fruit yield and quality, and in severe cases, tree death. Transmission occurs primarily through pear psyllids, which act as vectors by feeding on the sap and transferring the phytoplasma from infected to healthy trees (Blomquist and Kirkpatrick, 2007).

Within the various ***postharvest diseases*** that cause fruit decay and affect pome and stone fruit trees, there are a few worth mentioning: blue mold, caused by *Penicillium* spp.; gray mold, caused by *Botrytis cinerea*; *Phytophthora* rot, caused by *Phytophthora* spp.; bitter rot and other diseases caused by *Colletotrichum* spp.; bull's eye rot, caused by *Gloeosporium* spp.; brown rot, caused by *Monilinia* spp.; and, storage apple scab, caused by *Venturia inaequalis* (Giraud and Bompeix, 2012). Apart from diseases and rot decay, fruits under storage conditions can also succumb to postharvest disorders that might affect marketability. Such disorders include, for example, bitter pit and water core of apple.

Over the centuries, numerous efforts have been made to understand the dynamics of these diseases and pests to develop effective management strategies and promote sustainable fruit production. There are extensive descriptions of the diseases and pests affecting pome and stone fruit trees in books or reviews (Agrios, 2004; Bovey, 1989; Desvignes, 2004; Hadidi et al., 2011; Sutton et al., 2014). Based on their analysis, this section provides a brief but comprehensive overview of the major pome and stone fruits diseases by addressing their symptomatology, etiology, epidemiology, and management strategies. The reader should note that this thesis is not an extensive review of diseases and pests of pome and fruit trees. The facts and control strategies presented represent a fraction of the vast literature on them. New control methods and products are being developed and published, while the knowledge of pest biology and epidemiology is permanently improving.

In general, effective management of these diseases and pests often involves a combination of cultural practices (physical control), biological control methods, and chemical treatments. Integrated pest management (IPM) strategies are adopted to effectively control and minimize the impact of these diseases and pests while considering environmental and economic factors (Hoyt and Burts, 1974). Regular and timely monitoring is crucial for early detection, and implementing a holistic,

integrated approach. In recent years, there has been a shift in public and administrative opinion and concern regarding the application of synthetic pesticides in agriculture. In Europe, the legislation on plant protection products is essentially implemented via Regulation EC 1107/2009[1], which regulates the registration of pesticides on the market. Combining traditional and innovative strategies is essential for effective pest and disease control.

Moreover, using resistant, tolerant, or low-susceptibility cultivars combined with other control methods can be highly effective. It is important to note that for viral diseases of pome fruit trees, their management in orchards mainly relies on disease-free planting material and, in specific cases, removing infected trees to prevent further virus spread. Despite significant advances in research and disease management strategies, knowledge gaps remain in understanding the epidemiology and the host's genetic resistance to certain diseases.

1.3. Abiotic stresses

Environmental conditions not only have an impact on pathogens and pests of pome and stone fruit trees but also the growth and fruiting of the trees themselves. Temperature extremes pose a significant challenge, with late spring frost damaging blossoms and high temperatures during flowering reducing and even impeding pollination. Periods of low temperatures can result in freezing injuries, which include winter sunscald of thin-barked tree species, killing of dormant flower buds, or freezing of roots. If these periods occur during spring and fall, flowers and fruits are susceptible to frost damage (Yu and Lee, 2020). Younger trees are more susceptible to sudden changes in temperature as the cortex is thinner and cannot protect the cambium against solid insolation. Frost blankets can be used to protect trees from freezing injuries. Other effective preventive measures involve planting frost-resistant varieties or deploying windbreaks (Bovey, 1989).

When herbaceous parts of the tree are suddenly exposed to high temperatures and heat, they may develop sunburn. Heat stress often occurs during crucial stages of plant growth, such as flowering or fruit development. It can impact the growth and development of pome and stone fruit trees, as well as their cell structure, given that the structure of chloroplasts can be disrupted by heat (Liu et al., 2013). Putting a layer of mulch around the base can help keep the soil cool, and shade netting can protect the trees from direct sunlight (Sutton et al., 2014). Moreover, as heat stress is exacerbated when it is accompanied by drought periods, it becomes more challenging

[1] Consolidated text (21st of November 2022): http://data.europa.eu/eli/reg/2009/1107/2022-11-21

for the trees to reduce their temperature. Implementing strategic irrigation to alleviate heat stress can be an effective strategy.

Water stress, which ensues from periods of drought or saturated soils, manifests in reduced fruit size and overall tree vigor. Lack of water can impact the season where the drought happens and the following growing seasons, given that water stress may decrease the number of blooms and, therefore, the final number of fruits harvested. Apart from fruit yield, quality may also be impacted, including, for example, fruit size, color, or taste (sugar/acid ratio). On the other hand, excess humidity and water accumulation in the soil, or waterlogging, can also harm trees. The main effects include yellow, red, or purple leave colorations similar to the chlorosis observed during a nutrient deficiency (Bovey, 1989). Proactive strategies against water stress include meticulous irrigation systems and scheduling to reduce soil evaporation, summer pruning to decrease transpiration and water use/consumption, or additional shade produced by nets in the orchard, which can reduce the damages due to water stress (Lopez et al., 2012).

Hail damage can occur throughout the growing season to leaves, fruits, blooms, and limbs, although the level of injury depends on the size and density of the hail and the plant's developmental stage at the time of injury. Damage produced by hail can impact the harvest's quality and yield. Specifically, hail can bruise or scar developing fruits, which leads to deformities and renders them unmarketable. Depending on the developmental stage of the fruit at the time hail damage occurs, the wounds will look different. If damage occurs early in the season, the wounds heal and appear as large scars or deformed areas on the harvested fruit. However, if damage occurs later in the season, the wounds do not heal, and the injuries appear like bird damage. In severe cases, hail damage can impact branches and create entry points for various pathogens, such as fire blight (Sutton et al., 2014). Anti-hail nets are widely used to protect fruits against hailstorms and hail damage. Moreover, studies suggest that they can also have a beneficial effect on preventing attacks from pests (Nelson et al., 2023).

1.4. Climate change and its impact on plant health

Changes in the climate have occurred over hundreds of thousands of years, and evidence of their impact on biodiversity and shifts in ecosystems can be observed in fossil records and geological strata. Even though there is a long history of climate variations, the industrial revolution of the past centuries has increased greenhouse gas emissions into the atmosphere at a rate and level never seen. Aside from burning of fossil fuels and industrial activities, deforestation, agricultural practices, improper waste management, the use of certain synthetic gases in refrigeration and air conditioning, and land use changes are fundamental causes of the increase in the release of greenhouse gases into the atmosphere, which enhances the greenhouse effect and leads to global warming (Montzka et al., 2011).

The gradual increase in global temperatures alters growing seasons and precipitation patterns, directly affecting crop growth. This leads to shifts in optimal planting times

and geographical suitability for certain crops, challenging traditional agricultural practices and needing adjustments to new climate scenarios. Rising temperatures, altered precipitation patterns, and extreme weather events disrupt agricultural practices, affecting crop yields and quality. The changing dynamics of pests and diseases worsen plant health (Thornton et al., 2014). As temperatures rise, pests once confined to specific regions can migrate to new areas, affecting crops that are not protected against them.

Similarly, altered climate conditions create more favorable environments for the proliferation of specific pathogens, leading to an increased incidence of plant diseases (Chaloner et al., 2021; Raza and Bebber, 2022). For example, Glomerella leaf spot is an apple disease, typically found in humid subtropical climates, that is caused primarily by species of the *Colletotrichum gloesporioides* species complex, including *C. chrysophilum* and *C. fructicola* (Astolfi et al., 2022). Although the disease has not been reported in Europe, *C. chrysophilum* was detected in Spain (Cabrefiga et al., 2022a) and *C. fructicola* in France (Nodet et al., 2019) and Italy (Wenneker et al., 2021). Thus far, the effective control strategy relies on the use of fungicides when the weather conditions are favorable for disease development, from the fall of the petals until harvest (Villani and Hopper, 2018), which may result in the presence of fungicide residue on the surface of the fruit.

Temperature is crucial in determining phenological stages in pome and stone fruit trees. Dormancy is a period of suspended growth and low metabolic activity during winter, allowing pome and stone fruit trees to withstand harsh environmental conditions. During this phase, the trees accumulate chilling hours, a critical requirement for proper bud break and subsequent flowering in the spring. However, rising temperatures due to global warming and climate change can alter the chilling hour accumulation, thus disrupting the mechanism behind dormancy release (Fadón et al., 2020). Insufficient chilling can lead to delayed or uneven bud break, impacting fruit development and yield later in the season (El Yaacoubi et al., 2020).

Moreover, warmer winters might induce premature dormancy release, increasing vulnerability to late spring frosts. Warm temperatures during autumn and winter may cause flowering disorders, which result in irregular flowering and bud break (Guédon and Legave, 2008). It affects cold acclimation and freezing tolerance, dormancy progression, tree growth and flower initiation, and floral bud maturation. These conditions can also decrease the carbohydrate accumulation in shoots, thus leading to reduced tree vigor (Tominaga et al., 2022). Temperature changes can also lead to shifts in the timing of the emergence of pollinators, alter their distribution and abundance, and result in reduced pollination success, which is essential for fruit production (Schweiger et al., 2010). Temperature and precipitation patterns also influence pollinators' distribution (Potts et al., 2010) and the abundance and availability of freshwater (Rodell et al., 2018), further jeopardizing crop production. Addressing these challenges requires an integrated approach, including, for example,

the development of climate-resilient crops (Kole et al., 2015) and sustainable agricultural practices.

1.5. Importance of germplasm collections for conservation of genetic diversity

Aside from the loss of biodiversity linked to climate change, autochthonous varieties of fruit trees have been replaced by varieties better priced in national and international markets. Moreover, although demand for specific characteristics in fruits and vegetables changes from year to year, the intensive farming systems have, as well, dramatically reduced apple genetic diversity and the use of local varieties. Cultivated species and cultivars comprise a small fraction of the totality available, and there is a risk of losing ancient and local varieties and their genetic characteristics, which could include climatically and pathogenically resistant or tolerant genes from the cultivated trees (Zsögön et al., 2022).

Germplasm collections act as a reservoir of genetic diversity by storing living genetic resources to preserve genetic biodiversity and for animal and plant breeding. Plants can be kept as germplasm in collections, as stored seeds in cold storage, and as plants grown in nurseries or orchards (Acuña et al., 2019; Doekes et al., 2018; Marconi et al., 2018). Plant breeders can use the genetic diversity in germplasm collections to manipulate and improve the plant genome with desired traits such as higher yield, disease and pest resistance, better organoleptic properties, or resilience against abiotic stresses (Shahzad et al., 2021). Such collections contribute significantly to achieving a sustainable agriculture approach and mitigating climate change's impact on plant health.

International collaborations and exchanging plant material from germplasm collections among countries is a common practice and plays a crucial role in ensuring that the genetic diversity kept in the collections is accessible to more researchers and countries. The International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) is an international agreement that facilitates the exchange of plant genetic resources between countries. Organizations that promote and actively participate in the exchange of plant materials include, for example, the International Rice Research Institute (IRR), the Consultative Group on International Agricultural Research (CGIAR) centers, or national agricultural research institutions such as the Walloon Agricultural Research Centre (CRA-W).

During these exchanges of plant material between countries, it is essential to ensure that the plant materials are free from damaging pathogens, such as viruses and viroids, to prevent disease spread, protect agricultural systems, ensure research validity, comply with international regulations, and preserve the receiving germplasm collection(s) (Kumar et al., 2021). Various quarantine programs are set in place to prevent the spread of viruses between countries during international exchanges of plant material from germplasm collections. These phytosanitary measures are

overseen and managed by governments, international certification schemes, and guidelines to ensure the propagation material is free of quarantine and regulated organisms specified by national legislation and important nonquarantine pathogens (Barba et al., 2015). When working with clonally propagated crops, nurseries can harbor infected hosts that may be asymptomatic. Thus, the plants have the potential to be selected for cultivation by breeders and nurseries and to be shipped internationally (Gergerich et al., 2015). Compared to infections by pathogens such as nematodes of fungi, which can be treated, trees infected with a virus will remain infected.

2. Plant viruses

2.1. The beginnings of plant virology

Although it was not until the second half of the twentieth century that the study of plant viruses kicked off, recordings of plant viruses can be found throughout history. The oldest recording of a plant disease, most probably caused by a virus, dates from 752 CE in Japan. It consists of a poem by Empress Koken that describes plant fields during summer, showing yellowing on leaves. Later, the plant was identified as *Eupatorium lindleyanum,* which is an herbaceous perennial plant in the family *Asteraceae* susceptible to tobacco leaf curl virus (TLCV), which causes yellowing disease (Saunders et al., 2003). Later, during the events of Tulip Mania in the seventeenth century, numerous paintings and drawings of tulips showcasing color-breaking flowers were made. Later, it was found that the color-breaking was caused by several viruses belonging to the *Potyviridae* family (Lesnaw and Ghabrial, 2000). The catalyst for the first virus identification came centuries later, with a mosaic disease in tobacco plants. Adolf Mayer published a detailed description of the disease and its symptoms in a paper from 1886, which was followed by the observations and published works of Dimitrii Iwanowski in 1892 and Martinus Beijerinck in 1898 about the disease and its possible causal agent (Lecoq, 2001). As virology began gaining more traction in the twentieth century, tobacco mosaic virus (TMV) became one of the best-studied and characterized viruses, and its research remained at the forefront of virology (Harrison, 2009). For instance, in the late 1920s, cross-protection between different virus strains was observed, then in the late 1930s, TMV was confirmed to have rod-shaped particles by X-ray analysis and electron microscopy, and in 1960, its complete amino acid sequence for the coat protein was obtained (Harrison and Wilson, 1999; Okada, 1999). During the following decades and with the advent of new methods and technologies, scientists moved away from the “one pathogen to one disease” paradigm and recognized that, for the same host, viruses can have different strains that can produce different symptoms (or no symptoms), different viruses can cause similar symptoms, and a mixed viral infection causes some diseases.

2.2. Viruses, what are they?

By definition, viruses are microscopic infectious agents that are obligate intracellular parasites, thus requiring a host cell to replicate and reproduce because they lack the cellular machinery necessary to replicate and produce new virus particles (Hull, 2014). Viral particles, commonly known as virions, consist of a genome, either DNA or RNA, and a capsid made of proteins that shield and protect the genetic material. Sometimes, an external membrane composed of lipoproteins, called the envelope, is also a virion component. In addition to genes encoding structural components, viruses may carry genes responsible for encoding various regulatory active proteins and enzymes. Following infection, some viruses can enter a latent state by integrating their genetic information into the host cell's genome or maintaining it as an episomal form. Subsequently, specific cellular or external processes and influences can reactivate the integrated genomes or episomal forms, producing new infectious viruses within the host (Modrow et al., 2013).

Viruses can be classified as single-stranded and double-stranded, depending on the structure of their genetic material. In single-stranded viruses, their genetic material consists of a single strand of DNA or RNA. Single-stranded RNA viruses can be further categorized into positive-sense RNA or negative-sense RNA viruses (Figure 1-1). The RNA of positive-sense viruses can serve directly as messenger RNA, thus translating immediately the RNA into viral proteins. On the other hand, negative-sense RNA viruses require the synthesis of a complementary RNA strand before the protein synthesis can occur.

The genomic material of a virus is composed of several coding and non-coding regions. Coding regions express the proteins required to continue the infectious cycle, such as replication-associated proteins and coat proteins. These coding regions can also be identified as open reading frames (ORFs), usually starting with an AUG codon and stopping with one of the three stop codons. However, some cases of plant viruses with ORFs do not start with the AUG codon, such as Peach chlorotic mottle virus (PCMV), which starts with an AUC codon (James et al., 2007).

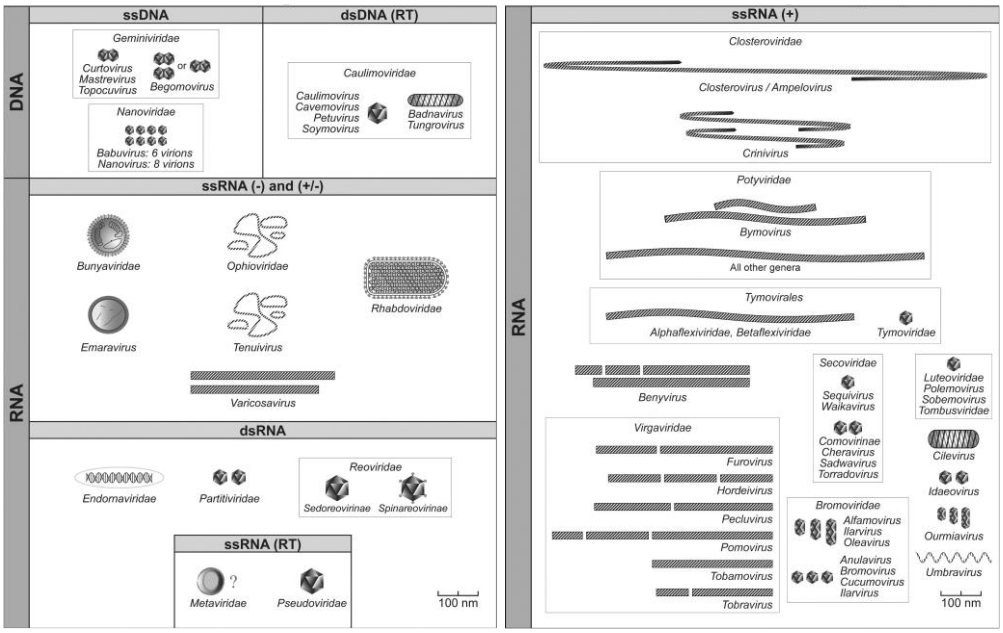

Figure 1-1. Diagram of the virus taxa that infect plants, showcasing their high structural diversity. Figure reproduced from the 9th Report of the ICTV (King et al., 2011) under the Creative Commons Attribution-ShareAlike 4.0 International Public License (CC BY-SA 4.0 DEED) terms.

2.3. Taxonomic classification of plant viruses

Classification of living creatures is a helpful tool for inferring characteristics and properties of individuals across populations based on their similarities and differences. However, it is ultimately an artifact created by humans. Since the discovery and description of viruses there have been several attempts to develop a reliable classification system for viruses. However, the evolutionary relationships of viruses are still an approximation given that there is no fossil record and viruses evolve at high and fluctuating rates (Gorbalenya and Lauber, 2017). Historically, viral taxonomy relied on phenotypic properties based on biology and other factors, including information on host range, replication cycle, and structure and properties of viral particles (Figure 1-2). The Baltimore classification is a widely used, although informal, classification of viruses developed in 1971 by David Baltimore. This classification divides viruses into seven groups depending on their genome type and replication method: group I includes double-stranded (ds) DNA (dsDNA) viruses; Group II includes positive-sense single-stranded (ss) DNA (+ssDNA) viruses; Group III includes dsRNA viruses; group IV, includes positive-sense ssRNA (+ssRNA); group V, includes negative-sense ssRNA (-ssRNA) viruses; group VI, includes positive-sense ssRNA viruses that replicate through a DNA intermediate (Retrovirus); and, group VIII, which includes dsDNA viruses that replicate through a single-stranded RNA intermediate (pararetroviruses) (Baltimore, 1971). However, the virus

taxonomy had to be adapted due to the high number of new viruses discovered by high-throughput sequencing (HTS) technologies (Kuhn, 2021).

Until 2017, the International Committee on Taxonomy of Viruses (ICTV) classified viruses in a five-rank hierarchy composed of species, genus, subfamily, family, and order, which was in concordance with a part of the Linnean hierarchical structure and classification scheme (Figure 1-3 left). As mentioned before, the fast-evolving field of HTS and virus discovery stimulated the adaptation of the formal virus classification scheme to introduce additional ranks to the virus taxonomy hierarchy. Currently, the formal virus classification hierarchy from ICTV is composed of 15 ranks, including eight primary ranks (species, genus, family, order, class, phylum, kingdom, and realm) and seven secondary ranks (subgenus, subfamily, suborder, subclass, subphylum, subkingdom, and subrealm) (Figure 1-3 right). Although relying on biological properties from phenotypic data and phylogeny or sequence distances from genomic data to classify a virus is still widely recommended, with the advent of metagenomic studies, using and integrating data obtained from metagenome analyses on the taxonomy and classification of viruses provides additional information on viral diversity, abundance, and prevalence and allows the inference of biological properties from genomic sequences (Simmonds et al., 2017). Nevertheless, accepting and classifying a new virus based solely on genomic data raises some questions regarding the validity of the reconstructed or assembled genome and poses some issues to harmonizing a classification based on genetic and structural properties with a partially phenetic classification advance towards a universal virus taxonomy (Simmonds et al., 2023).

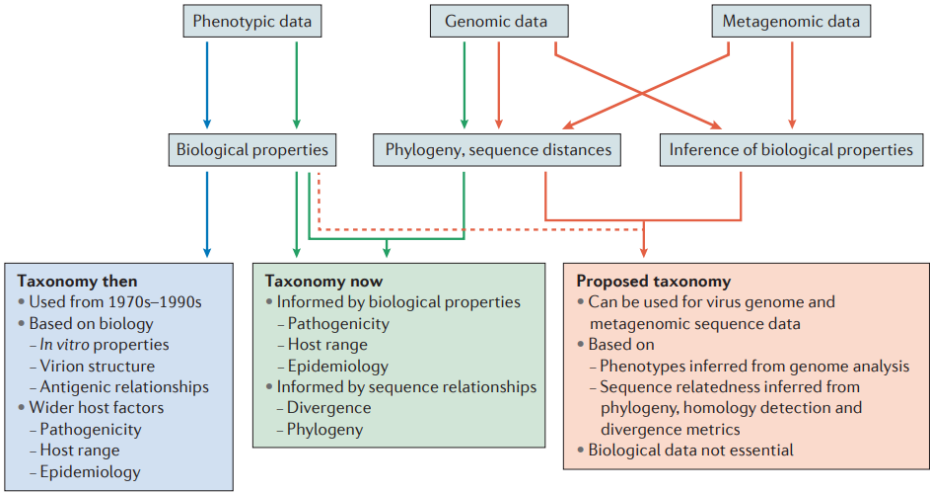

Figure 1-2. Classification pipelines used throughout the years to classify and organize the taxonomy of viruses. This diagram includes the recently proposed metagenomics-based taxonomy in the orange box and arrows, the taxonomy used in 2017 in the green box and arrows, and the previous taxonomy used between 1970s and 1990s in the blue box and arrows. Figure reproduced from Simmonds et al. (2017) under the Creative Commons Attribution License (CC BY) terms.

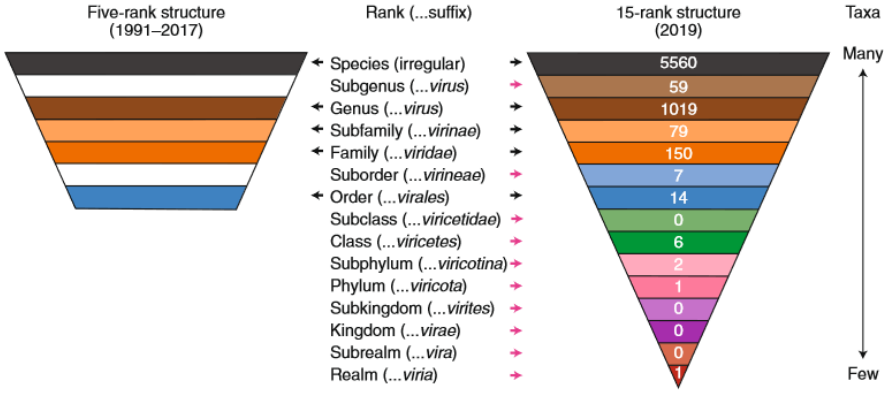

Figure 1-3. Comparison of the ICTV taxonomic rank hierarchy from 1991 to 2017 and since 2019. The number of taxa assigned to each rank from the new hierarchy is shown in white, based on the ICTV Master Species List released in 2018. Black arrows show the common taxonomic ranks between the five-rank (1991-2017) and the fifteen-rank (2019) structures. Figure reproduced from Gorbalenya et al. (2020) under the Creative Commons Attribution License (CC BY) terms.

2.1. Virus transmission

Overall, the transmission of plant viruses can be classified into two groups: vertical and horizontal. Horizontal transmission occurs via vectors, including arthropods, fungi, and nematodes, and mechanically or by contact, either in the aerial part of the plant (above ground) or in the soil through infection of the roots. The most studied type of horizontal transmission is by insect vectors, although it is not known to occur in pome fruit trees. However, horizontal transmission and spread of a virus within a pome fruit orchard may occur through root grafting and dagger nematodes feeding on the roots (Forer et al., 1984). Vertical transmission occurs through seeds, pollen, and vegetative and clonal propagation of infected material.

The major transmission modes via insect vectors can be defined as persistent or non-persistent and circulative or non-circulative (Fereres and Raccah, 2015). In circulative transmission, the virus circulates in the insect before being transmitted to another plant, presenting long acquisition and retention periods (persistent). If it replicates in the insect, it is called propagative; if it is not, it is called non-propagative. On the contrary, if the virus is transmitted in a non-circulative manner, it does not circulate in the insect's body as it stays in the stylet or the foregut. In this case, the viruses can be differentiated according to their acquisition and retention periods, which are short and vary from a few minutes (non-persistent) to a few hours (semi-persistent) (Whitfield et al., 2015). The acquisition phase is the time the insect needs to feed on an infected plant to acquire the virus, and the retention period is when the insect can transmit the virus to healthy plants. Other periods that can be considered for the categorization of the transmission modes via insect vectors are the latency period, which is the time between acquisition and transmission of the virus, and the

inoculation phase, which is the time needed to transmit the virus to a healthy plant (Bragard et al., 2013).

As mentioned before, aside from horizontal transmission, plant viruses can also be transmitted vertically from plant to plant. Vertical transmission of plant viruses involves transmission of viruses in the field via natural openings and wounds in the plant and its roots or through seeds where the virus is either in the coat of the seed or within the embryo and thus can be transmitted to the next generation of progeny. In pome fruit trees transmission of viruses is mainly done via vegetative propagation, including grafting infected material into healthy plants (Hull, 2014). In pome fruit trees, vegetative propagation can result in a genetic bottleneck that acts as a driving force to produce distinct populations of viruses and viroids (graft-transmissible agents) (Sano et al., 2008).

2.2. Pome fruit tree viruses

Pome fruit trees host a notable number of viruses with, generally, high host specificity, which could be explained by their vegetative mode of propagation and perennial nature (Table 1-1). One could say that a few viruses that are commonly and widely detected in pome fruit trees, especially apple and pear, have evolved together with their domestication, given that they do not have any known vector and are not seed transmitted either. These would have happened as the plant material used for propagation originates from trees showing no visible symptoms of diseases, and, thus, mild strains of viruses that produced no symptoms were artificially selected and propagated with the propagation and distribution of pome fruit trees over time. Viruses may cause diseases that severely affect yield and fruit quality, leading to substantial economic losses. Therefore, their identification, detection, and further characterization are essential for their management. Additionally, the elimination of viruses from pome fruit trees can be done via, for example, meristem culture and heat therapy (Vivek and Modgil, 2018). Nonetheless, viruses in pome fruit trees are still challenging due to their variable and low titer throughout the year and irregular distribution within the tree (Varveri et al., 1997; Zotto et al., 1999). Fruit trees can also accumulate multiple viruses in them because they are vegetatively propagated, resulting in inter- and intra-species mixed infections and complex and heterogeneous viral populations in a tree (Glasa et al., 2017; Jridi et al., 2006).

Apple mosaic disease was one of the first described economically significant and widespread disease of apple trees. This viral disease is caused by apple mosaic virus (ApMV), albeit apple necrotic mosaic virus (ApNMV) has also been associated with it. Both viruses belong to the genus *Ilarvirus* within the family *Bromoviridae* and comprise a tripartite genome and icosahedral viral particles (Manzoor et al., 2023). Even though ApMV receives its name from the first host where the disease was identified and described, this virus has been found in other plant hosts such as birch (*Betula* spp.), hop (*Humulus lupulus*), rose (*Rosa* spp.), or other woody hosts, as plum (*Prunus domestica*) and apricot (*Prunus armeniaca*) (Grimová et al., 2016).

Symptoms are variable among different hosts and the virus strain. Sensitive varieties present a range of leaf symptoms, including yellow or cream irregular spots that may become necrotic after sun exposure during summer (Dursunoglu and Ertunc, 2008) (Figure 1-4 C). ApMV is transmitted by grafting, vegetative propagation of infected material, and root grafting in nurseries and orchards (Hunter et al., 1958). This virus is widely distributed worldwide, although its prevalence is highly variable depending on the viral status of the plant material used for vegetative propagation. For instance, ApMV was found with a prevalence of 0.4% in birch trees from Germany (Grüntzig et al., 1996) and, in comparison, with a viral incidence of 97% in hazelnut samples collected from Spain (Aramburu and Rovira, 2000).

Apple topworking disease, also known as chlorotic leaf spot, is a viral disease of fruit trees caused by apple chlorotic leaf spot virus (ACLSV). ACLSV is found commonly on apples, pears, quince, peaches, apricots, almonds, sour and sweet cherries, and European and Japanese plums; that generally remains latent in most commercial cultivars and only shows symptoms of infection when grafted on susceptible cultivars or rootstocks, such as seedlings of *Malus sylvestris* (L.) Mill. cv. R12740-7A. Susceptible cultivars develop chlorotic rings and spots on leaves, pitting on wood, and a brown necrotic line at the graft union, which can lead to decline after 1 or 2 years of their top grafting (Barba et al., 2015; Sutton et al., 2014). ACLSV is often found in co-infections with apple stem pitting virus (ASPV) and apple stem grooving virus (ASGV), which cause stem pitting and apple stem grooving, respectively.

Apple stem grooving and ***stem pitting*** are two diseases, mostly latent and with variable symptoms. ASGV causes depressions in the wood cylinder and swollen graft union, presenting a brown necrotic line, which can lead to poor growth and a premature drop of the leaves. ASPV, when infected in susceptible cultivars, can cause pits to develop in the wood cylinder, which may impact the vascular tissue and thus lead to poor growth. Symptoms are exacerbated when combinations of these three viruses (ACLSV, ASPV, and ASGV) co-infect a tree. These viruses have also been associated with ***pear vein yellows***, which is a viral disease caused by ASPV and causes symptoms such as yellowing of the veins, chlorosis, and reduced vigor, and ***pear ring pattern mosaic***, caused by ACLSV and characterized by the appearance of ring patterns and mosaic symptoms on the leaves of pear trees (Sutton et al., 2014).

Apple russet ring is a disease affecting apple trees of, until recently, unknown etiology. This disease is graft-transmissible, and the appearance of ring-shaped rust lesions on the fruits characterizes it (Figure 1-4 B and E). In the past, ACLSV had been identified in trees showing symptoms of russet ring disease, but no definitive causal relationship could be established. Recently, a comprehensive study successfully determined a causal association between specific isolates of ACLSV and apple russet ring disease, although ASPV and ASGV could also be found in some of the diseased trees. This study analyzed by HTS the viruses in trees showing symptoms of the disease, followed by an amplification of the cDNA of the viral genomes

identified in the trees and an *in vitro* transcription of infectious viral RNAs. Then, these viruses were transmitted to trees of original hosts by back inoculation to reproduce the symptoms (Li et al., 2020).

In this same article, Li et al. used the same strategy to study the potential causal agent(s) of ***apple green crinkle*** disease, which mainly causes symptoms on fruits, although severe tree decline can also occur. Symptoms appear as depressions on the fruit that slowly grow around the original affected tissue and later develop into infoldings or crinkles (Figure 1-4 D). Viruses such as ASPV, ACLSV, and ASGV have been detected in trees showcasing symptoms of green crinkle disease. Furthermore, ASPV was further confirmed as one of the causal agents of green crinkle disease (Li et al., 2020).

Apple star crack is a graft-transmissible physiological disorder that affects the fruit. It is believed to be caused by a virus-like agent. It leads to the development of star-shaped cracks on the surface of the apples (Figure 1-4 B and E). These cracks compromise the fruit's appearance and marketability and can serve as entry points for secondary infections by fungi or bacteria, which further affect the tree (Sutton et al., 2014). So far, the virus causing the disease has not yet been identified.

Apple union necrosis, caused by the Tomato ringspot virus (ToRSV), is a serious disease that manifests as necrosis at the graft union. Affected trees exhibit pitting at the graft union, followed by a folding of the woody cylinder in on itself that forms a cavity decline in vigor and productivity, which may also be accompanied by symptoms such as leaf chlorosis and dieback. ToRSV is primarily transmitted by dagger nematodes (*Xiphinema* spp.), which feed on the roots and introduce the virus into the plant (Halbrendt, 2021).

Flat apple is a disease caused by the Cherry rasp leaf virus (CRLV) that flattens apples and often leads to irregularly shaped fruit. This condition negatively impacts fruit quality and marketability, as affected apples are less appealing to consumers. Infected trees may also experience premature fruit drop, reducing overall yield. Like ToRSV, CRLV is also transmitted by nematodes (James et al., 2001).

Rapid apple decline (RAD) is a complex and poorly understood disease that has been associated with the presence of apple luteovirus 1 (ALV-1), although definitive causal relationships have not been established, and it is believed that there are multiple causes that interact and contribute to this disease. RAD was first described in the United States in 2018, and it has been reported in other countries such as Canada and South Korea. First symptoms appear in young trees in the form of stunted growth, necrosis around the graft union, and canker, for example, and exhibit decline and collapse just a few weeks after the start of the symptoms (Lee et al., 2023).

Apple rubbery wood disease (ARWD) is associated with apple flat limb disease. Diseased trees were found to be infected with Apple rubbery wood virus 1 (ARWV-1) and Apple rubbery wood virus 2 (ARWV-2), although a conclusive causal

association has not been established yet. Furthermore, prior to the identification of ARWV-1 and ARWV-2, trees presenting symptoms of ARWD were analyzed by HTS and some contigs of viral origin were identified, albeit a definitive taxonomic assignation was not possible at the time, thus pointing towards a virus-like causal agent (Jakovljevic et al., 2016). Affected trees exhibit rubbery, flexible branches that lack the normal rigidity of healthy wood and are more susceptible to frost damage. Moreover, this condition can lead to reduced structural integrity of the trees and diminished fruit production (Rott et al., 2018).

Pear stony pit is a disease that causes the formation of hard, stone-like pits in the flesh of the pears (Figure 1-4 A), making them unmarketable and thus leading to significant economic losses. In addition to the pits, affected trees may display symptoms such as reduced vigor, stunted growth, and overall decline in health. The disease is believed to be associated with multiple viruses, though the exact causal agents remain a subject of ongoing research. The presence of stony pits disrupts the normal development of the fruit, interfering with cellular processes and leading to the characteristic hard inclusions (Kegler et al., 1976).

Pear vein yellows is a viral disease affecting pear trees, primarily caused by the Apple stem pitting virus (ASPV). This disease manifests through a distinctive yellowing of the leaf veins, accompanied by chlorosis and an overall reduction in tree vigor. Infected trees often exhibit stunted growth and diminished vitality, leading to significant yield losses and compromised fruit quality, resulting in weakened structural integrity and reduced resilience to environmental stresses. The transmission of ASPV occurs predominantly through grafting with infected plant material, underscoring the importance of using certified virus-free propagation sources to prevent the initial infection (Leone et al., 1998).

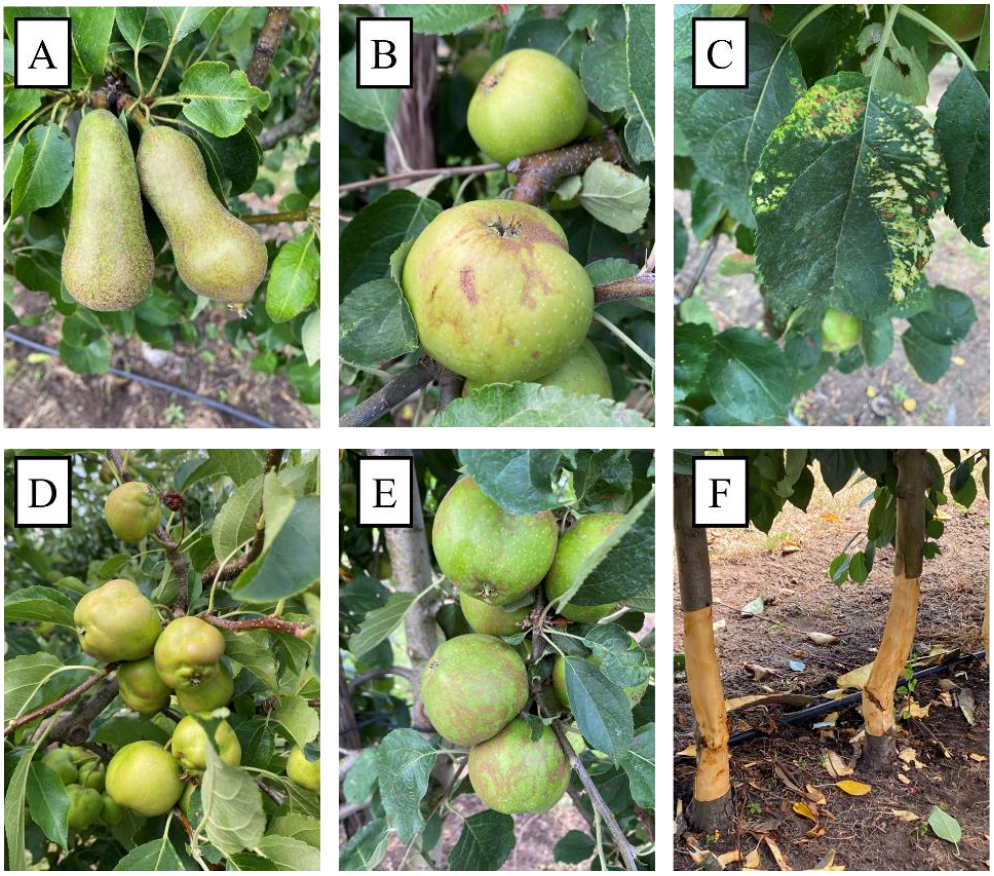

Figure 1-4. Symptoms associated with virus and viroid-like infections in pome fruit trees, taken in a collection created for the 25th International Conference on Virus and other graft transmissible diseases of Fruit crops (ICVF) held in Wageningen in July 2023. A) Pears showing stony pit symptoms. B and E) Apples with brownish corklike texture on their skin similar to apple star crack and apple russet ring. C) Mosaic symptoms on an apple tree leaf. D) Apples showing symptoms of apple green crinkle. F) Union incompatibility with a brown necrotic line.

Table 1-1. List of the viruses and viroids affecting the major cultivated pome fruit trees. *tentative member

Virus name	Abbreviation	Family	Genus	Reference
Apple associated luteovirus	AaLV	*Tombusviridae*	Luteovirus	Shen et al., 2018
Apple chlorotic leaf spot virus	ACLSV	*Betaflexiviridae*	Trichovirus	Luckwill and Campbell, 1959
Apple chlorotic fruit spot viroid	ACFSVd	*Pospiviroidae*	Apscaviroid	Leichtfried et al., 2019
Apple dimple fruit viroid	ADFVd	*Pospiviroidae*	Apscaviroid	Di Serio et al., 2001
Apple fruit crinkle viroid*	AFCVd	*Pospiviroidae*	Apscaviroid	Ito et al., 1993
Apple geminivirus	AGV	*Geminiviridae*	unclassified	Liang et al., 2015
Apple green crinkle associated virus	AGCaV	*Betaflexiviridae*	Foveavirus	James et al., 2013
Apple hammerhead viroid	AHVd	*Avsunviroidae*	Pelamoviroid	Serra et al., 2018; Zhang et al., 2014
Apple ilarvirus 1		*Bromoviridae*	Ilarvirus	Wright et al., 2020
Apple ilarvirus 2		*Bromoviridae*	Ilarvirus	Xiao et al., 2022
Apple latent spherical virus	ALSV	*Secoviridae*	Cheravirus	Li et al., 2000
Apple luteovirus 1	ALV-1	*Tombusviridae*	Luteovirus	Liu et al., 2018
Apple necrotic mosaic virus	ApNMV	*Bromoviridae*	Ilarvirus	Noda et al., 2017
Apple mosaic virus	ApMV	*Bromoviridae*	Ilarvirus	Bradford and Joly, 1933
Apple picorna-like virus 1				Wright et al., 2020
Apple rootstock virus A	ApRVA	*Rhabdoviridae*	Betanucleorhabdovirus	Baek et al., 2019
Apple rubbery wood virus 1	ARWV-1	*Phenuiviridae*	Rubdovirus	Rott et al., 2018
Apple rubbery wood virus 2	ARWV-2	*Phenuiviridae*	Rubdovirus	Rott et al., 2018
Apple scar skin viroid	ASSVd	*Pospiviroidae*	Apscaviroid	Koganezawa, 1986
Apple stem grooving virus	ASGV	*Betaflexiviridae*	Capillovirus	Lister et al., 1965
Apple stem pitting virus	ASPV	*Betaflexiviridae*	Foveavirus	Guengerich and Millikan, 1959
Apple tombus-like virus 1		*Tombusviridae*	Unassigned	Wright et al., 2020
Apple tombus-like virus 2		*Tombusviridae*	Unassigned	Wright et al., 2020
Apricot latent virus	ApLV	*Betaflexiviridae*	Foveavirus	Cho et al., 2016

Virus name	Abbreviation	Family	Genus	Reference
Blackberry chlorotic ringspot virus	BCRV	*Bromoviridae*	Ilarvirus	Tzanetakis et al., 2006
Cherry leaf roll virus	CLRV	*Secoviridae*	Nepovirus	Woo et al., 2012
Cherry rasp leaf virus	CRLV	*Secoviridae*	Cheravirus	Thompson et al., 2004
Citrus concave gum associated virus	CCGaV	*Phenuiviridae*	Coguvirus	Navarro et al., 2018a
Citrus virus A	CiVA	*Phenuiviridae*	Coguvirus	Navarro et al., 2018b
Eggplant mottled crinkle virus	EMCV	*Tombusviridae*	Tombusvirus	Russo et al., 2002
Hop stunt viroid	HSVd	*Pospiviroidae*	Hostuviroid	Sano et al., 1989; Sasaki and Shikata, 1977
Malus domestica virus A	MdoVA	*Closteroviridae*	Velarivirus	Koloniuk et al., 2020
Peach latent mosaic viroid	PLMVd	*Avsunviroidae*	Pelamoviroid	Flores and Llácer, 1988
Pear blister canker viroid	PBCVd	*Pospiviroidae*	Apscaviroid	Hernández et al., 1992
Pear chlorotic leaf spot-associated virus*	PCLSaV	*Fimoviridae*	Emaravirus	Liu et al., 2020
Pomes virus Greece*	PVGR	*Betaflexiviridae*	Robigovirus	Costa et al., 2022
Prunus necrotic ringspot virus	PNRSV	*Bromoviridae*	Ilarvirus	Chandel et al., 2008
Prunus virus T	PrVT	*Betaflexiviridae*	Tepovirus	Costa et al., 2022
Pyrus pyrifolia cryptic virus	PpCV	*Partitiviridae*	Deltapartitivirus	Osaki et al., 2017
Pyrus pyrifolia partitivirus 2	PpPV2	*Partitiviridae*	Alphapartitivirus	Osaki and Sasaki, 2018
Pyrus virus A*	PyVA	*Closteroviridae*	Velarivirus	Fontdevila et al., 2024
Solanum nigrum ilarvirus	SNIV	*Bromoviridae*	Ilarvirus	Xiao et al., 2022
Temperate fruit decay associated virus	TFDaV	*Amesuviridae*	Temfrudevirus	Basso et al., 2015
Tobacco mosaic virus	TMV	*Virgaviridae*	Tobamovirus	Kirkpatrick and Lindner, 1964
Tobacco ringspot virus	TRSV	*Secoviridae*	Nepovirus	Lana et al., 1983
Tomato black ring virus	TBRV	*Secoviridae*	Nepovirus	Mischke and Schuch, 1962
Tomato bushy stunt virus	TBSV	*Tombusviridae*	Tombusvirus	Allen, 1969
Tomato ringspot virus	ToRSV	*Secoviridae*	Nepovirus	Stouffer and Uyemoto, 1976
Tulare apple mosaic virus	TAMV	*Bromoviridae*	Ilarvirus	Yarwood, 1955

3. Detection methods

For sustainable and prosperous agriculture, the early identification and detection of plant pathogens is essential to ensure that timely and effective measures are implemented to prevent their spread. Detection methods for any known viral infection include serological assays such as ELISA test, molecular hybridization, polymerase chain reaction (PCR), real-time PCR, electron microscopy, or biological indexing. These serological and molecular techniques target known viruses; thus, they are not suited for identifying unknown pathogens. Techniques with a broader identification range include indexing or electron microscopy. However, they do not serve as a method of virus identification, and further analyses need to be carried out to identify the virus species. In comparison, HTS allows the identification of novel pathogens without any a priori knowledge of the pathogen from environmental and host tissue samples, as well as from asymptomatic infections or with no apparent symptoms (Massart et al., 2014). HTS has also signified an improvement in plant health diagnostics, the control of plant material importation and movement, and the associated pests (Adams et al., 2018).

Several metrics and parameters are key to ensuring a reliable result in a detection test. Such characteristics include analytical sensitivity, which measures the ability of a test to detect a virus present in low amounts; analytical specificity, which encompasses exclusivity (how accurately a test can detect a specific virus without detecting other viruses or sequences) and inclusivity (how accurately a test can detect all isolates and strains of a specific virus); accuracy, which is a metric that considers both the sensitivity and specificity of the detection test to represent the capacity of the test to obtain accurate results; and, reproducibility, which is the ability of a test to be reproducible and give consistent results under different conditions (i.e., operator, equipment, etc.) (Lebas et al., 2022; Massart et al., 2022). The protocol must be easy to perform, have clear instructions to increase the reproducibility of a test, and be cost-effective to increase the number of samples tested, consuming less time and resources.

3.1. Targeted molecular tests

Molecular tests are widely used to identify many viruses, given their high accuracy and sensitivity, although they are mainly used when the genetic information of the target(s) is available. They are usually able to detect a single target, although there are also universal and generic primers that amplify viruses belonging to a particular family or genus. Such protocols were widely used in the past for virus discovery (Zheng et al., 2010). Such techniques include polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR), and isothermal amplification. Several methods were developed based on the principle of (RT)-PCR, such as multiplex PCR (Elnifro et al., 2000), heminested and nested PCR (Pantaleo et al., 2001), co-operational PCR (Olmos et al., 2002), droplet digital PCR (Mehle et al., 2018) and real-time PCR (Watzinger et al., 2006). PCR is based on the use of naturally occurring nuclease

enzymes to catalyze the regeneration of DNA with a chain reaction that consists of three steps that take place in each cycle (Lodish et al., 2016):

I. Denaturation: the template DNA, together with the reaction mix containing the forward and reverse primers, the four deoxynucleotides (dNTPs), and the DNA polymerase (heat-stable) are heated to 95ºC when the DNA is submitted to this temperature, it is denatured, which separates the DNA double strand into two single strands.
II. Annealing: the template DNA mix is cooled to a temperature usually between 50-60ºC to allow the primers to anneal and hybridize to the single-stranded DNA. These synthetic oligonucleotides are complementary to the 3' ends of the target DNA. Once hybridized, it will act as a primer for DNA synthesis with the dNTPs present in the mix and the DNA polymerase.
III. Extension: the temperature is raised to 72ºC to allow the polymerase to add dNTPs to the target DNA and complete the amplification. Once the synthesis is complete, the mix is heated to 95ºC again to denature the duplicated DNA.

Repeated cycles of denaturation followed by hybridization (annealing) and synthesis (extension) amplify the targeted DNA sequence exponentially. On the other hand, given that PCR amplifies a DNA sequence, RT-PCR was created to amplify RNA. Commonly, RT-PCR is used to transform viral RNA targets to complementary DNA (cDNA), which is then amplified by conventional PCR. After amplification, the PCR product can be visualized using gel electrophoresis or processed using other techniques, such as colorimetric methods or sequencing. A fluorescent nucleic acid stain is added to visualize and analyze the PCR products with an agarose gel electrophoresis, which binds to DNA by intercalating between the bases and converting invisible ultraviolet light to visible light spectrum (Voytas, 2000).

Isothermal amplification can be achieved using different approaches, such as self-sustained sequence replication (3SR), transcription-mediated amplification (TMA), helicase-dependent amplification (HDA), recombinase polymerase amplification (RPA), strand displacement amplification (SDA), nucleic acid sequence-based amplification method (NASBA), or loop-mediated isothermal amplification (LAMP) (Glökler et al., 2021). Globally, isothermal amplification methods use a single temperature to amplify an RNA or DNA target. For instance, HDA uses a helicase to separate the double strands of DNA, which allows the primer to hybridize and allow the extension of the DNA by the DNA polymerase at a constant temperature of around 65ºC (Vincent et al., 2004). Another example is LAMP, which the reaction also takes place at between 60-65ºC. The denaturation step is performed by strand displacing polymerase, and it does not require a thermocycler, as a water bath is enough to keep the temperature. In addition, LAMP does not require the visualization of DNA via an

agarose gel electrophoresis, as it can be visualized by visual turbidity (Oliveira et al., 2021).

3.2. Protein-based detection by antibodies

Protein-based detection by antibodies was among the most used detection methods during the 20th century until more sensitive molecular diagnostic tools were developed. Such techniques are based on systems that use specific antibodies that respond to antigens. Before the development of enzyme-linked immunosorbent assays (ELISA), the techniques used were based on chloroplast agglutination tests (particularly for filamentous viruses) (Dijkstra and de Jager, 1998), tube precipitation tests (for viruses with various morphologies), and agar-gel double diffusion tests (for isometric viruses) (Torrance and Jones, 1981). Nonetheless, the development of ELISA was a revolution for plant virus diagnostics and research as it allowed for a more significant number of samples to be tested at a time by simplifying the detection method and shortening the time to obtain results (Torrance and Jones, 1981). Other serological techniques widely used for diagnostic purposes include tissue blot immunoassay (TBIA) (Garnsey et al., 1993) and lateral flow immunoassays (LFA) (Koczula and Gallotta, 2016).

ELISA is a commonly used laboratory technique that detects specific proteins and other substances in a sample. It is a versatile approach that can be applied to detect infectious agents, measure hormone levels, or identify allergens, among other uses. In this technique, the antigen is bound to a solid plate such as tubes or microplates of polystyrene, polyvinyl, and polypropylene (Engvall and Perlmann, 1971). Various types of ELISA developed, such as direct, indirect, double antibody sandwich (DAS), competitive, reverse, and quantitative ELISA. Generally, an ELISA is done in four main steps: coating (with either the antigen or antibody), blocking, detection (by adding a substrate that generates color), and final reading. In between the steps, there is a washing of the plate(s) using a specific buffer, such as phosphate-buffered saline (PBS), to remove the material that did not bind to any antigen or antibody).

The significant limitations of serological tests in plant virology are that many viruses are unstable and occur in low concentration in the host plant, which also contains phenolic compounds and other substances that can make the isolation of antigenically active viruses. Although ELISA successfully detects plant viruses in fruit trees, RT-PCR remains an approach with higher sensitivity and, therefore, is more recommended in certifying fruit trees (Çağlayan et al., 2006).

3.3. High-throughput sequencing

The development of sequencing technologies revolutionized once more the field of plant pathology as it allowed the determination of nucleotide sequences of any organism. This paradigm shift changed how we study pathogens and their classification, thus moving towards a more genomic-based taxonomy. Compared to

first-generation sequencing technologies, high-throughput sequencing (HTS), also called next-generation sequencing (NGS), is a powerful and efficient method that allows for simultaneous parallel processing of several sequences (Figure 1-5). Thus, generating a substantial amount of sequencing data in a relatively short time in a much more cost-effective manner.

3.3.1. Sample preparation

There are four main types of nucleic acids used as substrates for detecting viruses by HTS: total RNA or DNA, virion-associated nucleic acids (VANA), double-stranded RNAs (dsRNAs), and small interfering RNAs (siRNAs). Each one presents advantages and disadvantages; thus, a researcher must choose adequately according to the needs of each experiment (Maclot et al., 2020).

The most straightforward approach is the extraction of total RNA or DNA, which allows the detection of RNA or DNA viruses and viroids even in pooled samples. Nevertheless, the high background noise of ribosomal DNA, even if followed by ribodepletion, requires a high sequencing depth. Moreover, total RNA is not the most efficient for detecting viruses present in low concentrations, given its limited sensitivity (Gaafar and Ziebell, 2020). The total RNA extraction protocol offers the advantage of identifying a broad spectrum of RNA or DNA viruses and viroids without the need for prior enrichment of viral sequences. It applies to individual and pooled samples, although its sensitivity may be limited when detecting viruses in lower concentrations.

SiRNAs are produced when dicer-like proteins process intercellular dsRNA into tiny RNA fragments of varying lengths from 21 to 24 nucleotides (Kreuze et al., 2009; Pooggin, 2018). This approach allows the detection of any virus and viroid targeted by a silencing mechanism, and it is only suited for extracting individual plant samples. Genome reconstruction and sequence annotation are complex during the bioinformatic analysis of data produced from siRNA sequencing due to the small size of the sequences obtained (Massart et al., 2019). Additionally, in woody crops, many viruses may have low siRNA titer and require high sequencing depth, similar to total RNA.

VANA and dsRNA are two viral enrichment protocols used in virus discovery and detection by HTS. However, enriching viral sequences in the data introduces a technical bias for quantifying variants and species (Marais et al., 2018). The VANA approach is based on the enrichment of virus-like particles with the prior removal of host nucleic acids. In general, this method is divided into four main steps: (i) purification of viral particles followed by an enzymatic treatment to eliminate nonencapsidated nucleic acids, (ii) total nucleic acids extracted from resuspended virions, (iii) cDNA synthesis, purification, priming and extension by RT-PCR and Klenow fragmentation, and (iv) library preparation and amplification. Combining an RT-PCR with a Klenow fragmentation step allows the detection of RNA and DNA viruses in one sample, although still restricted to encapsidated viruses (Filloux et al.,

2015). The double-stranded RNA (dsRNA) approach also enriches the viral RNA before HTS, given that plants and other pathogens do not produce as much dsRNA as viruses. Specifically, it allows the detection of ssRNA and dsRNA viruses, and viroids, but it is unreliable for detecting DNA viruses and negative sense ssRNA viruses. Like total RNA, this method is suitable for the extraction of individual plants as well as pooled samples.

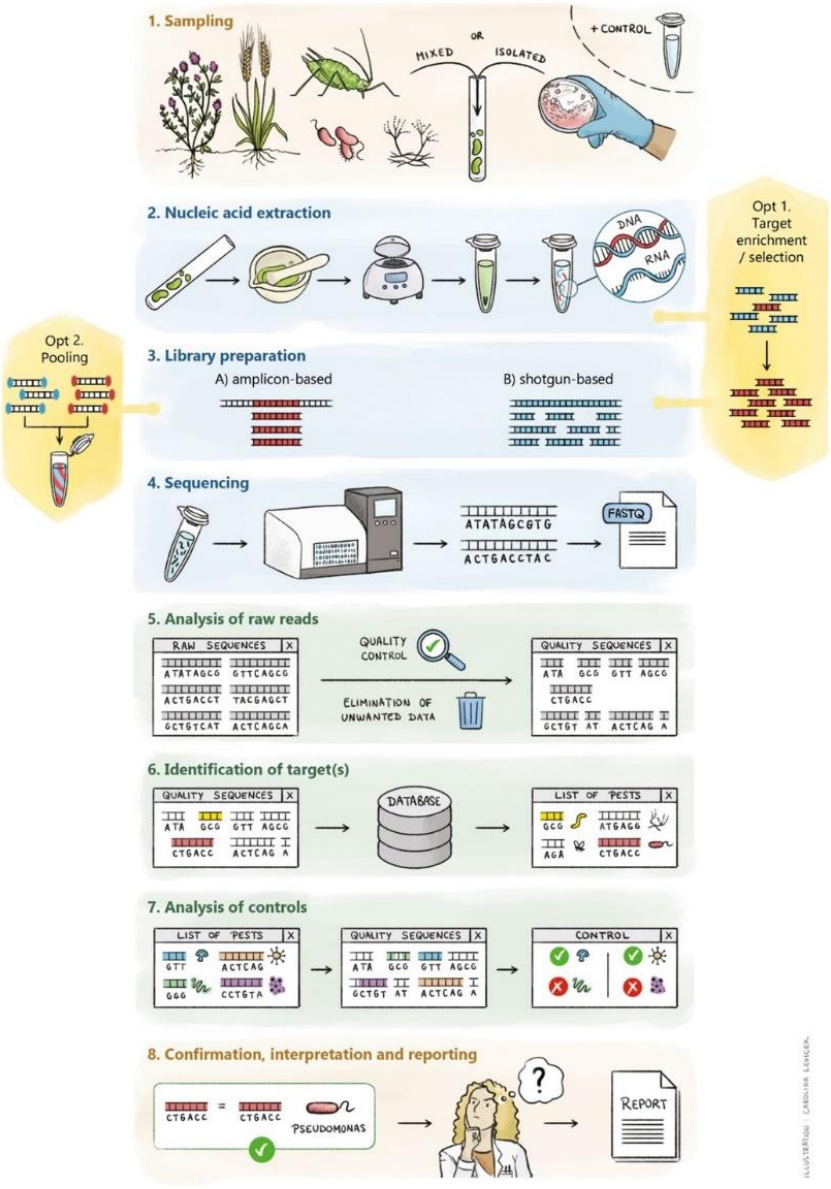

Figure 1-5. Schematic representation of the main steps of analyzing samples by high-throughput sequencing (HTS). Figure from Lebas et al. (2022) under the Creative Commons (CC) License.

3.3.2. Types of controls

According to the ISO/IEC 17025 (section 7.7) and EPPO standard PM 7/98 (2019), and as described as well by Massart et al. (2022), four types of controls can be used to validate and monitor the performance of an HTS test: positive, negative, alien, and internal positive (spike) controls.

- Positive control: is used to monitor the detection of the targets and is processed alongside the analyzed samples. This control usually contains a small but representative fraction of the possible targets that are close to the limit of detection of the HTS test, which can be used to control the analytical sensitivity of the sequencing run. Moreover, if they are in lower concentrations, they are less likely to contaminate the analyzed samples. At the same time, this control can also be used as a negative control if an unexpected target is detected, which may indicate a contamination event.
- Negative control: is used to monitor the presence of cross-contamination between samples given that it is either a matrix or purified water (blank). Like the positive control, it is processed alongside the analyzed samples.
- Alien control: is used to monitor the detection of the targets as the positive control and to check for cross-contamination between itself and the analyzed samples, similar to the negative control. In HTS, the positive and alien control(s) used must contain a quantity and concentration of nucleic acids like the one found in the analyzed samples. This will allow a better estimation of the cross-contamination between samples.
- Internal positive control: is used to monitor the analytical sensitivity and ensure the generated data is correct. The analyzed samples can be spiked with the control after or before extraction. This control is composed of a known target in low concentration near the limit of detection that is not expected in the analyzed samples.

3.3.3. Current technologies

Nowadays, sequencing technologies can be classified into two main categories: short and long-read. There are two approaches to short-read sequencing: sequencing by synthesis (SBS) and ligation (SBL). Companies like Illumina Sequencing[2] and Ion Torrent[3] use SBS to sequence DNA in which DNA polymerases and dNTPs are used to replicate and synthesize the new (complementary) strand. In Illumina Sequencing, the DNA polymerase synthesizes the complementary strand of the DNA placed in clusters into the flow cell by adding fluorescently labeled dNTPs. Each nucleotide is

[2] Illumina Sequencing: http://www.illumina.com/

[3] Ion Torrent: https://www.thermofisher.com/us/en/home/brands/ion-torrent.html

labeled with a different fluorescent color; thus, each time a nucleotide is added, the sequencing machine detects the fluorescence signal and records the base added in each position. After each round, the fluorescent label is washed and removed (Hu et al., 2021).

On the other hand, SBL uses DNA ligase to identify the nucleotides in the DNA sequence instead of a DNA polymerase to synthesize the complementary second strand. Like SBS, the DNA sequence is attached to an adaptor sequence bound to a bead, used as support, and labeled probes containing one or two known bases, several degenerate bases, and a fluorophore. Therefore, each labeled probe represents a nucleotide or a dinucleotide, as is the case for the SOLiD[4] platform. Once each fluorometric signal has been identified, the fluorophore is removed, and a new cycle begins using an adaptor sequence that is one nucleotide shorter (Garrido-Cardenas et al., 2017).

Long-read technologies can be divided into single-molecule real-time (SMRT) sequencing and synthetic approaches. Notably, synthetic approaches do not generate actual long reads, but they use barcodes between short read sequences to allow the computational assembly of a more extensive sequence. Unlike short-read sequencing, SMRT approaches (Pacific Biosciences[5], PacBio) do not rely on amplified DNA sequences to generate a detectable signal and chemical cycling for each dNTP added. Instead, they use a specialized flow cell with individual picolitre wells with transparent bottoms that fix the polymerase to the bottom of the well and allow the DNA strand to progress through the picolitre wells, called zero-mode waveguides (ZMW). A laser and camera system visualizes and records the color and duration of light emission while incorporating dNTPs (Goodwin et al., 2016). Alternatively, Oxford Nanopore Technologies[6] (ONT) uses synthetic nanopores to identify the nucleotides of a target DNA sequence by passing a single-stranded DNA sequence through a nanopore inside a membrane that has an attached enzyme that acts as a biosensor. This approach is similar to the SMRT from PacBio, except that the changes in the electrical signal are measured to determine the bases going through the nanopore (Mantere et al., 2019).

Nowadays, Illumina sequencing technology is still the most used in plant virology. However, more recent technologies, such as ONT or PacBio, which do not require a

[4] SOLiD Next-Generation Sequencing: https://www.thermofisher.com/es/es/home/life-science/sequencing/dna-sequencing/resequencing-applications/targeted-next-generation-sequencing-solid-sequencing.html

[5] Pacific Biosciences (PacBio): https://www.pacb.com/

[6] Oxford Nanopore Technologies (ONT): https://nanoporetech.com/

PCR to amplify the DNA before sequencing, are gaining traction. Such technologies reduce the preparation time and bias and error caused by PCR, and the signal is captured in real-time, whether it is fluorescent (PacBio) or electric signal (Oxford Nanopore). The significant advantage of PacBio and Nanopore technologies against Illumina and Ion Torrent is the maximum length of the fragment sequenced and that they do not need previous amplification of the nucleic acids. However, they produce fewer sequences (Nakano et al., 2017).

4. A new era of bioinformatics analyses

4.1. Analysis of sequencing reads

After receiving the data produced from the sequencing run, it is essential to check the associated quality metrics and clean the data accordingly. Quality control programs such as Trimmomatic (Bolger et al., 2014), Cutadapt (Martin, 2011), BBDuck[7], or Sickle[8] can be used to curate sequencing data, perform quality control of the initial reads, trim and remove residual adapter sequences, filter low quality reads, and trim reads with low-quality base pairs. When working with Illumina data, Q20 (1% error) and Q30 (0.1% error) are usually selected to trim the data. However, these values can vary depending on the use of the data and the sequencing platform (Kutnjak et al., 2021).

Once the data has been cleaned, sequencing reads can be aligned on reference sequences if the targeted virus is known or can be used for a taxonomic assignation. In the past few years, new tools such as Kraken2 (Wood et al., 2019) and Kaiju (Menzel et al., 2016) have been developed for taxonomic assignation without requiring prior assembly. Kaiju is a metagenome classification program that works with translated nucleotides and identifies matches at the protein level. At the same time, Kraken is a k-mer-based classification program that associates k-mers with the lowest common ancestor taxa. Alternatively, a more widely used approach is a de novo assembly strategy without prior alignment to a database using tools such as SPAdes (Bankevich et al., 2012) or Velvet (Zerbino and Birney, 2008). This strategy may be advantageous to detect divergent viral sequences by assembling overlapping reads into contigs, which may show a weak similarity but over a longer length in contrast to shorter reads (Khan et al., 2018).

[7] BBDuck: https://sourceforge.net/projects/bbmap/

[8] Sickle: https://github.com/najoshi/sickle

The taxonomic assignation of the contigs can be done with methods such as BLAST[9], which is a very accurate alignment method. However, it is slow, allows mismatches, and requires long queries to be efficient. The contigs are merged into scaffolds to reconstruct a full-length genome. However, often, the scaffold does not consist of the complete viral genome, either because of the lack of reference genome combined with insufficient coverage or because of the nucleic acids used as a template for sequencing. Once a potential novel virus genome's draft genome has been assembled, the next step is determining its phylogenetic relationships. Even if the genome lacks the 5' and 3' extremities or has gaps, the taxonomical position can be predicted by looking for the presence of conserved functional domain at the protein level, which can give hints about the taxonomic assignment, and by looking for the closely related virus through amino acid or nucleotide sequence similarity regarding molecular demarcation criteria (Maclot et al., 2021). Nevertheless, assigning a partial sequence genome lacking sufficient similarity with the existing taxa can be an issue. Provisional taxonomic assignation can be more complex if the newly discovered sequence belongs to a higher taxonomic unit, such as the genus or family.

4.2. Innovative bioinformatic tools

HTS has been around for over 10 years, and it has proven beneficial in obtaining the consensus genome sequences of known and unknown (novel) plant viruses. Researchers are turning towards a deeper understanding of what can be known based on genome sequences. For instance, Nyirakanani et al. (2023) published an innovative methodology based on single nucleotide polymorphisms (SNPs) to reconstruct viral haplotypes. This approach combined fixation index (F_{ST}) analysis and principal component analysis (PCA) to compute SNP frequencies, study the genome diversity of *Ugandan cassava brown streak virus* (UCBSV), and reconstruct its haplotypes, which allowed to discriminate between viral isolates showing >99% nt identity and characterize the haplotypes based on their clustering and geographical origin. Such methodologies can help to investigate the factors impacting virus evolution in specific areas and to create and design better management strategies (Nyirakanani et al., 2023).

Moreover, in recent years there have been advances in bioinformatic tools to characterize better and study RNA viruses, partially driven by the Covid-19 pandemic. For example, there are various efforts to create tools and models to predict future pandemics and adaptations of RNA viruses using machine learning (Li et al., 2020) or to predict potential hosts that act as viral reservoirs and vectors (Babayan et al., 2018). Another approach is screening public databases, mainly the sequence read archive (SRA), to mine new viruses. Serratus is a user-friendly web-based interface

[9] BLAST: https://blast.ncbi.nlm.nih.gov/Blast.cgi

that allows screening public databases, data deposited until January 2020, to look for viral RdRp motifs in SRA data (Edgar et al., 2022). This strategy can provide information about RNA viruses' host range and geographical distribution and enhance virus discovery. However, further confirmation needs to be done for such findings by, for example, reanalyzing the SRA datasets or contacting the laboratory responsible for publishing the dataset where the virus of interest was detected to test the plant material if available (Temple et al., 2022). New tools and approaches that go beyond the consensus sequence include the study and prediction of, for example, the secondary protein structure and the use of machine learning to find divergent and new viral sequences in a dataset (Jumper et al., 2021; Sukhorukov et al., 2022).

5. Control of pome fruit viruses

Severe symptoms of a viral infection in pome and stone fruit trees are highly variable and dependent on whether susceptible varieties are used. Therefore, it is essential to establish surveillance programs to detect viruses in plant material, mainly if used for breeding and commercial purposes. Diagnosis is the most critical aspect of controlling fruit plant viruses, as early detection of viruses in fruit trees or the propagative material is a prerequisite for their control and to guarantee sustainable and durable agriculture. The adopted control measures will depend on the identified viruses and their associated diseases. Moreover, given that the global movement of plant material has accelerated the spread of plant pathogens and increased the risk of new introductions, the creation of networks of scientists and risk managers is essential to be able to identify and respond to any potential emerging diseases on time (Barba et al., 2015).

5.1. Classification and regulation

To better manage and provide an adequate response, pests, including plant viruses, are classified into quarantine pests, regulated non-quarantine pests (RNQPs), and priority pests. International Standards for Phytosanitary Measures (ISPMs)[10] are standards adopted by the Commission on Phytosanitary Measures (CPM), which is the governing body of the International Plant Protection Convention (IPPC)[11]. Globally, the IPPC is an intergovernmental treaty that aims to protect plants, agricultural products, and natural resources worldwide from plant pests. It comprises

[10] List of adopted standards (ISPMs) by the IPPC: https://www.ippc.int/en/core-activities/standards-setting/ispms/

[11] IPPC: https://www.ippc.int/en/

ten Regional Plant Protection Organizations (RPPOs)[12] that cooperate to promote harmonized phytosanitary measures for plant protection. RPPOs also act as the coordinating body for National Plant Protection Organizations (NPPOs)[13] on a regional level. For example, the European and Mediterranean Plant Protection Organization (EPPO)[14] is the RPPO within the European and Mediterranean region. In the European Union (EU), the European Food Safety Authority (EFSA)[15] is the organization that conducts pest risk assessments and acts as a consulting body to provide scientific advice to policy-makers to create and implement the regulations for the control and management of plant pests within and into the designated area (Figure 1-6).

At the European level, Regulation (EU) 2019/2072 and later amendments establish the list of pests, prohibitions, and requirements for plants and plant products for importation into and movement within the European Union (EU). Moreover, certain plants, plant products, and other objects must have a phytosanitary certificate upon entering the EU, guaranteeing that they are correctly inspected, free from quarantine pests (within the requirements for regulated non-quarantine pests and practically free from other pests), and in line with the plant health requirements of the EU. The exporting country's national plant protection authorities are responsible for issuing the phytosanitary certificates. Once in the EU, a plant passport replaces the phytosanitary certificate. The approach to preventing pests' introduction into the EU is described in Regulation (EU) 2016/2031, which focuses on preventive measures, including surveillance of the territory and preparation for possible outbreaks. The key aspects of this regulation are to strengthen phytosanitary import controls on plants and plant products from third countries, harmonize the model of plant passports in the EU, and extend the list of plants (intended for planting) that a plant passport must accompany.

[12] RPPO: https://www.ippc.int/en/ippc-community/regional-plant-protection-organizations/

[13] Listo f NPPOs of IPPC Contracting parties: https://www.ippc.int/en/countries/nppos/list-countries/

[14] EPPO: https://www.eppo.int/index

[15] EFSA: https://www.efsa.europa.eu/en

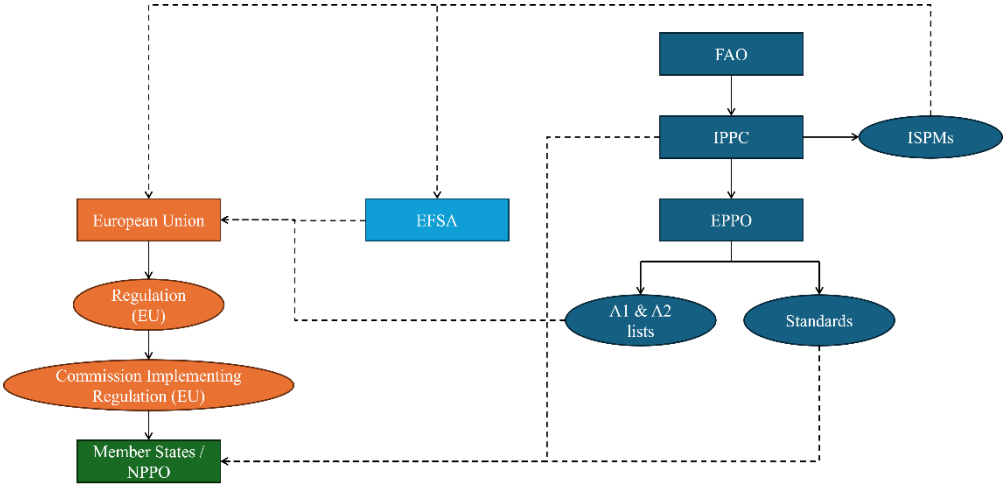

Figure 1-6. Schematic representation of the institutions and organizations involved in creating and regulating the laws and legislation related to plant health and plant protection in the European Union. Rectangular figures represent the institutions and organizations, and circles represent documents, laws, and regulations. Figures in dark blue show the organizations and documents that derive and depend on the IPPC; in orange, the organizations and documents from the European Union; in green, the member states, including their legislation; and in light blue, EFSA. Arrows with continuous lines represent the institutions and organizations that are directly linked and interacting, as well as the documents that they edit and produce. Arrows with discontinuous lines represent the institutions and organizations that communicate, give scientific advise, propose regulations, or act according to general guidelines set in the documents, such as ISPMs, for example.

5.2. Diagnostic and certification of plant material

Aside from compliance with RNQP legislation, certification serves other purposes like granting access to growers to plant material that is free from major pathogens and that has the proper varietal identification, facilitating the trade of plant material between countries albeit staying within quarantine legislation limits, contributing to commercial advantage, and leading to the collection of varietal royalties for breeding facilities.

Appropriate diagnostic techniques for virus detection and identification are essential to ensure the correct certification of plant material. Routine detection methods include visual inspection, biological indexing, microscopy, serological methods such as ELISA, and molecular methods such as PCR. Nevertheless, they are time-consuming when considering the significant number of plants and viruses to test. HTS has emerged as a viable alternative to reduce efforts and test samples on a larger scale while providing the potential to obtain a complete view of the sanitary status of a sample. Recent studies show that HTS is more inclusive and less time-consuming than biological indexing. Still, RT-PCR shows higher analytical sensitivity, although it is also restricted by the genetic diversity of the viruses, which is not an issue in HTS (Al

Rwahnih et al., 2015; Marais et al., 2024). Moreover, HTS allows the design of improved primers that are more inclusive for detecting viruses via molecular techniques.

Recently, in the United States, a strategy was developed to include HTS to fasten and reduce costs of screening and cleaning plant material that allows the provisional release and limited propagation of a selection of HTS-negative plant material in a designated area, which is followed by its official release only after completion of all bioassays and laboratory tests. If the official tests are positive, the material is destroyed. This approach allows to develop and accumulate plant material intended for commercial production before its official release, reducing the period that plants will be available to growers. The United States approved this strategy to replace biological indexing for quarantine release and certification of grapevine, *Prunus* spp., and rose by two rounds of testing by HTS and PCR after dormancy. Although not required, bioassays can also be used if preferred (FPS, 2021).

If the plant material is positive for the targeted viruses after testing, the next step is to eliminate the viruses from the infected mother propagation material to ensure that the produced material is healthy. The methods used include thermotherapy, meristem tissue culture, *in vitro* micrografting, *in vitro* chemotherapy, and cryotherapy of shoot tips followed by shoot-tip tissue culture or *in vitro* micrografting (Bettoni et al., 2024; Varveri et al., 2015).

5.3. Orchard management

Generally, pome fruit orchards are managed through IPM programs to control various diseases and pests (Jones et al., 2009). Specifically for viruses, the adopted strategy is based on the control of human-driven dissemination via certification programs and appropriate orchard structure to maintain the trees with enough spacing to avoid root grafting. Breeding for tolerance or partial resistance is another strategy to control viral diseases while maintaining commercial fruit production. The principle is that these cultivars present few symptoms, especially on fruits, after infection, thus maintaining fruit production levels (Barba et al., 2015).

Even though using certified plant material for the propagation and planting of pome fruit trees is a keystone in managing viruses in orchards, horizontal transmission can occur as well via dagger nematodes, for example, in ToRSV and CRLV, which are the causal agents of apple union necrosis and flat apple (see Chapter 1, section 2.2). Testing of soil samples for dagger nematodes can be routinely done to monitor the spread of certain viruses within the orchard. Dagger nematodes, or other nematodes for that matter, can be treated via various methods. Traditionally, a standard method to control nematodes in orchards is the use of nematicides such as fenamiphos, carbofuran, and carbosulfan (Rosenberger and Meyer, 1988). However, recent revisions of European Legislation have restricted the use of pesticides, including nematicides, in agricultural crops due to risks linked to environmental, human, and

animal health. Protection strategies against dagger nematodes may incorporate alternative approaches such as crop rotation, disinfestation using hot water and steam or soil solarization to increase the soil's temperature, and treatments using biopesticides or plant extracts (Sasanelli et al., 2021; Mulusa, 2023). Nonetheless, in fruit trees, strategies such as crop rotation or soil disinfestation and solarization are difficult to implement. Another approach would be to develop tolerant or resistant varieties to the damaging viruses (ToRSV and CRLV), given that their damage to the trees is greater than the damage caused by the feeding of the dagger nematodes, or tolerant rootstock varieties against nematodes. Moreover, there are preventive measures that can be done before planting the trees, such as the removal of roots that may be harboring nematodes or soil treatments using chemical nematicides and fumigants (DuPont et al., 2017).

Furthermore, temperate fruit tree viruses may also be transmitted by other vectors, such as insects or aphids. The most notable and studied example is Plum pox virus (PPV), which is why it is added as an example even though it is a virus affecting stone fruit trees. PPV is the causal agent of Sharka disease, and it can be transmitted by different aphid species. These aphids transmit the virus by feeding on infected leaves and then flying to healthy trees for feeding or probing. Insecticide treatments and chemical controls can be effective in controlling and limiting vector populations and preventing colonization of infected trees, especially in combination with other approaches. However, it is worth noting that, in the case of PPV and other non-persistent viruses, it does not completely prevent the spread of the virus (Barba et al., 2015). Long-distance spread from infected areas is caused when infected and uncertified planting material is used. Generally, the international movement of stone fruit trees and germplasm is regulated to prevent long-distant spread. Planting PPV-free material in orchards is helpful, as is using resistant and tolerant varieties (García et al., 2014). Regular monitoring for the detection and eradication of infected trees helps to reduce inoculum within the orchard and prevent the further spread of the virus. In recent years, there have been efforts to introduce resistance to PPV found in apricots and obtain resistance against PPV (Scorza et al., 2013), as well as to cross-protect trees with mild PPV strains by genetically engineering them to express the coat protein (CP) gene of PPV (Cambra et al., 2006).

References

Acuña, C.A., Martínez, E.J., Zilli, A.L., Brugnoli, E.A., Espinoza, F., Marcón, F., Urbani, M.H., Quarin, C.L., 2019. Reproductive Systems in Paspalum: Relevance for Germplasm Collection and Conservation, Breeding Techniques, and Adoption of Released Cultivars. Frontiers in Plant Science. https://doi.org/10.3389/fpls.2019.01377

Adams, I.P., Fox, A., Boonham, N., Massart, S., De Jonghe, K., 2018. The impact of high throughput sequencing on plant health diagnostics. European Journal of Plant Pathology 152, 909–919. https://doi.org/10.1007/s10658-018-1570-0

Agrios, G., 2004. Plant pathology: Fifth edition. Plant Pathology: Fifth Edition 1–922.

Al Rwahnih, M., Daubert, S., Golino, D., Islas, C., Rowhani, A., 2015. Comparison of Next-Generation Sequencing Versus Biological Indexing for the Optimal Detection of Viral Pathogens in Grapevine. https://doi.org/10.1094/PHYTO-06-14-0165-R

Allen, W.R., 1969. Ocurrence and seed transmission of Tomato bushy stunt virus in apple. Can. J. Plant Sci. 49, 797–799. https://doi.org/10.4141/cjps69-135

Aramburu, J., Rovira, M., 2000. Incidence and natural spread of apple mosaic ilarvirus in hazel in north-east Spain. Plant Pathology 49, 423–427. https://doi.org/10.1046/j.1365-3059.2000.00477.x

Astolfi, P., Velho, A.C., Moreira, V., Mondino, P.E., Alaniz, S.M., Stadnik, M.J., 2022. Reclassification of the Main Causal Agent of Glomerella Leaf Spot on Apple into Colletotrichum chrysophilum in Southern Brazil and Uruguay. Phytopathology® 112, 1825–1832. https://doi.org/10.1094/PHYTO-12-21-0527-SC

Babayan, S.A., Orton, R.J., Streicker, D.G., 2018. Predicting reservoir hosts and arthropod vectors from evolutionary signatures in RNA virus genomes. Science 362, 577–580. https://doi.org/10.1126/science.aap9072

Baek, D., Lim, S., Ju, H.-J., Kim, H.-R., Lee, S.-H., Moon, J.S., 2019. The complete genome sequence of apple rootstock virus A, a novel nucleorhabdovirus identified in apple rootstocks. Arch Virol 164, 2641–2644. https://doi.org/10.1007/s00705-019-04348-0

Baltimore, D., 1971. Expression of animal virus genomes. Bacteriological reviews 35, 235–241. https://doi.org/10.1128/mmbr.35.3.235-241.1971

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., Pyshkin, A.V., Sirotkin, A.V., Vyahhi, N., Tesler, G., Alekseyev, M.A., Pevzner, P.A., 2012. SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. J Comput Biol 19, 455–477. https://doi.org/10.1089/cmb.2012.0021

Barba, M., Ilardi, V., Pasquini, G., 2015. Control of Pome and Stone Fruit Virus Diseases. Advances in Virus Research 91, 47–83. https://doi.org/10.1016/BS.AIVIR.2014.11.001

Basso, M.F., da Silva, J.C.F., Fajardo, T.V.M., Fontes, E.P.B., Zerbini, F.M., 2015. A novel, highly divergent ssDNA virus identified in Brazil infecting apple, pear and grapevine. Virus Research 210, 27–33. https://doi.org/10.1016/j.virusres.2015.07.005

Bettoni, J.C., Wang, M.-R., Li, J.-W., Fan, X., Fazio, G., Hurtado-Gonzales, O.P., Volk, G.M., Wang, Q.-C., 2024. Application of Biotechniques for In Vitro Virus and Viroid Elimination in Pome Fruit Crops. Phytopathology® PHYTO-07-23-0232-KC. https://doi.org/10.1094/PHYTO-07-23-0232-KC

Blomquist, C. L., & Kirkpatrick, B. C. (2002). Frequency and Seasonal Distribution of Pear Psylla Infected with the Pear Decline Phytoplasma in California Pear Orchards. Phytopathology®, 92(11), 1218-1226. https://doi.org/10.1094/PHYTO.2002.92.11.1218

Bovey, R., 1989. La defensa de las plantas cultivadas, 2nd ed. Ediciones Omega, S.A., Barcelona.

Bradford, F.C., Joly, L., 1933. Infectious variegation in the apple. J. Agr. Res. 46, 901–908.

Bragard, C., Caciagli, P., Lemaire, O., Lopez-Moya, J.J., MacFarlane, S., Peters, D., Susi, P., Torrance, L., 2013. Status and Prospects of Plant Virus Control Through Interference with Vector Transmission. Annual Review of Phytopathology 51, 177–201. https://doi.org/10.1146/annurev-phyto-082712-102346

CABI. (2021). Phytoplasma mali (apple proliferation). CABI Compendium, CABI Compendium, 6502. https://doi.org/10.1079/cabicompendium.6502

Cabrefiga, J., Pizà, D., Vilardell, P., Luque, J., 2022a. First Report of Colletotrichum chrysophilum Causing Apple Bitter Rot in Spain. Plant Disease 106, 1752. https://doi.org/10.1094/PDIS-07-21-1578-PDN

Çağlayan, K., Serçe, Ç., Gazel, M., Jelkmann, W., 2006. Detection of Four Apple Viruses by ELISA and RT-PCR Assays in Turkey. Turkish Journal of Agriculture and Forestry 30, 241–246. https://doi.org/-

Cambra, M., Capote, N., Cambra, M.A., Llácer, G., Botella, P., López-Quílez, A., 2006. Epidemiology of sharka disease in Spain. EPPO Bulletin 36, 271–275. https://doi.org/10.1111/j.1365-2338.2006.00986.x

Casals, C., Torres, R., Teixidó, N., De Cal, A., Segarra, J., Usall, J., 2022. Brown rot on stone fruit: From epidemiology studies to the development of effective control strategies. Scientia Horticulturae 301, 111096. https://doi.org/10.1016/j.scienta.2022.111096

Chaloner, T.M., Gurr, S.J., Bebber, D.P., 2021. Plant pathogen infection risk tracks global crop yields under climate change. Nat. Clim. Chang. 11, 710–715. https://doi.org/10.1038/s41558-021-01104-8

Chandel, V., Rana, T., Handa, A., Thakur, P.D., Hallan, V., Zaidi, A.A., 2008. Incidence of Prunus necrotic ring spot virus on Malus domestica in India. Journal of Phytopathology 156, 382–384. https://doi.org/10.1111/j.1439-0434.2007.01361.x

Chane, T., Boyraz, N., 2017. Critical Review on Apple Scab (Venturia inaequalis) Biology, Epidemiology, Economic Importance, Management and Defense Mechanisms to the Causal Agent 5. https://doi.org/10.4172/2329-955X.1000166

Cho, I.-S., Igori, D., Lim, S., Choi, G.-S., Hammond, J., Lim, H.-S., Moon, J.S., 2016. Deep Sequencing Analysis of Apple Infecting Viruses in Korea. Plant Pathol J 32, 441–451. https://doi.org/10.5423/PPJ.OA.04.2016.0104

Costa, L.C., Hu, X., Malapi-Wight, M., Foster, J., McFarland, C., Hurtado-Gonzales, O.P., 2022. Identification of a novel robigovirus and a Prunus-infecting tepovirus in Pyrus communis and their transmissibility on Malus spp. Eur J Plant Pathol 162, 275–288. https://doi.org/10.1007/s10658-021-02402-9

Darbyshire, R., Webb, L., Goodwin, I., Barlow, S., 2011. Winter chilling trends for deciduous fruit trees in Australia. Agricultural and Forest Meteorology 151, 1074–1085. https://doi.org/10.1016/j.agrformet.2011.03.010

Desvignes, J.-C., 2004. Maladies à virus des arbres fruitiers. Centre Technique Interprofessionnel Des Fruits Et Légumes - Ctifl.

Di Serio, F., Malfitano, M., Alioto, D., Ragozzino, A., Desvignes, J.C., Flores, R., 2001. Apple dimple fruit viroid: Fulfillment of Koch's Postulates and Symptom Characteristics. Plant Disease 85, 179–182. https://doi.org/10.1094/PDIS.2001.85.2.179

Dijkstra, J., de Jager, C.P., 1998. Chloroplast Agglutination Test, in: Dijkstra, J., de Jager, C.P. (Eds.), Practical Plant Virology: Protocols and Exercises, Springer Lab Manual. Springer, Berlin, Heidelberg, pp. 333–335. https://doi.org/10.1007/978-3-642-72030-7_53

Doekes, H.P., Veerkamp, R.F., Bijma, P., Hiemstra, S.J., Windig, J., 2018. Value of the Dutch Holstein Friesian germplasm collection to increase genetic variability and improve genetic merit. Journal of Dairy Science 101, 10022–10033. https://doi.org/10.3168/jds.2018-15217

DuPont, T., Mazzola, M., & Ferris, H. (2017). Plant-Parasitic Nematodes in Pome and Stone Fruit Orchards: Biology and Management in Washington. WSU Tree Fruit. https://treefruit.wsu.edu/crop-protection/nematodes-2/

Dursunoglu, S., Ertunc, F., 2008. Distribution of apple mosaic ilarvirus (ApMV) in Turkey. Acta Hortic. 781, 131–134. https://doi.org/10.17660/ActaHortic.2008.781.19

Edgar, R.C., Taylor, J., Lin, V., Altman, T., Barbera, P., Meleshko, D., Lohr, D., Novakovsky, G., Buchfink, B., Al-Shayeb, B., Banfield, J.F., de la Peña, M., Korobeynikov, A., Chikhi, R., Babaian, A., 2022. Petabase-scale sequence alignment catalyses viral discovery. Nature 602, 142–147. https://doi.org/10.1038/s41586-021-04332-2

El Yaacoubi, A., El Jaouhari, N., Bourioug, M., El Youssfi, L., Cherroud, S., Bouabid, R., Chaoui, M., Abouabdillah, A., 2020. Potential vulnerability of Moroccan apple orchard to climate change–induced phenological perturbations: effects on yields and fruit quality. Int J Biometeorol 64, 377–387. https://doi.org/10.1007/s00484-019-01821-y

Elnifro, E.M., Ashshi, A.M., Cooper, R.J., Klapper, P.E., 2000. Multiplex PCR: Optimization and Application in Diagnostic Virology. Clinical Microbiology Reviews 13, 559–570. https://doi.org/10.1128/cmr.13.4.559

Engvall, E., Perlmann, P., 1971. Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. Immunochemistry 8, 871–874. https://doi.org/10.1016/0019-2791(71)90454-X

Fadón, E., Herrera, S., Guerrero, B.I., Guerra, M.E., Rodrigo, J., 2020. Chilling and Heat Requirements of Temperate Stone Fruit Trees (Prunus sp.). Agronomy 10, 409. https://doi.org/10.3390/agronomy10030409

Fereres, A., Raccah, B., 2015. Plant Virus Transmission by Insects, in: Encyclopedia of Life Sciences. John Wiley & Sons, Ltd (Ed.). https://doi.org/10.1002/9780470015902.a0000760.pub3

Filloux, D., Dallot, S., Delaunay, A., Galzi, S., Jacquot, E., Roumagnac, P., 2015. Metagenomics Approaches Based on Virion-Associated Nucleic Acids (VANA): An Innovative Tool for Assessing Without A Priori Viral Diversity of Plants, in: Lacomme, C. (Ed.), Plant Pathology: Techniques and Protocols, Methods in Molecular Biology. Springer, New York, NY, pp. 249–257. https://doi.org/10.1007/978-1-4939-2620-6_18

Flores, R., Llácer, G., 1988. Isolation of a viroid-like RNA associated with peach latent mosaic disease. Acta Horticulturae 235, 325–332. https://doi.org/10.17660/ActaHortic.1989.235.47

Fontdevila Pareta, N., Gailly, C., Blouin, A.G., Buchmann, B., Buenter, M., Candresse, T., Dubuis, N., Kutnjak, D., Lateur, M., Pecman, A., Steyer, S., Massart, S., in publication. Virome scanning of pear germplasm collections identifies a new Velarivirus and extends the geographical spread of three other pear viruses. Phytobiomes Journal.

Forer, L.B., Powell, C.A., Stouffer, R.F., 1984. Transmission of Tomato Ringspot Virus to Apple Rootstock Cuttings and to Cherry and Peach Seedlings by Xiphinema rivesi. Plant Disease 68, 1052–1054.

FPS, 2021. FPS Receives Approval for HTS- and PCR-Based Diagnostic Protocols for Quarantine Release and Certification of Grapevine, Prunus and Roses [WWW Document]. Foundation Plant Services. URL https://fps.ucdavis.edu/newsarticle.cfm?newsid=83 (accessed 3.19.24).

Gaafar, Y.Z.A., Ziebell, H., 2020. Comparative study on three viral enrichment approaches based on RNA extraction for plant virus/viroid detection using high-throughput sequencing. PLOS ONE 15, e0237951. https://doi.org/10.1371/journal.pone.0237951

García, J.A., Glasa, M., Cambra, M., Candresse, T., 2014. Plum pox virus and sharka: a model potyvirus and a major disease. Molecular Plant Pathology 15, 226–241. https://doi.org/10.1111/mpp.12083

Garnsey, S.M., Permar, T.A., Cambra, M., Henderson, C.T., 1993. Direct Tissue Blot Immunoassay (DTBIA) for Detection of Citrus Tristeza Virus (CTV). International Organization of Citrus Virologists Conference Proceedings (1957-2010) 12. https://doi.org/10.5070/C55t45z8fb

Garrido-Cardenas, J.A., Garcia-Maroto, F., Alvarez-Bermejo, J.A., Manzano-Agugliaro, F., 2017. DNA Sequencing Sensors: An Overview. Sensors (Basel) 17, 588. https://doi.org/10.3390/s17030588

Gergerich, R.C., Welliver, R.A., Gettys, S., Osterbauer, N.K., Kamenidou, S., Martin, R.R., Golino, D.A., Eastwell, K., Fuchs, M., Vidalakis, G., Tzanetakis, I.E., 2015. Safeguarding Fruit Crops in the Age of Agricultural Globalization. Plant Disease 99, 176–187. https://doi.org/10.1094/PDIS-07-14-0762-FE

Giraud, M., Bompeix, G., 2012. Postharvest diseases of pome fruits in Europe: perspectives for integrated control. IOBC-WPRS Bulletin 84, 257–263.

Glasa, M., Šoltys, K., Vozárová, Z., Predajňa, L., Sihelská, N., Šubr, Z., Candresse, T., 2017. High intra-host cherry virus a population heterogeneity in cherry trees in Slovakia. Journal of Plant Pathology 99, 745–752. https://doi.org/10.4454/JPP.V99I3.3947

Glökler, J., Lim, T.S., Ida, J., Frohme, M., 2021. Isothermal amplifications – a comprehensive review on current methods. Critical Reviews in Biochemistry and Molecular Biology 56, 543–586. https://doi.org/10.1080/10409238.2021.1937927

Goodwin, S., McPherson, J.D., McCombie, W.R., 2016. Coming of age: ten years of next-generation sequencing technologies. Nat Rev Genet 17, 333–351. https://doi.org/10.1038/nrg.2016.49

Gorbalenya, A.E., Krupovic, M., Mushegian, A., Kropinski, A.M., Siddell, S.G., Varsani, A., Adams, M.J., Davison, A.J., Dutilh, B.E., Harrach, B., Harrison, R.L., Junglen, S., King, A.M.Q., Knowles, N.J., Lefkowitz, E.J., Nibert, M.L., Rubino, L., Sabanadzovic, S., Sanfaçon, H., Simmonds, P., Walker, P.J., Zerbini, F.M., Kuhn, J.H., International Committee on Taxonomy of Viruses Executive Committee, 2020.

The new scope of virus taxonomy: partitioning the virosphere into 15 hierarchical ranks. Nature Microbiology 5, 668–674. https://doi.org/10.1038/s41564-020-0709-x

Gorbalenya, A.E., Lauber, C., 2017. Phylogeny of Viruses. Reference Module in Biomedical Sciences B978-0-12-801238-3.95723-4. https://doi.org/10.1016/B978-0-12-801238-3.95723-4

Grimová, L., Winkowska, L., Konrady, M., Ryšánek, P., 2016. Apple mosaic virus. Phytopathologia Mediterranea 55, 1–19.

Grüntzig, M., Fuchs, E., Hentsch, T., 1996. Occurrence and serological detection of cherry leaf roll nepovirus (CLRV) and apple mosaic ilarvirus (ApMV) in Betula spp. J. Plant Dis. Prot. 103, 571–581.

Guédon, Y., Legave, J.M., 2008. Analyzing the time-course variation of apple and pear tree dates of flowering stages in the global warming context. Ecological Modelling 219, 189–199. https://doi.org/10.1016/j.ecolmodel.2008.08.010

Guengerich, H.W., Millikan, D.F., 1959. Reaction of ownrooted trees of spy 227 and Virginia Crab to infection with the stem pitting virus. Plant Dis. Rep. 30–31.

Guo, L., Dai, J., Ranjitkar, S., Yu, H., Xu, J., Luedeling, E., 2014. Chilling and heat requirements for flowering in temperate fruit trees. Int J Biometeorol 58, 1195–1206. https://doi.org/10.1007/s00484-013-0714-3

Hadidi, A., Barba, M., Candresse, T., Jelkmann, W., 2011. Virus and Virus-Like Diseases of Pome and Stone Fruits, Virology. The American Phytopathological Society. https://doi.org/10.1094/9780890545010

Halbrendt,John M., 9781789247541.0029, CABI, doi:10.1079/9781789247541.0029, (207–214), CABI, A threat to stone fruit and grape production: tomato ringspot virus (ToRSV) transmission by X. americanum s.l. (sensu lato)., (2021)

Harrison, B.D., 2009. A Brief Outline of the Development of Plant Virology in the 20 Th Century. Journal of Plant Pathology 91, 509–520.

Harrison, B.D., Wilson, T.M., 1999. Milestones in the research on tobacco mosaic virus. Philos Trans R Soc Lond B Biol Sci 354, 521–529. https://doi.org/10.1098/rstb.1999.0403

Hernández, C., Elena, S.F., Moya, A., Flores, R., 1992. Pear Blister Canker Viroid is a Member of the Apple Scar Skin Subgroup (apscaviroids) and also has Sequence Homology with Viroids from other Subgroups. Journal of General Virology 73, 2503–2507. https://doi.org/10.1099/0022-1317-73-10-2503

Hoyt, S.C., Burts, E.C., 1974. Integrated Control of Fruit Pests. Annual Review of Entomology 19, 231–252. https://doi.org/10.1146/annurev.en.19.010174.001311

Hu, T., Chitnis, N., Monos, D., Dinh, A., 2021. Next-generation sequencing technologies: An overview. Human Immunology, Next Generation Sequencing and its Application to Medical Laboratory Immunology 82, 801–811. https://doi.org/10.1016/j.humimm.2021.02.012

Hull, R., 2014. Plant Virology, Fifth. ed. Academic Press, Boston. https://doi.org/10.1016/B978-0-12-384871-0.00001-7

Hunter, J.A., Chamberlain, E.E., Atkinson, J.D., 1958. Note on transmission of apple mosaic by natural root grafting. New Zealand Journal of Agricultural Research 1, 80–82. https://doi.org/10.1080/00288233.1958.10422062

Ito, T., Kanematsu, S., Koganezawa, H., Tsuchizaki, T., Yoshida, K., 1993. Detection of a Viroid Associated with Apple Fruit Crinkle Disease. Japanese Journal of Phytopathology 59, 520–527. https://doi.org/10.3186/jjphytopath.59.520

Jakovljevic, V., Otten, P., Berwarth, C., and Jelkmann, W. 2017. Analysis of the apple rubbery wood disease by next generation sequencing of total RNA. Eur J Plant Pathol 148, 637–646. https://doi.org/10.1007/s10658-016-1119-z

James, D., Howell, W.E., and Mink, G.I. 2001. Molecular evidence of the relationship between a virus associated with flat apple disease and *Cherry rasp leaf virus* as determined by RT-PCR. Plant Dis. 85:47-52.

James, D., Varga, A., Croft, H., 2007. Analysis of the complete genome of peach chlorotic mottle virus: identification of non-AUG start codons, in vitro coat protein expression, and elucidation of serological cross-reactions. Arch Virol 152, 2207–2215. https://doi.org/10.1007/s00705-007-1050-x

James, D., Varga, A., Jesperson, G.D., Navratil, M., Safarova, D., Constable, F., Horner, M., Eastwell, K., Jelkmann, W., 2013. Identification and complete genome analysis of a virus variant or putative new foveavirus associated with apple green crinkle disease. Arch Virol 158, 1877–1887. https://doi.org/10.1007/s00705-013-1678-7

Jones, V.P., Unruh, T.R., Horton, D.R., Mills, N.J., Brunner, J.F., Beers, E.H., Shearer, P.W., 2009. Tree fruit IPM programs in the western United States: the challenge of enhancing biological control through intensive management. Pest Management Science 65, 1305–1310. https://doi.org/10.1002/ps.1839

Jridi, C., Martin, J.-F., Marie-Jeanne, V., Labonne, G., Blanc, S., 2006. Distinct Viral Populations Differentiate and Evolve Independently in a Single Perennial Host Plant. Journal of Virology 80, 2349–2357. https://doi.org/10.1128/jvi.80.5.2349-2357.2006

Judd, W.S., Campbell, C.S., Kellogg, E., Stevens, P.F., Donoghue, J., 2002. Plant Systematics: A Phylogenetic Approach, 2nd ed, Systematic Biology. Oxford University Press (OUP), Sunderland, MA. https://doi.org/10.1080/10635150490445878

Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Žídek, A., Potapenko, A., Bridgland, A., Meyer, C., Kohl, S.A.A., Ballard, A.J., Cowie, A., Romera-Paredes, B., Nikolov, S., Jain, R., Adler, J., Back, T., Petersen, S., Reiman, D., Clancy, E., Zielinski, M., Steinegger, M., Pacholska, M., Berghammer, T., Bodenstein, S., Silver, D., Vinyals, O., Senior, A.W., Kavukcuoglu, K., Kohli, P., Hassabis, D., 2021. Highly accurate protein structure prediction with AlphaFold. Nature 596, 583–589. https://doi.org/10.1038/s41586-021-03819-2

Kegler, H., Kleinhempel, H. and Verderevskaja, T.D. (1976). Investigations on Pear stony pit virus. Acta Hortic. 67, 209-218. doi: 10.17660/ActaHortic.1976.67.26

Khan, A.R., Pervez, M.T., Babar, M.E., Naveed, N., Shoaib, M., 2018. A Comprehensive Study of De Novo Genome Assemblers: Current Challenges and Future Prospective. Evol Bioinform Online 14, 1176934318758650. https://doi.org/10.1177/1176934318758650

King, A.M.Q., Lefkowitz, E., Adams, M.J., Carstens, E.B. (Eds.), 2011. Virus taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses, First. ed. Elsevier, Amsterdam.

Kirkpatrick, H.C., Lindner, R.C., 1964. Recovery of Tobacco mosaic virus from apple. Plant Dis. Rep. 48, 855–857.

Koczula, K.M., Gallotta, A., 2016. Lateral flow assays. Essays Biochem 60, 111–120. https://doi.org/10.1042/EBC20150012

Koganezawa, H., 1986. Further evidence for viroid etiology of apple scar skin and dapple apple diseases. Acta Hortic. 193, 29–33. https://doi.org/10.17660/ActaHortic.1986.193.2

Kole, C., Muthamilarasan, M., Henry, R., Edwards, D., Sharma, R., Abberton, M., Batley, J., Bentley, A., Blakeney, M., Bryant, J., Cai, H., Cakir, M., Cseke, L.J., Cockram, J., Oliveira, A.C., Pace, C.D., Dempewolf, H., Ellison, S., Gepts, P., Greenland, A., Hall, A., Hori, K., Hughes, S., Humphreys, M.W., Iorizzo, M., Ismail, A.M., Marshall, A., Mayes, S., Nguyen, H.T., Ogbonnaya, F.C., Ortiz, R., Paterson, A.H., Simon, P.W., Tohme, J., Tuberosa, R., Valliyodan, B., Varshney, R.K., Wullschleger, S.D., Yano, M., Prasad, M., 2015. Application of genomics-assisted breeding for generation of climate resilient crops: progress and prospects. Frontiers in Plant Science 6.

Koloniuk, I., Přibylová, J., Fránová, J., Špak, J., 2020. Genomic characterization of Malus domestica virus A (MdoVA), a novel velarivirus infecting apple. Arch Virol 165, 479–482. https://doi.org/10.1007/s00705-019-04478-5

Kreuze, J.F., Perez, A., Untiveros, M., Quispe, D., Fuentes, S., Barker, I., Simon, R., 2009. Complete viral genome sequence and discovery of novel viruses by deep sequencing of small RNAs: A generic method for diagnosis, discovery and sequencing of viruses. Virology 388, 1–7. https://doi.org/10.1016/j.virol.2009.03.024

Kuhn, J.H., 2021. Virus Taxonomy. Encyclopedia of Virology 28–37. https://doi.org/10.1016/B978-0-12-809633-8.21231-4

Kumar, P.L., Cuervo, M., Kreuze, J.F., Muller, G., Kulkarni, G., Kumari, S.G., Massart, S., Mezzalama, M., Alakonya, A., Muchugi, A., Graziosi, I., Ndjiondjop, M.-N., Sharma, R., Negawo, A.T., 2021. Phytosanitary Interventions for Safe Global Germplasm Exchange and the Prevention of Transboundary Pest Spread: The Role of CGIAR Germplasm Health Units. Plants 10, 328. https://doi.org/10.3390/plants10020328

Kurokura, T., Mimida, N., Battey, N.H., Hytönen, T., 2013. The regulation of seasonal flowering in the Rosaceae. Journal of Experimental Botany 64, 4131–4141. https://doi.org/10.1093/jxb/ert233

Kutnjak, D., Tamisier, L., Adams, I., Boonham, N., Candresse, T., Chiumenti, M., De Jonghe, K., Kreuze, J.F., Lefebvre, M., Silva, G., Malapi-Wight, M., Margaria, P., Mavrič Pleško, I., McGreig, S., Miozzi, L., Remenant, B., Reynard, J.-S., Rollin, J., Rott, M., Schumpp, O., Massart, S., Haegeman, A., 2021. A Primer on the Analysis of High-Throughput Sequencing Data for Detection of Plant Viruses. Microorganisms 9, 841. https://doi.org/10.3390/microorganisms9040841

Lana, A.F., Peterson, J.F., Rouselle, G.L., Vrain, T.C., 1983. Association of Tobacco Ringspot Virus with a Union Incompatibility of Apple. Journal of Phytopathology 106, 141–148. https://doi.org/10.1111/j.1439-0434.1983.tb00037.x

Lebas, B., Adams, I., Al Rwahnih, M., Baeyen, S., Bilodeau, G.J., Blouin, A.G., Boonham, N., Candresse, T., Chandelier, A., De Jonghe, K., Fox, A., Gaafar, Y.Z.A., Gentit, P., Haegeman, A., Ho, W., Hurtado-Gonzales, O., Jonkers, W., Kreuze, J., Kutjnak, D., Landa, B., Liu, M., Maclot, F., Malapi-Wight, M., Maree, H.J., Martoni, F., Mehle, N., Minafra, A., Mollov, D., Moreira, A., Nakhla, M., Petter, F., Piper, A.M., Ponchart, J., Rae, R., Remenant, B., Rivera, Y., Rodoni, B., Roenhorst, J.W., Rollin, J., Saldarelli, P., Santala, J., Souza-Richards, R., Spadaro, D., Studholme, D.J.,

Sultmanis, S., van der Vlugt, R., Tamisier, L., Trontin, C., Vazquez-Iglesias, I., Vicente, C.S.L., Vossenberg, B.T.L.H., Wetzel, T., Ziebell, H., Massart, S., 2022. Facilitating the adoption of high-throughput sequencing technologies as a plant pest diagnostic test in laboratories: A step-by-step description. EPPO Bulletin 52, 394–418. https://doi.org/10.1111/epp.12863

Lecoq, H., 2001. Découverte du premier virus, le virus de la mosaïque du tabac: 1892 ou 1898? Comptes Rendus de l'Académie des Sciences - Series III - Sciences de la Vie 324, 929–933. https://doi.org/10.1016/S0764-4469(01)01368-3

Lee SY, Peter KA, Das K, Diane AR, Jung HY. The Rapid Apple Decline Phenomenon: Current Status and Expected Associated Factors in Korea. Plant Pathol J. 2023 Dec;39(6):538-547. doi: 10.5423/PPJ.RW.09.2023.0132. Epub 2023 Dec 1. PMID: 38081314; PMCID: PMC10721390.

Leichtfried, T., Dobrovolny, S., Reisenzein, H., Steinkellner, S., Gottsberger, R.A., 2019. Apple chlorotic fruit spot viroid: a putative new pathogenic viroid on apple characterized by next-generation sequencing. Arch Virol 164, 3137–3140. https://doi.org/10.1007/s00705-019-04420-9

Leone, G., Lindner, J.L., van der Meer, F.A., Schoen, C.D. and Jongedijk, G. (1998). Symptoms on apple and pear indicators after back-transmission from nicotiana occidentalis confirm the identity of apple stem pitting virus with pear vein yellows virus. Acta Hortic. 472, 61-66. doi: 10.17660/ActaHortic.1998.472.4

Lesnaw, J.A., Ghabrial, S.A., 2000. Tulip Breaking: Past, Present, and Future. Plant Disease 84, 1052–1060. https://doi.org/10.1094/PDIS.2000.84.10.1052

Li, C., Yoshikawa, N., Takahashi, T., Ito, T., Yoshida, K., Koganezawa, H., 2000. Nucleotide sequence and genome organization of Apple latent spherical virus: a new virus classified into the family Comoviridae. Journal of General Virology 81, 541–547. https://doi.org/10.1099/0022-1317-81-2-541

Li C, Yaegashi H, Kishigami R, Kawakubo A, Yamagishi N, Ito T and Yoshikawa N (2020) Apple Russet Ring and Apple Green Crinkle Diseases: Fulfillment of Koch's Postulates by Virome Analysis, Amplification of Full-Length cDNA of Viral Genomes, in vitro Transcription of Infectious Viral RNAs, and Reproduction of Symptoms on Fruits of Apple Trees Inoculated With Viral RNAs. Front. Microbiol. 11:1627. doi: 10.3389/fmicb.2020.01627

Li, J., Zhang, S., Li, B., Hu, Y., Kang, X.-P., Wu, X.-Y., Huang, M.-T., Li, Y.-C., Zhao, Z.-P., Qin, C.-F., Jiang, T., 2020. Machine Learning Methods for Predicting Human-Adaptive Influenza A Viruses Based on Viral Nucleotide Compositions. Molecular Biology and Evolution 37, 1224–1236. https://doi.org/10.1093/molbev/msz276

Liang, P., Navarro, B., Zhang, Z., Wang, H., Lu, M., Xiao, H., Wu, Q., Zhou, X., Di Serio, F., Li, S., 2015. Identification and characterization of a novel geminivirus with a monopartite genome infecting apple trees. Journal of General Virology 96, 2411–2420. https://doi.org/10.1099/vir.0.000173

Lister, R.M., Bancroft, J.B., Nadakavukaren, M., 1965. Some sap-transmissible viruses from apple. Phytopathology 55, 859–870.

Liu, D., Zhang, D., Liu, G., Hussain, S., Teng, Y., 2013. Influence of heat stress on leaf ultrastructure, photosynthetic performance, and ascorbate peroxidase gene expression of two pear cultivars (Pyrus pyrifolia). J. Zhejiang Univ. Sci. B 14, 1070–1083. https://doi.org/10.1631/jzus.B1300094

Liu, H., Wang, G., Yang, Z., Wang, Y., Zhang, Z., Li, L., Waqas, M., Hong, N., Liu, H., Wang, G., Hong, N., Hong, J., Zhang, J., Xu, L., Qi, L., 2020. Identification and Characterization of a Pear Chlorotic Leaf Spot-Associated Virus, a Novel Emaravirus Associated with a Severe Disease of Pear Trees in China. Plant Dis 104, 2786–2798. https://doi.org/10.1094/PDIS-01-20-0040-RE

Liu, H., Wu, L., Nikolaeva, E., Peter, K., Liu, Z., Mollov, D., Cao, M., Li, R., 2018. Characterization of a new apple luteovirus identified by high-throughput sequencing. Virol J 15, 85. https://doi.org/10.1186/s12985-018-0998-3

Lloret, A., Quesada-Traver, C., Ríos, G., 2022. Models for a molecular calendar of bud-break in fruit trees. Scientia Horticulturae 297, 110972. https://doi.org/10.1016/j.scienta.2022.110972

Lodish, H., Berk, A., Kaiser, C.A., Krieger, M., Bretscher, A., Ploegh, H., Amon, A., Martin, K.C., 2016. Molecular Genetic Techniques, in: Molecular Cell Biology. W. H. Freeman and Company, New York, NY, pp. 223–270.

Lopez, G., Hossein Behboudian, M., Girona, J., Marsal, J., 2012. Drought in Deciduous Fruit Trees: Implications for Yield and Fruit Quality, in: Aroca, R. (Ed.), Plant Responses to Drought Stress: From Morphological to Molecular Features. Springer, Berlin, Heidelberg, pp. 441–459. https://doi.org/10.1007/978-3-642-32653-0_17

Luckwill, L.C., Campbell, A.I., 1959. Malus Platycarpa as an Apple Virus Indicator. Journal of Horticultural Science 34, 248–252. https://doi.org/10.1080/00221589.1959.11513965

Mabberley, D.J., 1997. The Plant-Book. A portable dictionary of the vascular plants., 2nd ed, Feddes Repertorium. Cambridge University Press, Cambridge, New York, Melbourne. https://doi.org/10.1002/fedr.19981090507

Maclot, F., Candresse, T., Filloux, D., Malmstrom, C.M., Roumagnac, P., van der Vlugt, R., Massart, S., 2020. Illuminating an Ecological Blackbox: Using High Throughput Sequencing to Characterize the Plant Virome Across Scales. Frontiers in Microbiology 11, 2575. https://doi.org/10.3389/fmicb.2020.578064

Maclot, F.J., Debue, V., Blouin, A.G., Fontdevila-Pareta, N., Tamisier, L., Filloux, D., Massart, S., 2021. Identification, molecular and biological characterization of two novel secovirids in wild grass species in Belgium. Virus Research 198397. https://doi.org/10.1016/j.virusres.2021.198397

Mantere, T., Kersten, S., Hoischen, A., 2019. Long-Read Sequencing Emerging in Medical Genetics. Front. Genet. 10. https://doi.org/10.3389/fgene.2019.00426

Manzoor, S., Nabi, S.U., Baranwal, V.K., Verma, M.K., Parveen, S., Rather, T.R., Raja, W.H., Shafi, M., 2023. Overview on century progress in research on mosaic disease of apple (*Malus domestica* Borkh) incited by apple mosaic virus/apple necrotic mosaic virus. Virology 587, 109846. https://doi.org/10.1016/j.virol.2023.109846

Marais, A., Faure, C., Bergey, B., Candresse, T., 2018. Viral Double-Stranded RNAs (dsRNAs) from Plants: Alternative Nucleic Acid Substrates for High-Throughput Sequencing, in: Pantaleo, V., Chiumenti, M. (Eds.), Viral Metagenomics: Methods and Protocols, Methods in Molecular Biology. Springer, New York, NY, pp. 45–53. https://doi.org/10.1007/978-1-4939-7683-6_4

Marais, A., Gentit, P., Brans, Y., Renvoisé, J.P., Faure, C., Saison, A., Cousseau, P., Castaing, J., Chambon, F., Pion, A., Calado, G., Lefebvre, M., Garnier, S., Latour, F., Bresson, K., Grasseau, N., Candresse, T., 2024. Comparative performance evaluation of double-stranded RNA high-throughput sequencing for the detection of viral infection

in temperate fruit crops. Phytopathology. https://doi.org/10.1094/PHYTO-12-23-0480-R

Marconi, G., Ferradini, N., Russi, L., Concezzi, L., Veronesi, F., Albertini, E., 2018. Genetic characterization of the apple germplasm collection in central Italy: The value of local varieties. Frontiers in Plant Science 9. https://doi.org/10.3389/fpls.2018.01460

Martini, C., Mari, M., 2014. Monilinia fructicola, Monilinia laxa (Monilinia Rot, Brown Rot), in: Bautista-Baños, S. (Ed.), Postharvest Decay. Academic Press, San Diego, pp. 233–265. https://doi.org/10.1016/B978-0-12-411552-1.00007-7

Massart, S., Adams, I., Al Rwahnih, M., Baeyen, S., Bilodeau, G.J., Blouin, A.G., Boonham, N., Candresse, T., Chandellier, A., De Jonghe, K., Fox, A., Gaafar, Y.Z.A., Gentit, P., Haegeman, A., Ho, W., Hurtado-Gonzales, O., Jonkers, W., Kreuze, J., Kutjnak, D., Landa, B.B., Liu, M., Maclot, F., Malapi-Wight, M., Maree, H.J., Martoni, F., Mehle, N., Minafra, A., Mollov, D., Moreira, A.G., Nakhla, M., Petter, F., Piper, A.M., Ponchart, J.P., Rae, R., Remenant, B., Rivera, Y., Rodoni, B., Botermans, M., Roenhorst, J.W., Rollin, J., Saldarelli, P., Santala, J., Souza-Richards, R., Spadaro, D., Studholme, D.J., Sultmanis, S., van der Vlugt, R., Tamisier, L., Trontin, C., Vazquez-Iglesias, I., Vicente, C.S.L., van de Vossenberg, B.T.L.H., Westenberg, M., Wetzel, T., Ziebell, H., Lebas, B.S.M., 2022. Guidelines for the reliable use of high throughput sequencing technologies to detect plant pathogens and pests. Peer Community Journal 2. https://doi.org/10.24072/pcjournal.181

Massart, S., Chiumenti, M., De Jonghe, K., Glover, R., Haegeman, A., Koloniuk, I., Komínek, P., Kreuze, J., Kutnjak, D., Lotos, L., Maclot, F., Maliogka, V., Maree, H.J., Olivier, T., Olmos, A., Pooggin, M.M., Reynard, J.-S., Ruiz-García, A.B., Safarova, D., Schneeberger, P.H.H., Sela, N., Turco, S., Vainio, E.J., Varallyay, E., Verdin, E., Westenberg, M., Brostaux, Y., Candresse, T., 2019. Virus Detection by High-Throughput Sequencing of Small RNAs: Large-Scale Performance Testing of Sequence Analysis Strategies. Phytopathology® 109, 488–497. https://doi.org/10.1094/PHYTO-02-18-0067-R

Massart, S., Olmos, A., Jijakli, H., Candresse, T., 2014. Current impact and future directions of high throughput sequencing in plant virus diagnostics. Virus Research. https://doi.org/10.1016/j.virusres.2014.03.029

Mehle, N., Dobnik, D., Ravnikar, M., Pompe Novak, M., 2018. Validated reverse transcription droplet digital PCR serves as a higher order method for absolute quantification of Potato virus Y strains. Anal Bioanal Chem 410, 3815–3825. https://doi.org/10.1007/s00216-018-1053-3

Miller, A.J., Gross, B.L., 2011. From forest to field: Perennial fruit crop domestication. American Journal of Botany 98, 1389–1414. https://doi.org/10.3732/ajb.1000522

Mischke, W., Schuch, K., 1962. Untersuchungen über eine viröse Triebtachung des Pfirsichs. Journal of Phytopathology 44, 76–88. https://doi.org/10.1111/j.1439-0434.1962.tb02000.x

Modrow, S., Falke, D., Truyen, U., Schätzl, H., 2013. Viruses: Definition, Structure, Classification. Molecular Virology 17–30. https://doi.org/10.1007/978-3-642-20718-1_2

Moinina, A., Lahlali, R., Boulif, M., 2019. Important pests, diseases and weather conditions affecting apple production in Morocco: Current state and perspectives. Revue Marocaine des Sciences Agronomiques et Vétérinaires 7.

Montzka, S.A., Dlugokencky, E.J., Butler, J.H., 2011. Non-CO2 greenhouse gases and climate change. Nature 476, 43–50. https://doi.org/10.1038/nature10322

Mulusa, L. (2023). Dagger and Stubby Nematodes in Agricultural Crops and Their Bio-Management. En M. R. Khan (Ed.), Novel Biological and Biotechnological Applications in Plant Nematode Management (pp. 377-394). Springer Nature. https://doi.org/10.1007/978-981-99-2893-4_17

Nakano, K., Shiroma, A., Shimoji, M., Tamotsu, H., Ashimine, N., Ohki, S., Shinzato, M., Minami, M., Nakanishi, T., Teruya, K., Satou, K., Hirano, T., 2017. Advantages of genome sequencing by long-read sequencer using SMRT technology in medical area. Human Cell 30, 149–161. https://doi.org/10.1007/s13577-017-0168-8

Navarro, B., Minutolo, M., De Stradis, A., Palmisano, F., Alioto, D., Di Serio, F., 2018a. The first phlebo-like virus infecting plants: a case study on the adaptation of negative-stranded RNA viruses to new hosts. Molecular Plant Pathology 19, 1075–1089. https://doi.org/10.1111/mpp.12587

Navarro, B., Zicca, S., Minutolo, M., Saponari, M., Alioto, D., Di Serio, F., 2018b. A Negative-Stranded RNA Virus Infecting Citrus Trees: The Second Member of a New Genus Within the Order Bunyavirales. Frontiers in Microbiology 9, 2340. https://doi.org/10.3389/fmicb.2018.02340

Nelson, S.G.A., Klodd, A.E., Hutchison, W.D., 2023. Hail netting excludes key insect pests and protects from fruit damage in a commercial Minnesota apple orchard. J Econ Entomol 116, 2104–2115. https://doi.org/10.1093/jee/toad197

Noda, H., Yamagishi, N., Yaegashi, H., Xing, F., Xie, J., Li, S., Zhou, T., Ito, T., Yoshikawa, N., 2017. Apple necrotic mosaic virus, a novel ilarvirus from mosaic-diseased apple trees in Japan and China. J Gen Plant Pathol 83, 83–90. https://doi.org/10.1007/s10327-017-0695-x

Nodet, P., Chalopin, M., Crété, X., Baroncelli, R., Le Floch, G., 2019. First Report of Colletotrichum fructicola Causing Apple Bitter Rot in Europe. Plant Disease 103, 1767. https://doi.org/10.1094/PDIS-11-18-1915-PDN

Nyirakanani, C., Tamisier, L., Bizimana, J.P., Rollin, J., Nduwumuremyi, A., Bigirimana, V. de P., Selmi, I., Lasois, L., Vanderschuren, H., Massart, S., 2023. Going beyond consensus genome sequences: An innovative SNP-based methodology reconstructs different Ugandan cassava brown streak virus haplotypes at a nationwide scale in Rwanda. Virus Evolution 9, vead053. https://doi.org/10.1093/ve/vead053

Okada, Y., 1999. Historical overview of research on the tobacco mosaic virus genome: genome organization, infectivity and gene manipulation. Philos Trans R Soc Lond B Biol Sci 354, 569–582. https://doi.org/10.1098/rstb.1999.0408

Oliveira, B.B., Veigas, B., Baptista, P.V., 2021. Isothermal Amplification of Nucleic Acids: The Race for the Next "Gold Standard." Frontiers in Sensors 2.

Olmos, A., Bertolini, E., Cambra, M., 2002. Simultaneous and co-operational amplification (Co-PCR): a new concept for detection of plant viruses. Journal of Virological Methods 106, 51–59. https://doi.org/10.1016/S0166-0934(02)00132-5

Osaki, H., Sasaki, A., 2018. A novel alphapartitivirus detected in Japanese pear. Virus Genes 54, 149–154. https://doi.org/10.1007/s11262-017-1511-6

Osaki, H., Sasaki, A., Nakazono-Nagaoka, E., Ota, N., Nakaune, R., 2017. Genome segments encoding capsid protein-like variants of Pyrus pyrifolia cryptic virus. Virus Res 240, 64–68. https://doi.org/10.1016/j.virusres.2017.07.023

Pajač, I., Pejić, I., Barić, B., 2011. Codling Moth, Cydia pomonella (Lepidoptera: Tortricidae) – Major Pest in Apple Production: an Overview of its Biology, Resistance, Genetic Structure and Control Strategies. Agriculturae Conspectus Scientificus 76, 87–92.

Pantaleo, V., Saponari, M., Gallitelli, D., 2001. Development of a Nested Pcr Protocol for Detection of Olive-Infecting Viruses in Crude Extracts. Journal of Plant Pathology 83, 143–146.

Pedroncelli, A., Puopolo, G., 2023. This tree is on fire: a review on the ecology of Erwinia amylovora, the causal agent of fire blight disease. J Plant Pathol. https://doi.org/10.1007/s42161-023-01397-y

Pio, R., Souza, F.B.M. de, Kalcsits, L., Bisi, R.B., Farias, D. da H., 2018. Advances in the production of temperate fruits in the tropics. Acta Sci., Agron. 41, e39549. https://doi.org/10.4025/actasciagron.v41i1.39549

Pooggin, M.M., 2018. Small RNA-Omics for Plant Virus Identification, Virome Reconstruction, and Antiviral Defense Characterization. Frontiers in Microbiology 9, 2779. https://doi.org/10.3389/fmicb.2018.02779

Potter, D., Eriksson, T., Evans, R.C., Oh, S., Smedmark, E., Morgan, D.R., Kerr, M., Robertson, K.R., Arsenault, M., Dickinson, T.A., Campbell, C.S., 2007. Phylogeny and classification of Rosaceae. Plant Systematics and Evolution 266, 5–43.

Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O., Kunin, W.E., 2010. Global pollinator declines: trends, impacts and drivers. Trends in Ecology & Evolution 25, 345–353. https://doi.org/10.1016/j.tree.2010.01.007

Pusey, P.L., 2000. The Role of Water in Epiphytic Colonization and Infection of Pomaceous Flowers by Erwinia amylovora. Phytopathology® 90, 1352–1357. https://doi.org/10.1094/PHYTO.2000.90.12.1352

Raza, M.M., Bebber, D.P., 2022. Climate change and plant pathogens. Current Opinion in Microbiology 70, 102233. https://doi.org/10.1016/j.mib.2022.102233

Rodell, M., Famiglietti, J.S., Wiese, D.N., Reager, J.T., Beaudoing, H.K., Landerer, F.W., Lo, M.-H., 2018. Emerging trends in global freshwater availability. Nature 557, 651–659. https://doi.org/10.1038/s41586-018-0123-1

Rosenberger, D.A., Meyer, F.W., 1988. Control of dagger and lesion nematodes in apple and plum orchards with Fenamiphos, Carbofuran, and Carbosulfan. Plant Disease 72, 519–522.

Rott, M.E., Kesanakurti, P., Berwarth, C., Rast, H., Boyes, I., Phelan, J., Jelkmann, W., 2018. Discovery of Negative-Sense RNA Viruses in Trees Infected with Apple Rubbery Wood Disease by Next-Generation Sequencing. Plant Disease 102, 1254–1263. https://doi.org/10.1094/PDIS-06-17-0851-RE

Russo, M., Vovlas, C., Rubino, L., Grieco, F., & Martelli, G. P. (2002). Molecular characterization of a tombusvirus isolated from diseased pear trees in southern italy. Journal of Plant Pathology, 84(3), 161–166. http://www.jstor.org/stable/41998105

Sano, T., Hataya, T., Terai, Y., Shikata, E., 1989. Hop Stunt Viroid Strains from Dapple Fruit Disease of Plum and Peach in Japan. Journal of General Virology 70, 1311–1319. https://doi.org/10.1099/0022-1317-70-6-1311

Sano, T., Isono, S., Matsuki, K., Kawaguchi-Ito, Y., Tanaka, K., Kondo, K., Iijima, A., Bar-Joseph, M., 2008. Vegetative propagation and its possible role as a genetic bottleneck in the shaping of the apple fruit crinkle viroid populations in apple and hop plants. Virus Genes 37, 298–303. https://doi.org/10.1007/s11262-008-0270-9

Sasaki, M., Shikata, E., 1977. On Some Properties of Hop Stunt Disease Agent, a Viroid. Proceedings of the Japan Academy, Series B 53, 109–112. https://doi.org/10.2183/pjab.53.109

Sasanelli, Nicola, Alena Konrat, Varvara Migunova, Ion Toderas, Elena Iurcu-Straistaru, Stefan Rusu, Alexei Bivol, Cristina Andoni, and Pasqua Veronico. 2021. "Review on Control Methods against Plant Parasitic Nematodes Applied in Southern Member States (C Zone) of the European Union" Agriculture 11, no. 7: 602. https://doi.org/10.3390/agriculture11070602

Saunders, K., Bedford, I.D., Yahara, T., Stanley, J., 2003. The earliest recorded plant virus disease. Nature 422, 831–831. https://doi.org/10.1038/422831a

Schweiger, O., Biesmeijer, J.C., Bommarco, R., Hickler, T., Hulme, P.E., Klotz, S., Kühn, I., Moora, M., Nielsen, A., Ohlemüller, R., Petanidou, T., Potts, S.G., Pyšek, P., Stout, J.C., Sykes, M.T., Tscheulin, T., Vilà, M., Walther, G.-R., Westphal, C., Winter, M., Zobel, M., Settele, J., 2010. Multiple stressors on biotic interactions: how climate change and alien species interact to affect pollination. Biological Reviews 85, 777–795. https://doi.org/10.1111/j.1469-185X.2010.00125.x

Scorza, R., Callahan, A., Dardick, C., Ravelonandro, M., Polak, J., Malinowski, T., Zagrai, I., Cambra, M., Kamenova, I., 2013. Genetic engineering of Plum pox virus resistance: 'HoneySweet' plum—from concept to product. Plant Cell Tiss Organ Cult 115, 1–12. https://doi.org/10.1007/s11240-013-0339-6

Serra, P., Messmer, A., Sanderson, D., James, D., Flores, R., 2018. Apple hammerhead viroid-like RNA is a bona fide viroid: Autonomous replication and structural features support its inclusion as a new member in the genus Pelamoviroid. Virus Research 249, 8–15. https://doi.org/10.1016/j.virusres.2018.03.001

Shahzad, A., Ullah, S., Dar, A.A., Sardar, M.F., Mehmood, T., Tufail, M.A., Shakoor, A., Haris, M., 2021. Nexus on climate change: agriculture and possible solution to cope future climate change stresses. Environ Sci Pollut Res 28, 14211–14232. https://doi.org/10.1007/s11356-021-12649-8

Shen, P., Tian, X., Zhang, S., Ren, F., Li, P., Yu, Y., Li, R., Zhou, C., Cao, M., 2018. Molecular characterization of a novel luteovirus infecting apple by next-generation sequencing. Arch Virol 163, 761–765. https://doi.org/10.1007/s00705-017-3633-5

Shulaev, V., Korban, S.S., Sosinski, B., Abbott, A.G., Aldwinckle, H.S., Folta, K.M., Iezzoni, A., Main, D., Arús, P., Dandekar, A.M., Lewers, K., Brown, S.K., Davis, T.M., Gardiner, S.E., Potter, D., Veilleux, R.E., 2008. Multiple models for Rosaceae genomics. Plant Physiology 147, 985–1003. https://doi.org/10.1104/pp.107.115618

Simmonds, P., Adams, M.J., Benkő, M., Breitbart, M., Brister, J.R., Carstens, E.B., Davison, A.J., Delwart, E., Gorbalenya, A.E., Harrach, B., Hull, R., King, A.M.Q., Koonin, E.V., Krupovic, M., Kuhn, J.H., Lefkowitz, E.J., Nibert, M.L., Orton, R., Roossinck, M.J., Sabanadzovic, S., Sullivan, M.B., Suttle, C.A., Tesh, R.B., van der Vlugt, R.A., Varsani, A., Zerbini, F.M., 2017. Virus taxonomy in the age of metagenomics. Nat Rev Microbiol 15, 161–168. https://doi.org/10.1038/nrmicro.2016.177

Simmonds, P., Adriaenssens, E.M., Zerbini, F.M., Abrescia, N.G.A., Aiewsakun, P., Alfenas-Zerbini, P., Bao, Y., Barylski, J., Drosten, C., Duffy, S., Duprex, W.P., Dutilh, B.E., Elena, S.F., García, M.L., Junglen, S., Katzourakis, A., Koonin, E.V., Krupovic, M., Kuhn, J.H., Lambert, A.J., Lefkowitz, E.J., Łobocka, M., Lood, C., Mahony, J., Meier-Kolthoff, J.P., Mushegian, A.R., Oksanen, H.M., Poranen, M.M., Reyes-Muñoz, A., Robertson, D.L., Roux, S., Rubino, L., Sabanadzovic, S., Siddell, S.,

Skern, T., Smith, D.B., Sullivan, M.B., Suzuki, N., Turner, D., Doorslaer, K.V., Vandamme, A.-M., Varsani, A., Vasilakis, N., 2023. Four principles to establish a universal virus taxonomy. PLOS Biology 21, e3001922. https://doi.org/10.1371/journal.pbio.3001922

Singh, J., Sun, M., Cannon, S.B., Wu, J., Khan, A., 2021. An accumulation of genetic variation and selection across the disease-related genes during apple domestication. Tree Genetics & Genomes 17, 29. https://doi.org/10.1007/s11295-021-01510-1

Sorensen, J.T., 2009. Aphids, in: Resh, V.H., Cardé, R.T. (Eds.), Encyclopedia of Insects (Second Edition). Academic Press, San Diego, pp. 27–31. https://doi.org/10.1016/B978-0-12-374144-8.00008-4

Stouffer, R.F., Uyemoto, J.K., 1976. Association of Tomato ringspot virus with Apple union necrosis and decline. Acta Horticulturae 67, 203–208. https://doi.org/10.17660/ActaHortic.1976.67.25

Sukhorukov, G., Khalili, M., Gascuel, O., Candresse, T., Marais-Colombel, A., Nikolski, M., 2022. VirHunter: A Deep Learning-Based Method for Detection of Novel RNA Viruses in Plant Sequencing Data. Frontiers in Bioinformatics 2.

Sutton, T.B., Aldwinckle, H.S., Agnello, A.M., Walgenbach, J.F., 2014. Compendium of Apple and Pear Diseases and Pests, 2nd ed. The American Phytopathological Society.

Temple, C., Blouin, A.G., De Jonghe, K., Foucart, Y., Botermans, M., Westenberg, M., Schoen, R., Gentit, P., Visage, M., Verdin, E., Wipf-Scheibel, C., Ziebell, H., Gaafar, Y.Z.A., Kutnjak, D., Vučurović, A., Rivarez, M.P.S., Richert-Pöggeler, K.R., Ulrich, R., Zia, A., Yan, X.-H., Massart, S., 2022. Biological and genetic characterization of Physostegia chlorotic mottle virus in Europe based on host range, location, and time. Plant Disease. https://doi.org/10.1094/PDIS-12-21-2800-RE

Thompson JR, Perry KL, De Jong W. A new potato virus in a new lineage of picorna-like viruses. Arch Virol. 2004 Nov;149(11):2141-54. doi: 10.1007/s00705-004-0362-3. Epub 2004 Jul 23. PMID: 15503203.

Thornton, P.K., Ericksen, P.J., Herrero, M., Challinor, A.J., 2014. Climate variability and vulnerability to climate change: a review. Global Change Biology 20, 3313–3328. https://doi.org/10.1111/gcb.12581

Tominaga, A., Ito, A., Sugiura, T., Yamane, H., 2022. How Is Global Warming Affecting Fruit Tree Blooming? "Flowering (Dormancy) Disorder" in Japanese Pear (Pyrus pyrifolia) as a Case Study. Frontiers in Plant Science 12.

Torrance, L., Jones, R. a. C., 1981. Recent developments in serological methods suited for use in routine testing for plant viruses. Plant Pathology 30, 1–24. https://doi.org/10.1111/j.1365-3059.1981.tb01218.x

Tzanetakis, I.E., Susaimuthu, J., Gergerich, R.C., Martin, R.R., 2006. Nucleotide sequence of Blackberry yellow vein associated virus, a novel member of the Closteroviridae. Virus Research 116, 196–200. https://doi.org/10.1016/j.virusres.2005.10.003

Varveri, C., Holeva, R., Bem, F.P., 1997. Effect of sampling time and plant part on the detection of two viruses in apricot and one in almond by ELISA. Annales de l'Institut Phytopathologique Benaki 18, 25–33.

Varveri, C., Maliogka, V.I., Kapari-Isaia, T., 2015. Principles for Supplying Virus-Tested Material, in: Loebenstein, G., Katis, N.I. (Eds.), Advances in Virus Research, Control of Plant Virus Diseases. Academic Press, pp. 1–32. https://doi.org/10.1016/bs.aivir.2014.10.004

Villani, S., Hopper, B., 2018. Preparing for Glomerella Leaf Spot and Fruit Rot in 2018 [WWW Document]. URL go.ncsu.edu/readext?523205 (accessed 2.19.24).

Vincent, M., Xu, Y., Kong, H., 2004. Helicase-dependent isothermal DNA amplification. EMBO reports 5, 795–800. https://doi.org/10.1038/sj.embor.7400200

Vivek, M., Modgil, M., 2018. Elimination of viruses through thermotherapy and meristem culture in apple cultivar 'Oregon Spur-II.' Virusdisease 29, 75–82. https://doi.org/10.1007/s13337-018-0437-5

Voytas, D., 2000. Agarose Gel Electrophoresis. Current Protocols in Molecular Biology 51, 2.5A.1-2.5A.9. https://doi.org/10.1002/0471142727.mb0205as51

Watzinger, F., Ebner, K., Lion, T., 2006. Detection and monitoring of virus infections by real-time PCR. Molecular Aspects of Medicine, Real-time Polymerase Chain Reaction 27, 254–298. https://doi.org/10.1016/j.mam.2005.12.001

Webster, A.D., 1995. Temperate fruit tree rootstock propagation. New Zealand Journal of Crop and Horticultural Science 23, 355–372. https://doi.org/10.1080/01140671.1995.9513912

Webster, A.D., Palmer, J.W., 2017. Pome and Stone Fruit, in: Thomas, B., Murray, B.G., Murphy, D.J. (Eds.), Encyclopedia of Applied Plant Sciences (Second Edition). Academic Press, Oxford, pp. 193–202. https://doi.org/10.1016/B978-0-12-394807-6.00009-5

Wenneker, M., Pham, K.T.K., Kerkhof, E., Harteveld, D.O.C., 2021. First Report of Preharvest Fruit Rot of 'Pink Lady' Apples Caused by Colletotrichum fructicola in Italy. Plant Disease 105, 1561. https://doi.org/10.1094/PDIS-11-20-2404-PDN

Whitfield, A.E., Falk, B.W., Rotenberg, D., 2015. Insect vector-mediated transmission of plant viruses. Virology, 60th Anniversary Issue 479–480, 278–289. https://doi.org/10.1016/j.virol.2015.03.026

Woo, E.N.Y., Clover, G.R.G., Pearson, M.N., 2012. First report of Cherry leaf roll virus (CLRV) in Malus domestica. Australasian Plant Dis. Notes 7, 151–156. https://doi.org/10.1007/s13314-012-0072-8

Wright, A.A., Cross, A.R., Harper, S.J., 2020. A bushel of viruses: Identification of seventeen novel putative viruses by RNA-seq in six apple trees. PLOS ONE 15, e0227669. https://doi.org/10.1371/journal.pone.0227669

Xiao, H., Hao, W., Storoschuk, G., MacDonald, J.L., Sanfaçon, H., 2022. Characterizing the Virome of Apple Orchards Affected by Rapid Decline in the Okanagan and Similkameen Valleys of British Columbia (Canada). Pathogens 11, 1231. https://doi.org/10.3390/pathogens11111231

Yarwood, C., 1955. Mechanical Transmission of an apple mosaic virus. Hilgardia 23, 613–628. https://doi.org/10.3733/hilg.v23n15p613

Yu, D.J., Lee, H.J., 2020. Evaluation of freezing injury in temperate fruit trees. Hortic. Environ. Biotechnol. 61, 787–794. https://doi.org/10.1007/s13580-020-00264-4

Zeng, Q., Puławska, J., Schachterle, J., 2021. Early events in fire blight infection and pathogenesis of Erwinia amylovora. J Plant Pathol 103, 13–24. https://doi.org/10.1007/s42161-020-00675-3

Zerbino, D.R., Birney, E., 2008. Velvet: Algorithms for de novo short read assembly using de Bruijn graphs. Genome Res. 18, 821–829. https://doi.org/10.1101/gr.074492.107

Zhang, Z., Qi, S., Tang, N., Zhang, X., Chen, S., Zhu, P., Ma, L., Cheng, J., Xu, Y., Lu, M., Wang, H., Ding, S.-W., Li, S., Wu, Q., 2014. Discovery of Replicating Circular RNAs

by RNA-Seq and Computational Algorithms. PLOS Pathogens 10, e1004553. https://doi.org/10.1371/journal.ppat.1004553

Zheng, L., Rodoni, B.C., Gibbs, M.J. and Gibbs, A.J. (2010), A novel pair of universal primers for the detection of potyviruses. Plant Pathology, 59: 211-220. https://doi.org/10.1111/j.1365-3059.2009.02201.x

Zotto, A.D., Nome, S.F., Di Rienzo, J.A., Docampo, D.M., 1999. Fluctuations of Prunus Necrotic Ringspot Virus (PNRSV) at Various Phenological Stages in Peach Cultivars. Plant Disease 83, 1055–1057. https://doi.org/10.1094/PDIS.1999.83.11.1055

Zsögön, A., Peres, L.E.P., Xiao, Y., Yan, J., Fernie, A.R., 2022. Enhancing crop diversity for food security in the face of climate uncertainty. The Plant Journal 109, 402–414. https://doi.org/10.1111/tpj.15626

Chapter 2

Objectives

1. Objectives and research context

The thesis was divided into three major research topics:

I. Create a new scientific and regulatory framework for the biological characterization and risk analysis of novel plant viruses and viroids discovered by HTS.

Traditionally plant virologists start to characterize a virus from the symptoms it produces on the host, but it was a long process to characterize a plant virus. With the advent of HTS, the speed of virus discovery increased considerably as not only symptomatic tissue samples of crops of economic importance were being analyzed. Many viruses were discovered from symptomatic, symptomless, cultivated, and wild plants. Nonetheless, even if HTS changed how plant virologists discover new viruses from symptom-based to genome-based, the characterization of these novel viruses remains a cumbersome and lengthy process. This bottleneck due to the struggle of biological experimentation leads to poor biological characterization of the novel viruses discovered by HTS. Therefore, it is complicated to evaluate the threat that these novel viruses pose for plant health, as it is essential to have a complete view of viral populations, distribution, severity, host range, transmission, and diversity of plant viruses to apply adequate management strategies.

II. Scan using HTS technologies the virome of apple and pear from the germplasm collection at the CRA-W (Gembloux, Belgium) which hosts a wide diversity of cultivars, including local and ancient cultivars.

Some pome fruit tree viruses that are associated with economically significant diseases have been vastly studied and characterized. The research was usually triggered by the observation of disease symptoms, So, there is already an abundant and extensive literature about their host range, geographical distribution, transmission modes, and symptomatology. However, there is a gap in the knowledge for viruses which are not strongly associated with apparent symptoms on the cultivar they are currently infecting. Some are not yet discovered while, for others, nor their biological characteristics neither their diversity are known. HTS technologies allow plant pathologists to study and characterize the plant virome without a priori knowledge and represent a new tool for identifying and characterizing novel viruses, including those that are not associated with symptoms.

III. Following the virome study performed in the germplasm collection at the CRA-W, a novel velarivirus was identified in pear trees, which was tentatively named Pyrus virus A (PyVA). The biological characterization of this novel virus was performed following the framework designed as part of objective I.

Chapter 3

Managing the deluge of newly discovered plant viruses and viroids

Synopsis

This chapter is dedicated to creating a new scientific and regulatory framework. This framework, a result of the revision of the 2017 framework, is envisioned to aid and guide during the biological characterization and risk analysis of novel plant viruses and viroids, especially those discovered by HTS. It addresses the significant difficulties in assessing the risks posed by these novel entities, which are becoming increasingly complex. The revision and creation of this new framework was a result of an international effort and collaboration in the frame of the Innovative Network for Next Generation Training and Sequencing of Virome (INEXTVIR) Horizon 2020 Project, which included twenty-two co-authors from various backgrounds, such as plant virologists and plant protection authorities.

This chapter starts with an introduction to the reasons behind the need for a revision of the previous framework, which involves findings from two reviews that were recently published where it was evidenced that the old framework was not well adapted to the current rate of virus discovery and what is feasible in terms of time and resources (Hou et al., 2020; Rivarez et al., 2021). Moreover, given that these two reviews focused on novel viruses of tomato and fruit trees, a review was done, following the process of Hou et al. (2020), to analyze the data and information provided in the publications of a novel virus identified from Poaceae (data presented in Supplementary Material 1).

The chapter's main body describes the newly created framework, emphasizing the use of innovative tools that are becoming a staple in plant virus research. These tools, such as Serratus (https://serratus.io/), are integral to the new framework's adaptability and effectiveness, ensuring it remains at the forefront of plant virus research. Supplementary Material 2 provides an example of the potential of Serratus as a tool to characterize novel viruses better. This tool was used to study the potential geographic distribution and host range of the emergent virus PhCMoV.

Chapter 3:

Managing the deluge of newly discovered plant viruses and viroids: an optimized scientific and regulatory framework for their characterization and risk analysis

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Nuria Fontdevila Pareta [1], Maryam Khalili [2,3], Ayoub Maachi [4], Mark Paul S. Rivarez [5,6], Johan Rollin [1,7], Ferran Salavert [8], Coline Temple [1], Miguel A. Aranda [9], Neil Boonham [8], Marleen Botermans [10], Thierry Candresse [2], Adrian Fox [8,11], Yolanda Hernando [4], Denis Kutnjak [5], Armelle Marais [2], Françoise Petter [12], Maja Ravnikar [5], Ilhem Selmi [1], Rachid Tahzima [1,13], Charlotte Trontin [12], Thierry Wetzel [14], and Sebastien Massart [1,15]

[1] *Plant Pathology Laboratory, Gembloux Agro-Bio Tech, University of Liège, Gembloux, Belgium,*

[2] *Univ. Bordeaux, INRAE, UMR BFP, Villenave d'Ornon, France,*

[3] *EGFV, Univ. Bordeaux, INRAE, ISVV, Villenave d'Ornon, France,*

[4] *Abiopep S.L., Murcia, Spain,*

[5] *Department of Biotechnology and Systems Biology, National Institute of Biology, Ljubljana, Slovenia,*

[6] *College of Agriculture and Agri-Industries, Caraga State University, Butuan, Philippines,*

[7] *DNAVision (Belgium), Charleroi, Belgium,*

[8] *School of Natural and Environmental Sciences, Faculty of Science, Agriculture and Engineering, Newcastle University, Newcastle upon Tyne, United Kingdom,*

[9] *Department of Stress Biology and Plant Pathology, Center for Edaphology and Applied Biology of Segura, Spanish National Research Council (CSIC), Murcia, Spain,*

[10] *Netherlands Institute for Vectors, Invasive Plants and Plant Health (NIVIP), Wageningen, Netherlands,*

[11] *Fera Science Ltd, York Biotech Campus, York, United Kingdom,*

[12] *European and Mediterranean Plant Protection Organization, Paris, France,*

[13] *Plant Sciences Unit, Institute for Agricultural, Fisheries and Food Research (ILVO), Merelbeke, Belgium,*

[14] *DLR Rheinpfalz, Institute of Plant Protection, Neustadt an der Weinstrasse, Germany,*

[15] *Bioversity International, Montpellier, France*

Abstract

The advances in high-throughput sequencing (HTS) technologies and bioinformatic tools have provided new opportunities for virus and viroid discovery and diagnostics. Hence, new sequences of viral origin are being discovered and published at a previously unseen rate. Therefore, a collective effort was undertaken to write and propose a framework for prioritizing the biological characterization steps needed after discovering a new plant virus to evaluate its impact at different levels. Even though the proposed approach was widely used, a revision of these guidelines was prepared to consider virus discovery and characterization trends and integrate novel approaches and tools recently published or under development. This updated framework is more adapted to the current rate of virus discovery and provides an improved prioritization for filling knowledge and data gaps. It consists of four distinct steps adapted to include a multi-stakeholder feedback loop. Key improvements include better prioritization and organization of the various steps, earlier data sharing among researchers and involved stakeholders, public database screening, and exploitation of genomic information to predict biological properties.

Keywords: plant viruses and viroids, high throughput sequencing (HTS), biological characterization, plant health, regulatory agencies, Pest Risk Analysis (PRA), virus disease

1. Introduction

Advances in high-throughput sequencing (HTS) technologies and bioinformatic analyses have created new opportunities for the discovery and unbiased diagnosis of plant viruses and viroids (together referred to hereafter as viruses) (Massart et al., 2014). This exponential growth in the application of HTS technologies and the improvement of the bioinformatics algorithms have generated a steep increase in the discovery and publication of new sequences of viral origin (Shi et al., 2016; Chiapello et al., 2020; Edgar et al., 2022; Zayed et al., 2022; Rivarez et al., 2023).

A collective framework was published in 2017 to address the difficulties in assessing risks that these novel detections might pose. The framework aimed to suggest guidelines for researchers, policymakers, plant health authorities, and plant inspection services. It proposed an approach for prioritizing the biological characterization steps for newly identified plant viruses and evaluating their impact at biosecurity, commercial, regulatory and scientific levels (Massart et al., 2017). The first notification to the other plant health stakeholders in the framework was recommended after targeted methods (i.e., PCR or RT-PCR) confirmation of the novel virus detection by HTS. Then, if the novel virus was considered a phytosanitary priority, it was recommended to study its local prevalence and epidemiology (i.e., in the sampled field and surrounding area or in the batch of intercepted plants). Then a second communication with the regulatory authorities was proposed before further biological characterization of the novel virus, including fulfillment of Koch's postulates, study of the mode of transmission, identification of potential vectors, evaluation of host range, symptomatology, and, if possible, global distribution. Finally, additional communication with authorities was recommended whenever considered relevant for the development of a Pest Risk Analysis (PRA) (Massart et al., 2017).

This framework was widely used to guide the characterization of newly identified plant viruses. However, recent reviews have shown that there is rarely a follow-up after the first report of novel viruses except for viruses that cause an immediate and obvious threat to production. Hou et al. (2020) reviewed 78 publications describing the discovery of novel viruses from 32 fruit tree species since 2011 and 933 citing publications. They observed interesting trends related to the characterization efforts carried out when publishing the discovery of a new fruit tree virus. The design of diagnostic primers and the completion of the genome sequence were done in more than 90% of the publications, underlining the importance but also the ease to obtain these two pieces of information. At large and local scales, infectivity assays and confirmation of a mixed infection were done in between 30 and 49% of the articles reviewed. Association with symptoms, studies on herbaceous indicators or other potential hosts, gene and genome diversity, latent infection and transmission assays were studied for 25% or less of the novel viruses.

Another publication by Rivarez et al. analyzed 53 published discovery and post-discovery studies on novel tomato viruses for the 2011–2020 period. It assessed how the framework by Massart et al. was fulfilled after the initial discovery (Rivarez et al., 2021). In most cases, a complete genome was provided and in approximately 80% of the articles, virus-specific primers were designed for diagnostic purposes. At the same time, more than 50% of the publications performed a local survey and gave information on the presence or absence of a co-infection with other viruses. However, less than 50% of the original publications studied the novel virus diversity, symptomatology, or association with symptoms in field samples, infectivity on original and indicator hosts, or did a large-scale survey. A study on the natural host range of the novel virus was done only in less than 20% of the citing publications or post-discovery studies. Nevertheless, the framework's criteria were fulfilled relatively quickly for novel viruses perceived as posing a considerable threat to crop production. For example, less than 4 years after the discovery of tomato brown rugose fruit virus (ToBRFV), which was discovered using non-HTS methods, 13 out of the 14 proposed characterization criteria had been fulfilled. In comparison, for tomato mottle mosaic virus and tomato necrotic stunt virus, that had been discovered through HTS, 11/14 criteria were fulfilled within 4–8 years.

Here, a similar analysis for 28 publications reporting 42 novel viruses identified by HTS in Poaceae was performed. The analysis is summarized in Figure 3-1 and further detailed in Sup. material 1. Similar to the pattern observed for fruit tree and tomato viruses, the complete genome was published for all involved viruses, and in 95% of cases specific primers were designed. In contrast, further biological characterization studies such as the association with symptoms (43%) or electron microscopy (10%) were done much less often. Interestingly, gene and genome diversity were studied for 34 and 44% of the new Poaceae infecting viruses respectively, while for fruit tree viruses, they were mentioned in only 18 and 11% of cases and for tomato viruses in approximately 30 and 40%, respectively.

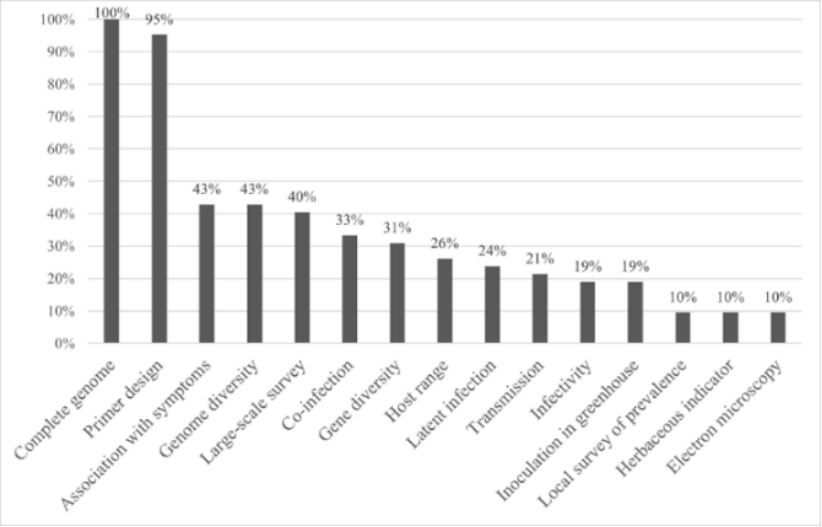


Figure 3-1. Percentage of newly identified *Poaceae* viruses for which data was developed for each characterization category, as defined by Hou et al. (2020).

These three studies exemplify the exponential growth in plant viruses' discovery due to HTS and the scarcity of biological characterization efforts for the identified novel viruses. The probable reasons for such an observation are the extended time and resources required for characterization experiments, including host range testing, large-scale surveys, and the technical difficulty of working with novel viruses for which little or no information is available. Nevertheless, there are some exceptions. For example, chestnut mosaic virus (ChMV) was identified by HTS technologies in symptomatic plants and was proposed as the potential causal agent of chestnut mosaic disease (ChMD). After obtaining a complete genome sequence from two chestnut disease sources, the genomes of ChMV were used to determine the phylogenetic relationships with other badnaviruses. New isolates were identified from publicly available chestnut HTS data. Incidence and genetic variability of ChMV were studied using samples from France and Italy (Marais et al., 2021). Another example is papaya virus X (PapVX), first identified in diseased papaya crops from northwest Argentina using HTS. Viral particles were confirmed with electron microscopy, and after obtaining a complete genome sequence, the genome organization and provisional taxonomic assignation were done. In addition, publicly available transcriptome datasets were also explored for other isolates of PapVX. The phylogenetic relationships were studied at nucleotide and amino acid levels for the RNA replicase (RdRp) and coat protein (CP) sequences and the complete genome. Mechanical inoculations were done to study the host range of PapVX, and a local survey in the northern region of Argentina was conducted to determine the distribution of the novel virus (Cabrera Mederos et al., 2022).

Data on the geographic distribution, incidence, severity, symptomatology, host range, transmission mode, and genetic diversity of these novel viruses are necessary to support a proper risk assessment. Therefore, the previous framework is revised here to adapt it to the current rate of virus discovery through HTS, and add clarity on the prioritization of knowledge gaps (Figure 3-2). Furthermore, because of the recent reconsideration of the conceptual framework addressing the causal association between symptoms and the presence of a virus (Fox, 2020), this revision moves the evaluation of causal association at an earlier stage, as well as integrating the impact of HTS on plant health diagnostics and management (Adams et al., 2018; Olmos et al., 2018). The overall aim was to better adapt the framework to what is feasible, realistic, and efficient, while considering the limitation in time and resources that constrain the ability to fully characterize any newly discovered virus.

Data-driven virus discovery through scanning of large public sequencing datasets is a major recent development. Re-examining existing datasets for the presence of known and novel viruses has become accessible for virologists, through new web-based platforms like Serratus (www.serratus.io) (Edgar et al., 2022), RVMT (www.riboviria.org) (Neri et al., 2022) and ViroidDB (www.viroids.org) (Lee et al., 2022a,b). Nevertheless, virologists and plant health stakeholders should consider the consequences, not only benefits, of these data-driven virus discovery approaches (Lauber and Seitz, 2022).

In a short timeframe, these revolutionary high-throughput sequencing and data-driven approaches have extended the need to reconsider and adapt the current framework through a multi-stakeholder consultation. We thus propose an improved and adapted framework for plant health stakeholders, which could include researchers, policymakers, plant health authorities [also referred to as National Plant Protection Organizations (NPPOs)], plant inspection services, funding bodies, grower associations, technical extension services, seed traders, and breeding companies. It details the prioritization process to be followed for novel plant viruses and viroids identified by HTS technologies or datamining of HTS datasets.

2. Revised framework

2.1. Detection test, confirmation of detection and genome sequence

Importantly, international guidelines were proposed to improve the reliability of data generation by HTS technologies and their analysis by bioinformatics pipelines. These guidelines are generic and do not depend on the plant pest or pathogen being detected, sequencing protocol, or platform. These guidelines are advised to be implemented when applying HTS tests to detect viruses, whatever protocol is selected (EPPO PM 7/151 (1), 2022; Lebas et al., 2022; Massart et al., 2022).

In selected cases, the complete genome sequence is not obtained because of insufficient coverage (low read numbers), which can also depend on the library preparation protocol (ribosomal RNA depleted RNA, virion-associated nucleic acid (VANA), double-stranded RNA (dsRNA), small interfering RNA (siRNA), and total DNA with or without rolling circle amplification (RCA)) (Boonham et al., 2014; Hall et al., 2014; Roossinck et al., 2015; Claverie et al., 2019; Maclot et al., 2020) and the choice of the sequencing platform (i.e., Illumina sequencing, which generate short reads with large volume of sequences; or Oxford Nanopore Technologies, which generate longer but fewer reads) (Pfeiffer et al., 2018; Bester et al., 2021; Delahaye and Nicolas, 2021).

Nevertheless, once the draft genome of a potential novel virus was assembled, essential succeeding steps concern the annotation of the ORFs, the evaluation of percent pairwise identity, and phylogenetic relationships with known species. This information will be compared with the demarcation criteria established by the International Committee on Taxonomy of Viruses (ICTV) to evaluate if the assembled sequence belongs to a recognized species or to a new one. Even if the assembled genome lacks the UTRs or still has some gaps, the taxonomical position can still often be predicted (Lefkowitz et al., 2018). Furthermore, the construction of a phylogenetic tree should validate or help with the taxonomic assignation of the assembled genome into a genus or a family (Pagán, 2018). This work should be carried out with at least one representative from each closely related genus and one outgroup. However, each case may be different, depending on the particular ICTV demarcation criteria applying to the virus under consideration. Thus, the analysis can/should be done using the RdRp, CP, or any other ORFs that are included in the relevant ICTV demarcation criteria. For example, for the family Closteroviridae, ICTV advises using RdRp, CP and HSP70h (70 kDA heat shock protein homolog) amino acid sequences to distinguish viral species (Candresse and Fuchs, 2020).

Provisional taxonomic assignment can be more complex if the newly discovered sequence is significantly divergent from known viruses or shows identity levels on the borderline with known taxa (Maclot et al., 2021). The viral sequences detected may correspond to a plant virus or any virus that infects an organism associated with the plant sample, such as bacteria, fungi, or insects (Al Rwahnih et al., 2011). For instance, the viral family Partitiviridae includes viruses that can infect plants, fungi, or protozoa (Vainio et al., 2018) so that determining whether a detected Partitiviridae infects the sampled plant or an associated organism may be complicated. Nevertheless, phylogenetic and taxonomic relationships to known viruses can facilitate the discrimination between plant, fungal, bacterial or insect viruses.

When detecting a potentially new viral species, the previous framework recommended confirmation of detection by a second test (Massart et al., 2017). This step remains essential, the common procedure being to use validated generic PCR tests if available. However, if a laboratory has a validated HTS test, this new finding could be tested using HTS (performing a new nucleic acid extraction) instead of PCR

(Massart et al., 2022). For example, confirmation of detection of a novel virus is valid if the novel virus is identified in two independent laboratories using proper controls and validated HTS tests. Nevertheless, given the potential cross-contamination at each step of sample processing (i.e., sample collection, nucleic acid extraction, library preparation, and sequencing) (Rong et al., 2022), especially for viruses present in high concentration, it is advised to confirm the presence of the novel virus in the host using plant material of the original sample (back-up sample) for a new nucleic acid extraction.

Amplifying fragments of viruses/genomes using generic primers for a genus (or family) could also help in verifying the taxonomic assignment of the virus. It is possible that generic primers are not available or fail to amplify the novel virus, which can be because there are mismatches between the primer and the sequence to amplify, meaning that there will be a need to develop a more specific diagnostic tool that can detect the novel virus (Maree et al., 2018). The obtained amplicon may be further sequenced to confirm its viral origin. Usually, RT-PCR detection tests are developed using primers designed based on assembled sequences or a reference-based assembly to account for variability. If the genetic diversity of closely related viral species is well characterized, it is worth checking the scientific literature and aligning the existing sequences to evaluate the less variable ORF/regions within the taxonomic group as a way to design primers targeting a conserved genomic region to allow for detection of the most virus variants from the species. The ability of the designed primers to detect only the targeted new viral species and not related species should be checked by comparing their sequences to databases using primer BLAST, for example, or the genome alignments already developed. It is worth nothing that the specific test will be used as a diagnostic test in the following steps of the framework to complete the biological characterization of the new or poorly characterized virus (i.e., greenhouse assays and field surveys), as well as in managing the disease if the virus is causing symptoms to economically important crops. The test's degree of specificity and sensitivity are therefore of prime importance.

When the full or near complete genome sequence is obtained from the initial HTS test, only the completeness of the genome sequence needs to be confirmed. For instance, viruses from the genus Tenuivirus have almost complementary 3′ and 5′ genome ends (Gaafar et al., 2021), which can provide an indication on genome completeness. Similarly, if the novel genome contains a 3′ poly-A tail or the assembled genome length is very similar to that of closely related viruses, it provides an indication that the UTRs are likely complete or only missing a few nucleotides (Kwibuka et al., 2021). However, if an incomplete genome assembly is obtained, it is a good practice to carry out additional analyses to complete the genome, such as iterative mapping of unassembled reads (Olmedo-Velarde et al., 2022). Nevertheless, obtaining the whole genome should not be the priority in an outbreak situation as long as primers can be designed for diagnostic purposes, and thus should not impede progression of the proposed framework. The genome sequence of the novel virus can

be completed by filling the sequence gaps between contigs and determining the sequences of both extremities, usually using a rapid amplification of cDNA ends (RACE) (Marais et al., 2020; Maclot et al., 2021). Not all publications evaluate the genome completeness of a novel virus, and ICTV no longer recommends it as long as the complete set of ORFs are detected (Simmonds et al., 2017).

There are also specific cases that deserve particular attention, such as the case of some plant DNA viruses of families Caulimoviridae and Geminiviridae. These viruses can exist as endogenous viral elements integrated into the plant genome (i.e., endogenous pararetroviruses (EPRVs) for caulimoviruses or endogenous geminivirus-like (EGV) elements for geminiviruses) and/or as episomal forms that are contagious and can cause pathogenic infections (Sharma et al., 2020). Therefore, further investigation is necessary to verify whether the detected viral sequence corresponds to an infective episomal form or not. Endogenous viral sequences also represent a challenge for diagnostics and disease management, as a few endogenous viruses can revert to an infective episomal form (Rong et al., 2022). For example, several integrated banana streak viruses (BSV), tobacco vein clearing virus (TVCV), and petunia vein clearing virus (PVCV) can be activated to infectious episomal forms in specific plants hosts as a response to stress (Harper et al., 2002). Nonetheless, most endogenous viral sequences are not able to revert to episomal viruses, despite being transcribed. This could be verified by observing viral particles by electron microscopy (Chabannes and Iskra-Caruana, 2013) or with southern hybridization (Staginnus et al., 2007). Immunocapture PCR (IC-PCR) could be used if there are antibodies available, which, because the sequence in question belongs to a new virus, there probably are not (Le Provost et al., 2006). Rolling-Circle Amplification (RCA) is sometimes also used to distinguish between endogenous and episomal viral sequences (James et al., 2011). However, it is not recommended as it is not an absolute enrichment in circular sequences.

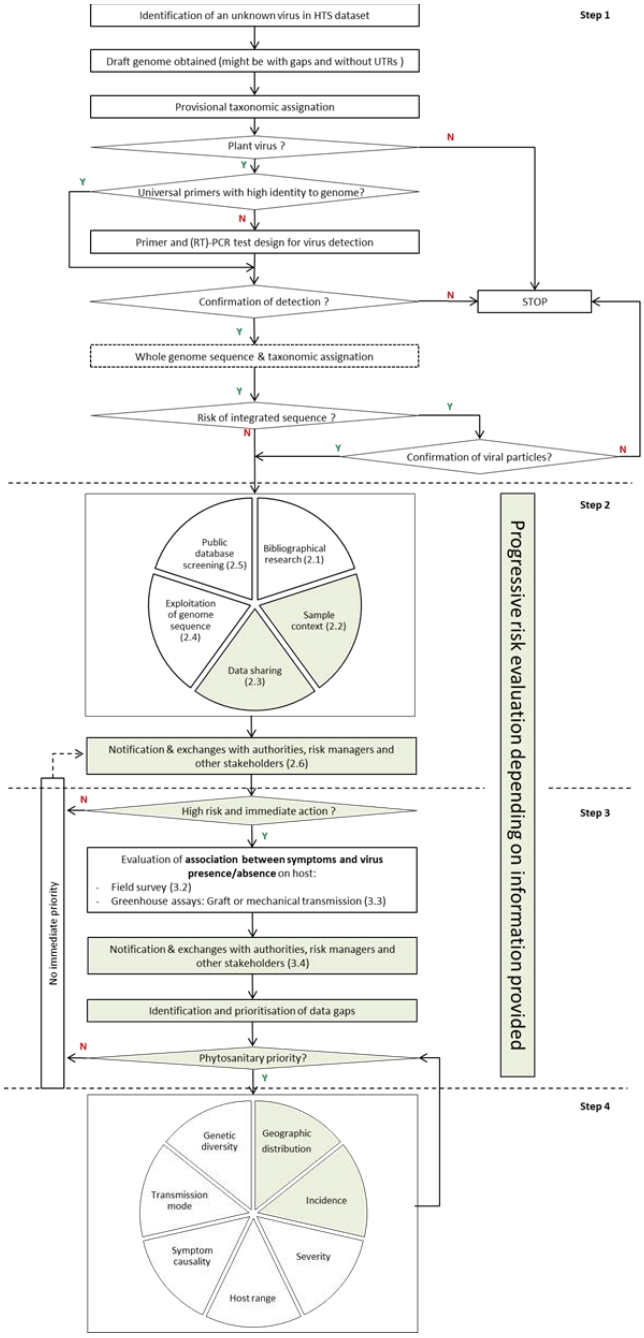


Figure 3-2. Proposed framework following the discovery of a novel virus or viroid. Y means positive response (yes) and N means negative response (no). Multi-stakeholders are involved in green-highlighted actions, and researchers in white-highlighted actions. Actions belonging to each step are separated with a dotted line, and numbers in brackets correspond to subchapters in the text.

2.2. Contextual information gathering and notification to stakeholders

2.2.1. Bibliographical research on the biology of related viruses

After confirmation of the presence of a novel virus and its provisional taxonomic classification, the next step is the bibliographical research on the biology of related viruses (within the same family or genus). However, one should keep in mind that extrapolating the biological properties of a novel virus based on the viruses in the same taxa is associated with significant uncertainties.

At this stage, the main focus of the bibliographical research should be on (i) the putative modes of vertical and horizontal transmission and candidate vectors, if any, to assess the potential spread of the disease (Massart et al., 2017); (ii) the potential host range broadness and its botanical scope (Moury et al., 2017); (iii) the potential pathogenicity of the virus in its host(s), including symptomatology and the potential existence of helper or satellite viruses that may have an impact on symptoms and transmission, and (iv) in the case where broad-spectrum resistance is known against related viruses, the potential existence of resistance or tolerance to the novel virus in the identified host plant(s), keeping in mind that resistance or tolerance is often species-specific and, even with a broader spectrum, might still be lost for a closely related viral species. For example, the gene Tm-22 confers resistance against several tobamoviruses in tomatoes, but it does not protect against the newly discovered tomato brown rugose fruit virus (ToBRFV) (Hak and Spiegelman, 2021).

Even though information on closely related viruses can only give clues about the most probable mode of transmission of the novel virus or point to potential vectors, this information can be biased. For example, all members of the genus Tenuivirus are transmitted by a particular planthopper species, except maize yellow stripe virus (MYSV) that is transmitted by leafhoppers (Ammar et al., 2007; King et al., 2011). Viruses within the family Geminiviridae can be transmitted by whiteflies (Begomovirus genus), by leafhoppers (genera Mastrevirus, Curtovirus, Becurtovirus, Mulcrilevirus, and Turncurtovirus), by aphids (genus Capulavirus), or by treehoppers (genera Topocuvirus and Grablovirus) (Zerbini et al., 2017). Differences may also exist within a genus: torradoviruses are generally whitefly-transmitted (Vlugt et al., 2015), although some non-tomato infecting torradoviruses are aphid-transmitted (Rozado-Aguirre et al., 2016; Verbeek et al., 2017).

The information gathered from this bibliographical research will assist in elaborating possible epidemiological scenarios and hypotheses, from which further investigation on the host and vector range can be defined. This information can also help in formulating provisional tentative control measures included in the first notification to regulatory authorities, risk managers and other stakeholders. However, as mentioned before, this information is extrapolated from that of related viruses and should therefore be treated with caution.

2.2.2. Documentation of sample context

At this stage and to assist the risk assessment process, as much information as possible, whenever possible, should be collected regarding the original sample (or pool of samples) where the novel virus was detected. This includes the plant species and cultivar, sample accession number, description of the symptoms observed at the time of sampling, plant tissue collected, the viral status of the neighboring plants (if known either by onsite testing or from previous records), the incidence in the affected crop, other crops affected, recent meteorological conditions, sample collection date, geographical origin of the sample with specific map coordinates, and growth conditions of the plants (Massart et al., 2017). Collecting this information at the time of sampling can facilitate and minimize efforts later on. Additionally, the documentation could include the economic importance and geographical distribution of the crop species affected, globally or domestically (Kwibuka et al., 2021). Optionally, plants that are taxonomically related to the infected hosts, including other crops and wild plants that could potentially be threatened by the virus or be a reservoir or alternate host, could also be documented. This set of information could help make better preliminary assessment of the potential threat (García-Arenal and Zerbini, 2019; Hasiów-Jaroszewska et al., 2021; Rivarez et al., 2023). The information-gathering step, if done well, is critical and can decrease the burden during the submission of the dataset to public repositories such as the European nucleotide archive (ENA) or the sequence read archive (SRA) of GenBank.

2.2.3. Data sharing among research groups

Communication between stakeholders and the scientific community is essential for a quick decision-making process. Pre-publication data sharing between research groups that independently detected the novel virus is highly encouraged, because it can provide valuable information on the presence, distribution, host range, and impact of the novel virus (Koloniuk et al., 2018; Sõmera et al., 2019; Kwibuka et al., 2021; Temple et al., 2022). For example, actinidia virus X (AVX) was first reported as a novel virus infecting kiwifruit and blackcurrant, although it was later found to be synonymous with plantain virus X (PlVX) (Hammond et al., 2021); or potato virus V (PVV), which was confused with potato virus Y (PVY) since it caused similar symptoms when inoculated to PVY-sensitive cultivars (Fuentes et al., 2022). Nevertheless, data sharing is mainly done through informal contact between groups and is limited by the network of each researcher. The lack of communication and cooperation may lead to the multiplication of parallel efforts on the same issue (Giovani et al., 2020). Creating and improving networks, such as the global surveillance system (GSS), could enhance collaboration between stakeholders, nationally and internationally (Carvajal-Yepes et al., 2019). For example, a Euphresco (European phytosanitary research coordination) data-sharing project aims to improve pre-publication data-sharing approaches with a focus on documentation of sample context (step 2.2.2) to explore similar findings from different research groups, thus providing access to distribution and host range data on novel virus detections. Data

sharing could also be useful when research groups have many unpublished findings, which they may not be able to publish or disseminate on their own.

2.2.4. An unexplored path: exploitation of structural features from genomic sequence toward predictive sequence-to-function viral proteomics

In animal virology, many publications used machine learning approaches on databases of genomic features and biological properties from known viruses to predict the taxonomy or key biological properties of new viruses, such as host range and vector. Most of these approaches focus on nucleotide features like CG bias, CpG bias, di-codon, or dinucleotide bias (Young et al., 2020; Giovani et al., 2022). For example, dinucleotide bias was used to identify host reservoirs and vector candidates for mammalian RNA viruses (Babayan et al., 2018), to predict hosts of coronaviruses (Tang et al., 2015), or to identify the human or avian origin of influenza A viruses (IAV) using random forest analysis (Eng et al., 2016, 2017; Li et al., 2019).

Recent research used an original approach to identify new proteomic features potentially involved in plant virus-vector transmission, i.e., intrinsically disordered proteins/regions, and to understand how their biophysical properties and regulation might arise from these interactions (Tahzima et al., 2021). As a result, it was shown that most encoded plant virus proteins contain multiple disordered features that are phylogenomically preserved and can be associated with structural, bio-physical, and evolutionary strategies.

This opens a new focus for predicting the biological properties of the new plant virus from in-depth structural and functional analyses of protein sequences.

Nevertheless, interpreting all these features and results should still currently be done with much caution, given the uncertainty attached to such predictions and the sometimes limited accuracy of these databases. In the future, integrating these powerful emerging approaches to the framework could represent a significant step toward gathering relevant biological predictions from a genomic sequence. Therefore, it might ultimately support regulatory and phytosanitary decisions linked to discovering novel viruses.

2.2.5. Public database screening and consideration on careful use of related metadata

Valuable information can be gained by screening public databases of HTS data, such as the SRA of GenBank (https://www.ncbi.nlm.nih.gov/sra), for the presence of newly identified or poorly characterized viruses (Hily et al., 2020). SRA is srapredicted to surpass 50 petabytes of data by 2023 [Sequence Read Archive (SRA) Data Working Group | DPCPSI, 2021; Katz et al., 2022] and mining such an enormous amount of information for virus presence previously required heavy computational power unaffordable for most virology laboratories as well as expertise in data science.

The recent development of a practical and user-friendly web-based interface called Serratus (Edgar et al., 2022) represents a major advancement toward a more generalized public database screening (see text footnote 1 for more details). Serratus uses a pre-screening strategy to look for viral RdRp motifs in SRA data (deposited until January 2020). It has the potential to provide hints about the host range or geographical distribution of specific RNA viruses present in the sequencing datasets. Following the pre-screening of SRA, Serratus provides a database of potential viral RdRp sequences (known and unknown), which is publicly available and can be used for exploratory and further diversity or phylogenetic analyses. This database also contains the link between each RdRp (SRA origin) and their associated "palmprint," (https://github.com/rcedgar/palmdb) which is an RdRp "barcode" classified by taxonomy and clustered in operational taxonomic units (OTUs) with 90% identity threshold (Babaian and Edgar, 2021). Through Serratus, the SRA datasets deposited until January 2020 can be mined by looking for an RNA virus (via a family/Genbank/SRA_id search or a taxonomic tree exploration) or by searching for the sequence (protein or nucleic) of an RdRp using the palmID search tool (www.serratus.io/palmid). The palmID search tool allows finding the palmprint sequence within the provided RdRp and match it, with a minimal threshold of pairwise identity, to palmprint sequences in the palmprint database. The palmprint OTU is necessary to avoid heavy computational requirements, although it lowers the confidence in the results, thus only giving hints to the presence of the target virus, which needs further validation. Thus, the sequencing reads of the identified SRA dataset should be reanalyzed using existing bioinformatic approaches to confirm the presence of the virus of interest. Sup. material 2 presents a practical example of the additional information that can be gained by using palmID for an emerging virus (physostegia chlorotic mottle alphanucleorhabdovirus).

Nevertheless, Serratus has some limitations, mainly when the virus of interest is not detected from public SRA datasets. Even in case of detection, the verification step (assembly and mapping) is time-consuming and a computational burden, and sometimes inefficient, depending for example on virus representation and on sample identity (i.e., fragmented genome or pooled sample). Another limitation is the potential misassignment of the host, in particular when the new finding involves a metagenomic dataset with unexpected virome content (i.e., a plant virus found in a human clinical dataset or animal viruses found in plant datasets). In addition, metadata information such as the host or the country of origin of the sequenced material should also be investigated, keeping in mind that the metadata may not be accurate. If biological material is still available, contacting the authors may allow the confirmation of the detection. It should be stressed that there could also be some implications for trade and related policies when relying on SRA mining for reporting the detection of pathogens in a country where it is not currently known to be present. In such a case, the conduct of confirmatory tests in the wet lab should be encouraged and given a very high priority. The ethics of reporting the presence of a pathogen in a territory without prior notification to its NPPO should also be considered. Whenever

possible, it is advised to contact the dataset's original authors and notify the country's NPPO before the publication of a new country record.

2.2.6. Notification and exchanges with other stakeholders

Based on the studies described in steps 2.2.1 to 2.2.5, researchers should have a better idea of whether a newly identified virus might threaten plant health and whether the new finding(s) should be reported to other stakeholders. However, it is crucial not to overburden relevant stakeholders with non-relevant information that might raise unnecessary concerns. For example, the detection of a plant virus, belonging to a family of pathogenic viruses with high horizontal transmission rates, on a sample of a critical crop should be communicated as soon as possible to the NPPO. In contrast, detecting a partitiviridae in an asymptomatic wild plant has lower significance and, therefore, priority. The participation of plant virology experts is therefore crucial at this stage to support well-informed decision making. As mentioned before, when a virus is considered a potential threat to plant health, researchers should report the finding to the relevant NPPO, engage in discussions with risk managers and assist them in efforts to determine if the novel virus should be considered a priority, and whether immediate action (i.e., destruction of consignment) or specific management measures (i.e., disinfection or rouging) should be taken and to evaluate whether further research is needed. Given the potential impact of the management decision taken by the NPPO, the uncertainties associated with the discovery of the novel virus and its potential impacts should be highlighted in a transparent fashion.

Further on, if the NPPO analysis confirms the potential threat following consultations, the main challenge for scientists is to efficiently characterize the biological properties through short, mid- and long-term strategies. This is while creating appropriate communication channels with the regulatory authorities and other stakeholders including grower associations, technical extension centers, or seed companies (Massart et al., 2017; Fiallo-Olivé and Navas-Castillo, 2019).

2.3. Evaluation of the association between symptoms and virus presence

2.3.1. General background

After the first notification to the regulatory agencies, if a novel virus is considered a priority or has potential risks, further evaluation of the association between symptoms and virus presence must be carried out via field surveys or greenhouse assays. Field surveys and greenhouse assays can provide helpful information regarding symptomatology, infectivity, causal association, virus genetic diversity, geographic distribution, incidence, host range, transmission mode, and disease severity, as discussed in step 2.4. Nevertheless, at this stage, it is essential to focus the survey's aim and assays on the symptom(s) causation issue if the novel virus is considered a priority. These efforts should not be hindered by those toward the

completion of previous steps, since obtaining a complete genome sequence might be time-consuming and could delay the needed surveys or assay actions.

In conventional plant pathology approaches, when trying to establish a causal association between a disease and a pathogen, causation is demonstrated by isolating the putative pathogenic agent and subjecting it to the experimental demonstration of Koch's postulates (Rivers, 1937; Evans, 1976). Nevertheless, this strategy has downsides because not all diseases are caused by a single pathogen, and pathogen complexes, timing of infection and influence of abiotic factors may also play a role in disease development. There are examples of situations in which causation could not be shown by fulfilling Koch's postulates, such as different virus strains causing a variable array of symptoms on the same host (Blystad et al., 2015), environmental conditions affecting the disease (Fraile and García-Arenal, 2016), the importance of the time passed after the infection (Chikh-Ali et al., 2020), or pathogens in an active mixed infection (Murphy and Bowen, 2006).

In recent years, there has been an ongoing discussion among researchers to find possible alternative and systematic approaches that overcome the limitations of Koch's postulates in plant virology (Di Serio et al., 2018; Fox, 2020). These efforts follow numerous previous attempts (Ehrlich, 1913; Rivers, 1937; Huebner, 1957; Hill, 1965; Johnson and Gibbs, 1974; Falkow, 1988; Evans, 1991; Fredericks and Relman, 1996). As a consequence of the most recent efforts, a simplification of criteria needed to establish causal association was proposed, mainly focusing on four key considerations: experimental evidence, the strength of the relationship, consistency of the relationship, and a binary evaluation of coherence and plausibility (Fox, 2020). A simplified hierarchical approach was thus proposed when considering a causal relationship in plant virology based on four criteria: (i) experimental, which complies with Koch's third postulate; (ii) strength, which is based on field/glasshouse observations, confirmation of a single pathogen infection by HTS and statistical analysis, considering the prevalence of the virus and eventually co-infecting species on both symptomatic and asymptomatic individuals; (iii) consistency, following the same principle and approach as for the "strength" criteria but adding the variable of multiple geographic locations and over time; and (iv) coherence and plausibility, which account for any confounding factors and similar effects that have been reported in other pathosystems.

2.3.2. Field survey

Surveys at small- and/or large scales allow a better understanding of the key factors associated with a disease. These epidemiological field surveys should include both symptomatic and asymptomatic plants since an asymptomatic individual could be in the incubation or latent phase at the time of sampling. In field surveys, caution should be taken in generating and analyzing data on the virus variability to ensure that the viral populations in crops and weeds are sufficiently similar to support the hypothesis of the role of weeds as a potential reservoir. Neighboring plants from the same or

different species that present similar symptoms may also be tested for presence or absence of the novel virus, without the need for viral enrichment extraction protocols, and instead using commercial extraction kits or even crude extracts (Massart et al., 2009). Because mixed infections are common, field surveys can be based on targeted tests, such as RT-PCR, or HTS. Whenever possible, especially for viruses detected in perennial hosts, the survey should be carried out at different time points of the year on the same individual plants and on more than one plant tissue. This is because there are seasonal fluctuations of the viral titer that can impact the detection of the virus and because viruses in many woody hosts show an uneven distribution in host tissues (Katsiani et al., 2018; Tahzima et al., 2019; Beaver-Kanuya and Harper, 2021). This multiple sampling approach allows more time for the disease and symptoms to develop on previously healthy-looking but already infected plants. In addition, following the virus spread over the years in the fields where it has been detected can provide information on how rapidly the prevalence is changing. Depending on the interim risk assessment conclusions, plants positive for the novel virus may represent a risk requiring their prompt removal to avoid further spread of the disease. If supported by statistical analyses, surveys allow sound evaluation of the association between virus presence and disease development (Adams et al., 2014).

2.3.3. Greenhouse assays

Greenhouse assays are commonly used to assess symptom causality and symptomatology. Since mixed infection can occur in the source material, working on single viral species during greenhouse assays is essential. In many cases, particularly with graft inoculation, these techniques cannot separate viruses in a mixed infection. One solution to this limitation is the use of infectious clones during the biological characterization of the virus as proposed in the previous framework (Massart et al., 2017). Nevertheless, constructing infectious clone can be a complex and time-consuming step that is not possible for all plant viruses. Other approaches include use of differential hosts (i.e., with known virus resistance or preference to viruses), vector or seed transmission, and thermotherapy. The novel virus can be inoculated to indicator plants, host plant candidates, or other cultivars of the original host species by mechanical or graft inoculation (Wu et al., 2020). Identifying the mode(s) of transmission of the virus would facilitate the greenhouse assays, which the bibliographical research (elaborated in step 2.2.1) may give clues before being experimentally tested. Other factors to consider when designing a greenhouse assay are availability of detection tests for the novel virus, host range, choice of indicator plant, developmental stage of the plant during the inoculation, greenhouse climate conditions, availability of space, and greenhouse biosecurity or biosafety level required for the experiment, as well as ensuring that the host and indicator plants are virus- or pathogen-free (da Silva et al., 2020; Panno et al., 2020; EPPO PM 7/153 (1), 2022).

2.3.4. Notification and exchanges with other stakeholders

The additional biological information obtained will progressively feed the risk evaluation. It should be shared with stakeholders via, for example, a reporting system or ad hoc meetings with relevant plant health authorities. A meeting between the involved parties can be organized to analyze the new information obtained since the last notification (step 2.2.3), to assess the status of the novel virus as a pest, and if it needs to be regulated (EPPO, 2012; IPPC ISPM 11, 2019; IPPC ISPM 2, 2019; IPPC ISPM 21, 2021). This discussion will help identify further data gaps and to prioritize the research focus as the assessment moves forward. At this point, exchanges with the authorities, risk managers, and stakeholders will allow re-evaluation of risks posed by the virus and reach a provisional decision on its phytosanitary status.

2.4. Completion of data gaps to strengthen the risk evaluation process

At this point, knowledge/data gaps can remain uncompleted resulting in significant uncertainties, with an ensuing need for strengthening and refining the risk evaluation for the virus, especially if it is still considered a phytosanitary priority. During discussions with stakeholders and plant health authorities, these data gaps, which can be of various kinds, should be identified and filled through further field surveys (small- or large-scale) and greenhouse assays. As shown in Figure 3-3, a well-designed field survey can provide missing information on genetic diversity, geographic distribution, incidence and prevalence, severity of the disease (if any), host range, and symptom causality (if any); and a well-designed greenhouse assay can provide additional information on severity of the disease, host range, symptom causality, and transmission mode. Additionally, the researcher may focus on filling more specific data gaps such as the effect of mixed infections, susceptibility of different cultivars and other economically important host plants, the effect of other biotic/abiotic stressors and, if possible, variability of pathogenicity between isolates (Chinnaraja and Viswanathan, 2015).

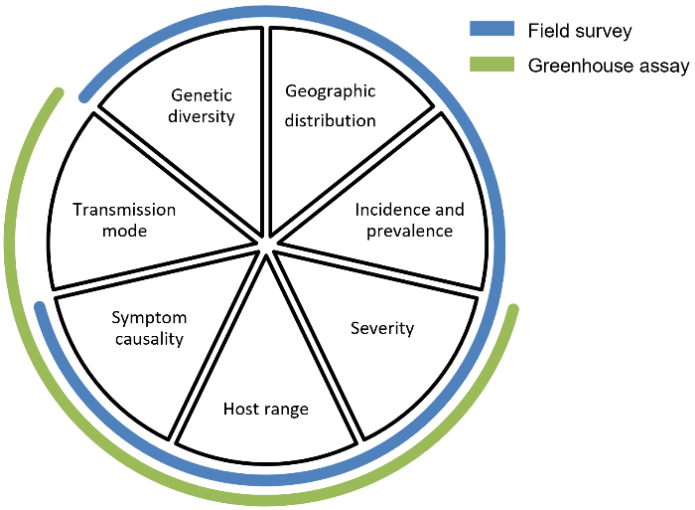


Figure 3-3. Pie chart diagram summarizing the data gaps to be filled in step 2.4 of the framework (adapted from Figure 3-2).

It is possible that despite the efforts described in step 2.3, causation issues were not solved. In that case, further field surveys or greenhouse assays informed by the partial or negative outcome of the early efforts can be envisioned to fill the remaining data gaps on disease causation. This is necessary to assess the priority status of novel viruses and demonstrate their role in disease development. Once the association between presence/absence of the virus and symptoms in the host is confirmed, the aim is to determine the severity of the disease symptoms on host plant species and to estimate the potential yield and economic losses due to said disease, which can be done by (i) surveys to assess the impact on infected plants (Gent et al., 2004), and (ii) greenhouse (or field) inoculation assays (Nancarrow et al., 2021). For practical experimental reasons, the impact on yield and quality might prove challenging to estimate in greenhouse trials. Noticeably, not all viruses will cause a disease, while some may even be beneficial for the host (Roossinck, 2015; Aguilar et al., 2017). Severity and symptoms may vary depending on the other viruses infecting the host or environmental and other external factors (Bertazzon et al., 2017).

It is also essential to study the potential spread of the virus and its geographic distribution, to assess the situation's urgency and the measures to be taken, particularly when considering the need to restrict plant commodities' circulation. For this, large-scale field surveys (both nationally and internationally) are necessary, as well as collecting symptomatic and asymptomatic plants and test them with the diagnostic protocols designed in step 2.1 or with generic tests such as HTS, to better determine the presence of mixed infections. Ideally, this large-scale evaluation could be supported by a network of collaboration with other stakeholders (i.e., plant virologists or plant inspection services) that could facilitate the exchange of samples.

Whenever possible, sampling of geographically and phylogenetically related wild and domesticated plant species should be considered to expand the knowledge of the potential host range, study its prevalence and identify potential reservoirs, since earlier efforts may have provided incomplete information (Wintermantel et al., 2009). However, it is worth noting that the host range is never fully known as novel natural hosts are frequently described after the initial discovery. Closely related crop species of known hosts can potentially become hosts themselves (Xing et al., 2020), thus maybe experimental evolution assays or untargeted virome surveys could be conducted.

Accounting for the genetic variability of a viral population when designing the experiments is essential to improve the inclusiveness of detection tests and study the origin, dynamics, evolution, and phylogenetic relationships of the novel virus (Kutnjak et al., 2014; Kawakubo et al., 2021). This diversity can be studied through whole genome sequencing of isolates obtained from field surveys or by partial genome sequencing of a specific genomic region showing a level of variability.

Knowing the primary transmission mechanism of a virus is advised to properly design a successful greenhouse assay, as well as to evaluate risks and design an efficient disease/pest management strategy. Although difficult to accomplish, from a risk assessment perspective it is important to know about all transmission mechanisms as it can influence the fitness of the novel virus, selection pressures driving resistance and tolerance genes in the host, and viral population structure (Stewart et al., 2005; Pagán et al., 2014; da Silva et al., 2020).

3. Conclusion

The recent reviews of Hou et al. (2020) and Rivarez et al. (2021), as well as the similar analysis done here for Poaceae, highlighted the need for a revision of the previous characterization framework. Conducting similar systematic reviews in other crops might give additional insights on what is the current landscape on plant virus characterization after a first identification in an HTS dataset. Essentially, this revision emphasizes adapting a progressive feedback approach during the risk evaluation process, highlights the growing importance of database mining, proposes as a keystone the disease causal association, and underlines the importance and benefits of data and effort sharing, as well as the advantages to collaborate with other researchers. It is worth noting that this is not a substitution for any decision-support scheme for a pest risk analysis or pest categorization but a complementary document, especially useful for cases where the virus is considered as non-priority or where communication with plant health authorities may be more limited. Researchers may be overwhelmed with the number of findings in HTS datasets, or have a lack of resources and time, so this revision serves as an outline of the prioritization steps to go further than the genomic and molecular characterization of a novel virus, which will produce more useful and practical information for plant health authorities and producers/grower

associations. In the long term, advances in artificial intelligence, machine learning technologies and bioinformatic tools will facilitate the process of characterization of a novel virus and reduce the resources and time needed. For example, SRA mining tools have the potential to complement conventional global surveys although a discussion around the technical and ethical considerations of using such methods should be held between the scientific community and stakeholders. Similar to the aim of the previous framework, this work should be regularly adapted by authorities to help rationalize and accelerate decisions on the most relevant actions at the different stages of virus discovery and characterization. In addition, this characterization framework can also be adapted for different countries not only for plant viruses but also for animal viruses and even other pathogens.

References

Adams, I. P., Fox, A., Boonham, N., Massart, S., and De Jonghe, K. (2018). The impact of high throughput sequencing on plant health diagnostics. Eur. J. Plant Pathol. 152, 909–919. doi: 10.1007/s10658-018-1570-0

Adams, I. P., Skelton, A., Macarthur, R., Hodges, T., Hinds, H., Flint, L., et al. (2014). Carrot yellow leaf virus is associated with carrot internal necrosis. PLoS One 9:e109125. doi: 10.1371/journal.pone.0109125

Aguilar, E., Cutrona, C., del Toro, F. J., Vallarino, J. G., Osorio, S., Pérez-Bueno, M. L., et al. (2017). Virulence determines beneficial trade-offs in the response of virus-infected plants to drought via induction of salicylic acid. Plant Cell Environ. 40, 2909–2930. doi: 10.1111/pce.13028

Al Rwahnih, M., Daubert, S., Úrbez-Torres, J. R., Cordero, F., and Rowhani, A. (2011). Deep sequencing evidence from single grapevine plants reveals a virome dominated by mycoviruses. Arch. Virol. 156, 397–403. doi: 10.1007/s00705-010-0869-8

Ammar, E.-D., Khlifa, E. A., Mahmoud, A., Abol-Ela, S. E., and Peterschmitt, M. (2007). Evidence for multiplication of the leafhopper-borne maize yellow stripe virus in its vector using ELISA and dot-blot hybridization. Arch. Virol. 152, 489–494. doi: 10.1007/s00705-006-0877-x

Babaian, A., and Edgar, R. C. (2021). Ribovirus classification by a polymerase barcode sequence. bioRxiv. doi: 10.1101/2021.03.02.433648

Babayan, S. A., Orton, R. J., and Streicker, D. G. (2018). Predicting reservoir hosts and arthropod vectors from evolutionary signatures in RNA virus genomes. Science 362, 577–580. doi: 10.1126/science.aap9072

Beaver-Kanuya, E. E., and Harper, S. J. (2021). Seasonal fluctuation and host species affect tobacco ringspot virus detection. doi: 10.21203/rs.3.rs-369469/v1 [preprint].

Bertazzon, N., Forte, V., Filippin, L., Causin, R., Maixner, M., and Angelini, E. (2017). Association between genetic variability and titre of grapevine pinot gris virus with disease symptoms. Plant Pathol. 66, 949–959. doi: 10.1111/ppa.12639

Bester, R., Cook, G., Breytenbach, J. H. J., Steyn, C., De Bruyn, R., and Maree, H. J. (2021). Towards the validation of high-throughput sequencing (HTS) for routine plant virus diagnostics: measurement of variation linked to HTS detection of citrus viruses and viroids. Virol. J. 18:61. doi: 10.1186/s12985-021-01523-1

Blystad, D.-R., van der Vlugt, R., Alfaro-Fernández, A., del Carmen Córdoba, M., Bese, G., Hristova, D., et al. (2015). Host range and symptomatology of Pepino mosaic virus strains occurring in Europe. Eur. J. Plant Pathol. 143, 43–56. doi: 10.1007/s10658-015-0664-1

Boonham, N., Kreuze, J., Winter, S., van der Vlugt, R., Bergervoet, J., Tomlinson, J., et al. (2014). Methods in virus diagnostics: from ELISA to next generation sequencing. Virus Res. 186, 20–31. doi: 10.1016/j.virusres.2013.12.007

Cabrera Mederos, D., Debat, H., Torres, C., Portal, O., Jaramillo Zapata, M., Trucco, V., et al. (2022). An unwanted association: the threat to papaya crops by a novel Potexvirus in Northwest Argentina. Viruses 14:2297. doi: 10.3390/v14102297

Candresse, T., and Fuchs, M. (2020). Closteroviridae. In: Chinchester: eLS. John Wiley & Sons, Ltd, pp. 1–10.

Carvajal-Yepes, M., Cardwell, K., Nelson, A., Garrett, K. A., Giovani, B., Saunders, D. G. O., et al. (2019). A global surveillance system for crop diseases. Science 364, 1237–1239. doi: 10.1126/science.aaw1572

Chabannes, M., and Iskra-Caruana, M.-L. (2013). Endogenous pararetroviruses—a reservoir of virus infection in plants. Curr. Opin. Virol. 3, 615–620. doi: 10.1016/j.coviro.2013.08.012

Chiapello, M., Rodríguez-Romero, J., Nerva, L., Forgia, M., Chitarra, W., Ayllón, M. A., et al. (2020). Putative new plant viruses associated with Plasmopara viticola-infected grapevine samples. Ann. Appl. Biol. 176, 180–191. doi: 10.1111/aab.12563

Chikh-Ali, M., Tran, L. T., Price, W. J., and Karasev, A. V. (2020). Effects of the age-related resistance to potato virus Y in potato on the systemic spread of the virus, incidence of the potato tuber necrotic ringspot disease, tuber yield, and translocation rates into progeny tubers. Plant Dis. 104, 269–275. doi: 10.1094/PDIS-06-19-1201-RE

Chinnaraja, C., and Viswanathan, R. (2015). Variability in yellow leaf symptom expression caused by the sugarcane yellow leaf virus and its seasonal influence in sugarcane. Phytoparasitica 43, 339–353. doi: 10.1007/s12600-015-0468-z

Claverie, S., Ouattara, A., Hoareau, M., Filloux, D., Varsani, A., Roumagnac, P., et al. (2019). Exploring the diversity of Poaceae-infecting mastreviruses on Reunion Island using a viral metagenomics-based approach. Sci. Rep. 9:12716. doi: 10.1038/s41598-019-49134-9

da Silva, W., Kutnjak, D., Xu, Y., Xu, Y., Giovannoni, J., Elena, S. F., et al. (2020). Transmission modes affect the population structure of potato virus Y in potato. PLoS Pathog. 16:e1008608. doi: 10.1371/journal.ppat.1008608

Delahaye, C., and Nicolas, J. (2021). Sequencing DNA with nanopores: troubles and biases. PLoS One 16:e0257521. doi: 10.1371/journal.pone.0257521

Di Serio, F., Ambrós, S., Sano, T., Flores, R., and Navarro, B. (2018). Viroid diseases in pome and stone fruit trees and Koch's postulates: a critical assessment. Viruses 10:612. doi: 10.3390/v10110612

Edgar, R. C., Taylor, J., Lin, V., Altman, T., Barbera, P., Meleshko, D., et al. (2022). Petabase-scale sequence alignment catalyses viral discovery. Nature 602, 142–147. doi: 10.1038/s41586-021-04332-2

Ehrlich, P. (1913). Address in pathology, ON CHEMIOTHERAPY: delivered before the seventeenth international congress of medicine. Br. Med. J. 2, 353–359. doi: 10.1136/bmj.2.2746.353

Eng, C. L. P., Tong, J. C., and Tan, T. W. (2016). Distinct host tropism protein signatures to identify possible zoonotic influenza a viruses. PLoS One 11:e0150173. doi: 10.1371/journal.pone.0150173

Eng, C. L. P., Tong, J. C., and Tan, T. W. (2017). Predicting zoonotic risk of influenza a viruses from host tropism protein signature using random forest. Int. J. Mol. Sci. 18:1135. doi: 10.3390/ijms18061135

EPPO (2012). Decision-support scheme for an express Pest risk analysis. EPPO Bull. 42, 457–462. doi: 10.1111/epp.2591

EPPO PM 7/151 (1) (2022). Considerations for the use of high throughput sequencing in plant health diagnostics1. EPPO Bull. 52, 619–642. doi: 10.1111/epp.12884

EPPO PM 7/153 (1) (2022). Mechanical inoculation of test plants. EPPO Bull. 52, 693–703. doi: 10.1111/epp.12901

Evans, A. S. (1976). Causation and disease: the Henle-Koch postulates revisited. Yale J. Biol. Med. 49, 175–195.

Evans, A. S. (1991). Causation and disease: effect of technology on postulates of causation. Yale J. Biol. Med. 64, 513–528.

Falkow, S. (1988). Molecular Koch's postulates applied to microbial pathogenicity. Rev. Infect. Dis. 10, S274–S276. doi: 10.1093/cid/10.supplement_2.s274

Fiallo-Olivé, E., and Navas-Castillo, J. (2019). Tomato chlorosis virus, an emergent plant virus still expanding its geographical and host ranges. Mol. Plant Pathol. 20, 1307–1320. doi: 10.1111/mpp.12847

Fox, A. (2020). Reconsidering causal association in plant virology. Plant Pathol. 69, 956–961. doi: 10.1111/ppa.13199

Fraile, A., and García-Arenal, F. (2016). Environment and evolution modulate plant virus pathogenesis. Curr. Opin. Virol. 17, 50–56. doi: 10.1016/j.coviro.2016.01.008

Fredericks, D. N., and Relman, D. A. (1996). Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. Clin. Microbiol. Rev. 9, 18–33. doi: 10.1128/CMR.9.1.18

Fuentes, S., Gibbs, A. J., Adams, I. P., Hajizadeh, M., Kreuze, J., Fox, A., et al. (2022). Phylogenetics and evolution of potato virus V: another Potyvirus that originated in the Andes. Plant Dis. 106, 691–700. doi: 10.1094/PDIS-09-21-1897-RE

Gaafar, Y. Z. A., Rabenstein, F., Zia, A., Gaafar, A.-R. Z. A., and Ziebell, H. (2021). Molecular characterisation of a new tenuivirus from Festuca sp. Virus Res. 304:198509. doi: 10.1016/j.virusres.2021.198509

García-Arenal, F., and Zerbini, F. M. (2019). Life on the edge: geminiviruses at the interface between crops and wild plant hosts. Annu. Rev. Virol. 6, 411–433. doi: 10.1146/annurev-virology-092818-015536

Gent, D. H., Schwartz, H. F., and Khosla, R. (2004). Distribution and incidence of Iris yellow spot virus in Colorado and its relation to onion plant population and yield. Plant Dis. 88, 446–452. doi: 10.1094/PDIS.2004.88.5.446

Giovani, B., Blümel, S., Lopian, R., Teulon, D., Bloem, S., Galeano Martínez, C., et al. (2020). Science diplomacy for plant health. Nat. Plants 6, 902–905. doi: 10.1038/s41477-020-0744-x

Giovani, B., Boutigny, A.-L., Djelouah, K., Fox, A., and D'onghia, A. M. (2022). Plant health research collaboration in the Mediterranean region: case studies on citrus tristeza virus, tomato brown rugose fruit virus and Xylella fastidiosa. Phytopathol. Mediterr. 61, 525–530. doi: 10.36253/phyto-14085

Hak, H., and Spiegelman, Z. (2021). The tomato brown rugose fruit virus movement protein overcomes tm-22 resistance in tomato while attenuating viral transport. Mol. Plant Microbe Interact. 34, 1024–1032. doi: 10.1094/MPMI-01-21-0023-R

Hall, R. J., Wang, J., Todd, A. K., Bissielo, A. B., Yen, S., Strydom, H., et al. (2014). Evaluation of rapid and simple techniques for the enrichment of viruses prior to metagenomic virus discovery. J. Virol. Methods 195, 194–204. doi: 10.1016/j.jviromet.2013.08.035

Hammond, J., Adams, I. P., Fowkes, A. R., McGreig, S., Botermans, M., van Oorspronk, J. J. A., et al. (2021). Sequence analysis of 43-year old samples of Plantago lanceolata show that plantain virus X is synonymous with Actinidia virus X and is widely distributed. Plant Pathol. 70, 249–258. doi: 10.1111/ppa.13310

Harper, G., Hull, R., Lockhart, B., and Olszewski, N. (2002). Viral sequences integrated into plant genomes. Annu. Rev. Phytopathol. 40, 119–136. doi: 10.1146/annurev.phyto.40.120301.105642

Hasiów-Jaroszewska, B., Boezen, D., and Zwart, M. P. (2021). Metagenomic studies of viruses in weeds and wild plants: a powerful approach to characterise variable virus communities. Viruses 13:1939. doi: 10.3390/v13101939

Hill, A. B. (1965). The environment and disease: association or causation? Proc. R. Soc. Med. 58, 295–300. doi: 10.1177/003591576505800503

Hily, J.-M., Poulicard, N., Candresse, T., Vigne, E., Beuve, M., Renault, L., et al. (2020). Datamining, genetic diversity analyses, and phylogeographic reconstructions redefine the worldwide evolutionary history of grapevine pinot gris virus and grapevine berry inner necrosis virus. Phytobiomes J. 4, 165–177. doi: 10.1094/PBIOMES-10-19-0061-R

Hou, W., Li, S., and Massart, S. (2020). Is there a "Biological Desert" with the discovery of new plant viruses? A retrospective analysis for new fruit tree viruses. Front. Microbiol. 11:2953. doi: 10.3389/fmicb.2020.592816

Huebner, R. J. (1957). The virologist's dilemma. Ann. N. Y. Acad. Sci. 67, 430–438. doi: 10.1111/j.1749-6632.1957.tb46066.x

IPPC ISPM 11 (2019). Pest risk analysis for quarantine pests. Rome: IPPC, FAO.

IPPC ISPM 2 (2019). Framework for pest risk analysis. Rome: IPPC, FAO.

IPPC ISPM 21 (2021). Pest risk analysis for regulated non-quarantine pests. Rome: IPPC, FAO.

James, A. P., Geijskes, R. J., Dale, J. L., and Harding, R. M. (2011). Development of a novel rolling-circle amplification technique to detect banana streak virus that also discriminates between integrated and episomal virus sequences. Plant Dis. 95, 57–62. doi: 10.1094/PDIS-07-10-0519

Johnson, R. T., and Gibbs, C. J. Jr. (1974). Koch's postulates and slow infections of the nervous system. Arch. Neurol. 30, 36–38. doi: 10.1001/archneur.1974.00490310038006

Katsiani, A. T., Pappi, P., Olmos, A., Efthimiou, K. E., Maliogka, V. I., and Katis, N. I. (2018). Development of a real-time RT-PCR for the universal detection of LChV1 and study of the seasonal fluctuation of the viral titer in sweet cherry cultivars. Plant Dis. 102, 899–904. doi: 10.1094/PDIS-01-17-0107-RE

Katz, K., Shutov, O., Lapoint, R., Kimelman, M., Brister, J. R., and O'Sullivan, C. (2022). The sequence read archive: a decade more of explosive growth. Nucleic Acids Res. 50, D387–D390. doi: 10.1093/nar/gkab1053

Kawakubo, S., Gao, F., Li, S., Tan, Z., Huang, Y.-K., Adkar-Purushothama, C. R., et al. (2021). Genomic analysis of the brassica pathogen turnip mosaic potyvirus reveals its spread along the former trade routes of the silk road. Proc. Natl. Acad. Sci. 118:e2021221118. doi: 10.1073/pnas.2021221118

King, A., Lefkowitz, E. J., Adams, M. J., and Carstens, E. B. (2011). "Genus tenuivirus" in Virus taxonomy (San Diego, CA: Elsevier)

Koloniuk, I., Thekke-Veetil, T., Reynard, J.-S., Mavrič Pleško, I., Přibylová, J., Brodard, J., et al. (2018). Molecular characterization of divergent closterovirus isolates infecting ribes species. Viruses 10:369. doi: 10.3390/v10070369

Kutnjak, D., Silvestre, R., Cuellar, W., Perez, W., Müller, G., Ravnikar, M., et al. (2014). Complete genome sequences of new divergent potato virus X isolates and discrimination between strains in a mixed infection using small RNAs sequencing approach. Virus Res. 191, 45–50. doi: 10.1016/j.virusres.2014.07.012

Kwibuka, Y., Bisimwa, E., Blouin, A. G., Bragard, C., Candresse, T., Faure, C., et al. (2021). Novel ampeloviruses infecting cassava in Central Africa and the South-West Indian Ocean Islands. Viruses 13:1030. doi: 10.3390/v13061030

Lauber, C., and Seitz, S. (2022). Opportunities and challenges of data-driven virus discovery. Biomol. Ther. 12:1073. doi: 10.3390/biom12081073

Le Provost, G., Iskra-Caruana, M.-L., Acina, I., and Teycheney, P.-Y. (2006). Improved detection of episomal banana streak viruses by multiplex immunocapture PCR. J. Virol. Methods 137, 7–13. doi: 10.1016/j.jviromet.2006.05.021

Lebas, B., Adams, I., Al Rwahnih, M., Baeyen, S., Bilodeau, G. J., Blouin, A. G., et al. (2022). Facilitating the adoption of high-throughput sequencing technologies as a plant pest diagnostic test in laboratories: a step-by-step description. EPPO Bull. 52, 394–418. doi: 10.1111/epp.12863

Lee, B. D., Neri, U., Oh, C. J., Simmonds, P., and Koonin, E. V. (2022a). ViroidDB: a database of viroids and viroid-like circular RNAs. Nucleic Acids Res. 50, D432–D438. doi: 10.1093/nar/gkab974

Lee, B. D., Neri, U., Roux, S., Wolf, Y. I., Camargo, A. P., Krupovic, M., et al. (2022b). A vast world of viroid-like circular RNAs revealed by mining metatranscriptomes. bioRxiv. doi: 10.1101/2022.07.19.500677

Lefkowitz, E. J., Dempsey, D. M., Hendrickson, R. C., Orton, R. J., Siddell, S. G., and Smith, D. B. (2018). Virus taxonomy: the database of the international committee on taxonomy of viruses (ICTV). Nucleic Acids Res. 46, D708–D717. doi: 10.1093/nar/gkx932

Li, J., Zhang, S., Li, B., Hu, Y., Kang, X., Wu, X., et al. (2019). Machine learning methods for predicting human-adaptive influenza a viruses based on viral nucleotide compositions. Mol. Biol. Evol. 37, 1224–1236. doi: 10.1093/molbev/msz276

Maclot, F., Candresse, T., Filloux, D., Malmstrom, C. M., Roumagnac, P., van der Vlugt, R., et al. (2020). Illuminating an ecological blackbox: using high throughput sequencing to characterize the plant virome across scales. Front. Microbiol. 11:2575. doi: 10.3389/fmicb.2020.578064

Maclot, F. J., Debue, V., Blouin, A. G., Fontdevila-Pareta, N., Tamisier, L., Filloux, D., et al. (2021). Identification, molecular and biological characterization of two novel secovirids in wild grass species in Belgium. Virus Res. 298:198397. doi: 10.1016/j.virusres.2021.198397

Marais, A., Murolo, S., Faure, C., Brans, Y., Larue, C., Maclot, F., et al. (2021). Sixty years from the first disease description, a novel badnavirus associated with chestnut mosaic disease. Phytopathology 111, 1051–1058. doi: 10.1094/PHYTO-09-20-0420-R

Marais, A., Umber, M., Filloux, D., Gomez, R.-M., Faure, C., Pavis, C., et al. (2020). Yam asymptomatic virus 1, a novel virus infecting yams (Dioscorea spp.) with significant prevalence in a germplasm collection. Arch. Virol. 165, 2653–2657. doi: 10.1007/s00705-020-04787-0

Maree, H. J., Fox, A., Al Rwahnih, M., Boonham, N., and Candresse, T. (2018). Application of HTS for routine plant virus diagnostics: state of the art and challenges. Front. Plant Sci. 9:1082. doi: 10.3389/fpls.2018.01082

Massart, S., Adams, I., Al Rwahnih, M., Baeyen Steve, S., Bilodeau Guillaume, J., Blouin, G. J., et al. (2022). Guidelines for the reliable use of high throughput sequencing technologies to detect plant pathogens and pests. Zenodo 2. doi: 10.5281/zenodo.7142136

Massart, S., Brostaux, Y., Barbarossa, L., Batlle, A., Cesar, V., Dutrecq, O., et al. (2009). Interlaboratory evaluation of two reverse-transcriptase polymeric chain reaction-based methods for detection of four fruit tree viruses. Ann. Appl. Biol. 154, 133–141. doi: 10.1111/j.1744-7348.2008.00281.x

Massart, S., Candresse, T., Gil, J., Lacomme, C., Predajna, L., Ravnikar, M., et al. (2017). A framework for the evaluation of biosecurity, commercial, regulatory, and scientific

impacts of plant viruses and viroids identified by NGS technologies. Front. Microbiol. 8:45. doi: 10.3389/fmicb.2017.00045

Massart, S., Olmos, A., Jijakli, H., and Candresse, T. (2014). Current impact and future directions of high throughput sequencing in plant virus diagnostics. Virus Res. 188, 90–96. doi: 10.1016/j.virusres.2014.03.029

Moury, B., Fabre, F., Hébrard, E., and Froissart, R. (2017). Determinants of host species range in plant viruses. J. Gen. Virol. 98, 862–873. doi: 10.1099/jgv.0.000742

Murphy, J. F., and Bowen, K. L. (2006). Synergistic disease in pepper caused by the mixed infection of cucumber mosaic virus and pepper mottle virus. Phytopathology 96, 240–247. doi: 10.1094/PHYTO-96-0240

Nancarrow, N., Aftab, M., Hollaway, G., Rodoni, B., and Trębicki, P. (2021). Yield losses caused by barley yellow dwarf virus-PAV infection in wheat and barley: a three-year field study in south-eastern Australia. Microorganisms 9:645. doi: 10.3390/microorganisms9030645

Neri, U., Wolf, Y. I., Roux, S., Camargo, A. P., Lee, B., Kazlauskas, D., et al. (2022). A five-fold expansion of the global RNA virome reveals multiple new clades of RNA bacteriophages. bioRxiv. doi: 10.1101/2022.02.15.480533

Olmedo-Velarde, A., Loristo, J., Kong, A., Waisen, P., Wang, K.-H., Hu, J., et al. (2022). Examination of the virome of taro plants affected by a lethal disease, the alomae-bobone virus complex, in Papua New Guinea. Viruses 14:1410. doi: 10.3390/v14071410

Olmos, A., Boonham, N., Candresse, T., Gentit, P., Giovani, B., Kutnjak, D., et al. (2018). High-throughput sequencing technologies for plant pest diagnosis: challenges and opportunities. EPPO Bull. 48, 219–224. doi: 10.1111/epp.12472

Pagán, I. (2018). The diversity, evolution and epidemiology of plant viruses: a phylogenetic view. Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis. 65, 187–199. doi: 10.1016/j.meegid.2018.07.033

Pagán, I., Montes, N., Milgroom, M. G., and García-Arenal, F. (2014). Vertical transmission selects for reduced virulence in a plant virus and for increased resistance in the host. PLoS Pathog. 10:e1004293. doi: 10.1371/journal.ppat.1004293

Panno, S., Caruso, A. G., Barone, S., Lo Bosco, G., Rangel, E. A., and Davino, S. (2020). Spread of tomato brown rugose fruit virus in sicily and evaluation of the spatiotemporal dispersion in experimental conditions. Agronomy 10:834. doi: 10.3390/agronomy10060834

Pfeiffer, F., Gröber, C., Blank, M., Händler, K., Beyer, M., Schultze, J. L., et al. (2018). Systematic evaluation of error rates and causes in short samples in next-generation sequencing. Sci. Rep. 8:10950. doi: 10.1038/s41598-018-29325-6

Rivarez, M. P. S., Pecman, A., Bačnik, K., Maksimović, O., Vučurović, A., Seljak, G., et al. (2023). In-depth study of tomato and weed viromes reveals undiscovered plant virus diversity in an agroecosystem. Microbiome. 11:60. doi: 10.1186/s40168-023-01500-6

Rivarez, M. P. S., Vučurović, A., Mehle, N., Ravnikar, M., and Kutnjak, D. (2021). Global advances in tomato virome research: current status and the impact of high-throughput sequencing. Front. Microbiol. 12:1064. doi: 10.3389/fmicb.2021.671925

Rivers, T. M. (1937). Viruses and Koch's Postulates1. J. Bacteriol. 33, 1–12. doi: 10.1128/jb.33.1.1-12.1937

Rong, W., Rollin, J., Hanafi, M., Roux, N., and Massart, S. (2022). Validation of high throughput sequencing as virus indexing test for Musa germplasm: performance

criteria evaluation and contamination monitoring using an alien control. PhytoFrontiers™. doi: 10.1094/PHYTOFR-03-22-0030-FI

Roossinck, M. (2015). A new look at plant viruses and their potential beneficial roles in crops. Mol. Plant Pathol. 16, 331–333. doi: 10.1111/mpp.12241

Roossinck, M., Martin, D. P., and Roumagnac, P. (2015). Plant virus metagenomics: advances in virus discovery. Phytopathology 105, 716–727. doi: 10.1094/PHYTO-12-14-0356-RVW

Rozado-Aguirre, Z., Adams, I., Collins, L., Fox, A., Dickinson, M., and Boonham, N. (2016). Detection and transmission of carrot torrado virus, a novel putative member of the torradovirus genus. J. Virol. Methods 235, 119–124. doi: 10.1016/j.jviromet.2016.05.018

Sequence Read Archive (SRA) Data Working Group | DPCPSI. (2021). [WWW Document]. Available at: https://dpcpsi.nih.gov/council/sradwg (accessed 11.9.22)

Sharma, V., Lefeuvre, P., Roumagnac, P., Filloux, D., Teycheney, P.-Y., Martin, D. P., et al. (2020). Large-scale survey reveals pervasiveness and potential function of endogenous geminiviral sequences in plants. Virus Evol. 6:veaa071. doi: 10.1093/ve/veaa071

Shi, M., Lin, X.-D., Tian, J.-H., Chen, L.-J., Chen, X., Li, C.-X., et al. (2016). Redefining the invertebrate RNA virosphere. Nature 540, 539–543. doi: 10.1038/nature20167

Simmonds, P., Adams, M. J., Benkő, M., Breitbart, M., Brister, J. R., Carstens, E. B., et al. (2017). Virus taxonomy in the age of metagenomics. Nat. Rev. Microbiol. 15, 161–168. doi: 10.1038/nrmicro.2016.177

Sõmera, M., Kvarnheden, A., Desbiez, C., Blystad, D.-R., Sooväli, P., Kundu, J. K., et al. (2019). Sixty years after the first description: genome sequence and biological characterization of European wheat striate mosaic virus infecting cereal crops. Phytopathology 110, 68–79. doi: 10.1094/PHYTO-07-19-0258-FI

Staginnus, C., Gregor, W., Mette, M. F., Teo, C. H., Borroto-Fernández, E. G., Machado, M. L. D. C., et al. (2007). Endogenous pararetroviral sequences in tomato (Solanum lycopersicum) and related species. BMC Plant Biol. 7:24. doi: 10.1186/1471-2229-7-24

Stewart, A. D., Logsdon, J. M., and Kelley, S. E. (2005). An empirical study of the evolution of virulence under both horizontal and vertical transmission. Evolution 59, 730–739. doi: 10.1111/j.0014-3820.2005.tb01749.x

Tahzima, R., Foucart, Y., Peusens, G., Beliën, T., Massart, S., and De Jonghe, K. (2019). New sensitive and fast detection of little cherry virus 1 using loop-mediated isothermal amplification (RT-LAMP). J. Virol. Methods 265, 91–98. doi: 10.1016/j.jviromet.2018.12.019

Tahzima, R., Haegeman, A., Massart, S., and Hébrard, E. (2021). Flexible spandrels of the global plant virome: Proteomic-wide evolutionary patterns of structural intrinsic protein disorder elucidate modulation at the functional virus-host interplay. Progress in molecular biology and translational science, 183, 355–409. doi: 10.1016/bs.pmbts.2021.06.007

Tang, Q., Song, Y., Shi, M., Cheng, Y., Zhang, W., and Xia, X.-Q. (2015). Inferring the hosts of coronavirus using dual statistical models based on nucleotide composition. Sci. Rep. 5:17155. doi: 10.1038/srep17155

Temple, C., Blouin, A. G., De Jonghe, K., Foucart, Y., Botermans, M., Westenberg, M., et al. (2022). Biological and genetic characterization of physostegia chlorotic mottle virus

in Europe based on host range, location, and time. Plant Dis. 106, 2797–2807. doi: 10.1094/PDIS-12-21-2800-RE

Vainio, E. J., Chiba, S., Ghabrial, S. A., Maiss, E., Roossinck, M., Sabanadzovic, S., et al. (2018). 2018. ICTV virus taxonomy profile: Partitiviridae. J. Gen. Virol. 99, 17–18. doi: 10.1099/jgv.0.000985

Verbeek, M., Dullemans, A. M., and van der Vlugt, R. A. A. (2017). Aphid transmission of lettuce necrotic leaf curl virus, a member of a tentative new subgroup within the genus Torradovirus. Virus Res. 241, 125–130. doi: 10.1016/j.virusres.2017.02.008

Vlugt, R., Verbeek, M., Dullemans, A., Wintermantel, W., Cuellar, W., Fox, A., et al. (2015). Torradoviruses. Annu. Rev. Phytopathol. 53, 485–512. doi: 10.1146/annurev-phyto-080614-120021

Wintermantel, W. M., Hladky, L. L., Cortez, A. A., and Natwick, E. T. (2009). A new expanded host range of cucurbit yellow stunting disorder virus includes three agricultural crops. Plant Dis. 93, 685–690. doi: 10.1094/PDIS-93-7-0685

Wu, J., Zhang, S., Atta, S., Yang, C., Zhou, Y., Di Serio, F., et al. (2020). Discovery and survey of a new mandarivirus associated with leaf yellow mottle disease of citrus in Pakistan. Plant Dis. 104, 1593–1600. doi: 10.1094/PDIS-08-19-1744-RE

Xing, F., Hou, W., Massart, S., Gao, D., Li, W., Cao, M., et al. (2020). RNA-Seq reveals hawthorn tree as a new natural host for apple necrotic mosaic virus, possibly associated with hawthorn mosaic disease. Plant Dis. 104, 2713–2719. doi: 10.1094/PDIS-11-19-2455-RE

Young, F., Rogers, S., and Robertson, D. L. (2020). Predicting host taxonomic information from viral genomes: a comparison of feature representations. PLoS Comput. Biol. 16:e1007894. doi: 10.1371/journal.pcbi.1007894

Zayed, A. A., Wainaina, J. M., Dominguez-Huerta, G., Pelletier, E., Guo, J., Mohssen, M., et al. (2022). Cryptic and abundant marine viruses at the evolutionary origins of Earth's RNA virome. Science 376, 156–162. doi: 10.1126/science.
abm5847

Zerbini, F. M., Briddon, R. W., Idris, A., Martin, D. P., Moriones, E., Navas-Castillo, J., et al. (2017). ICTV virus taxonomy profile: geminiviridae. J. Gen. Virol. 98, 131–133. doi: 10.1099/jgv.0.000738

Supplementary Material 1

The present review provides in depth analysis of information provided by a representative set of publications reporting the discovery of new viruses infecting Poaceae species. The 28 publications reporting newly discovered Poaceae viruses were screened according to several categories of information adapted from Hou et al. (2020): genome completeness, primer design, association with symptoms, gene and genome diversity, large-scale survey, co-infection, host range, latent infection, transmission, infectivity, inoculation in greenhouse, local survey of prevalence, and herbaceous indicator (Table 3-1).

A total of 42 complete genomes of new viral species detected by HTS were reported, among of which, 40 (95%) have been confirmed by designing of specific primers, amplifying the targeted genomic region and sequencing of the amplicon. For panicum ecklonii associated virus (PeaV), lolium erenne associated virus (LpaV), holcus lanatus associated virus (HlaV), and stipagrostic associated virus (SaV) only the complete genome and specific primers were reported (Richet et al., 2019). Furthermore, the diversity of the genome or specific genes was analysed for 43% (18) and 31% (13) of new viruses respectively, including, for example, rice latent virus 1 (RLV1) and 2 (RLV2) (Kraberger et al., 2017) and barley yellow dwarf virus OYV (BYDV-OYV) (Sõmera et al., 2021). This diversity analysis is sometimes impossible to achieve. Indeed, one publication, having discovered wheat umbra-like virus (WULV) and wheat-associated vipovirus (WaVPV), did not report information on genome or gene diversity because only a single isolate was detected (Redila et al., 2021).

This analysis showed that majority of samplings was carried out on symptomatic wild and cultivated Poaceae species (17/28 studies). While for 7 studies, samples were collected regardless of whether they were symptomatic or asymptomatic. Co-infection has been reported for 33% of viruses and 13% were present as a single infection, including for example maize yellow dwarf virus (MYDV)-like polerovirus (Massawe et al., 2018), maize-associated pteridovirus (MaPV) (Read et al., 2019a) and morogoro maize-associated virus (MMaV) (Read et al., 2019b). For the rest of the viruses, it was not stated in the publications. On the other hand, latent infections were reported for 24% of new viral species, for example for sorghum mastrevirus-associated alphasatellite (SMasA) (Claverie et al., 2020). Nevertheless, results for silvergrass cryptic virus 1 (SgCV-1) are not yet conclusive because no obvious symptoms were observed and its impact in mixed infections is unclear (Costa et al., 2022). Large-scale field surveys were conducted for 40% (17) of the viruses, including, for example, bromus-associated circular DNA viruses 1-4 (BasCV1-4) and trifolium-associated circular DNA virus 1 (TasCV-1) (Kraberger et al., 2015).

Inoculation to herbaceous indicators was carried out for 4 (10%) viruses, although it was only successfull for tall oatgrass mosaic virus (TOgMV) (Hassan et al., 2014). For wheat yellow stunt associated betaflexivirus (WYSaBV) herbaceous indicators

remained symptomless and it was not detected by RT-PCR at 30 days post inoculation (Fu et al., 2021). Association with symptoms was demonstrated for 43% (18) of viruses: wheat yellow stunt-associated betaflexivirus (WYSaBV), sugarcane striate virus (SCStV) (Boukari et al., 2017), wheat dwarf India virus, maize streak Réunion virus (Pande et al., 2012), TOgMV, stentaphrum nepovirus (SteNV) (Tran et al., 2021), festuca stripe-associated virus (FSaV) (Gaafar et al., 2021), wheat virus Q (WVQ), maize-associated totivirus (MATV) (Chen et al., 2016), wheat stripe mosaic virus (WhSMV), sugarcane umbra-like virus (SULV) (Tahir et al., 2021), alopecurus myosuroides partitivirus 1 and 2 (AMPV1-2), alopecurus myosuroides varisacovirus (AMVV1), wheat leaf yellowing associated virus (WLYaV) (Zhang et al., 2017), wheat yellow striate virus (WYSV) (Liu et al., 2018), wheat yellow dwarf virus (WYDV) (Guo et al., 2022) and miscanthus yellow fleck virus (Bolus et al., 2020).

Studies to identify alternative hosts have been carried out for 11 virus species (26%). A single additional host has been identified for 3 viruses: Digitaria didactyla for SteNV (Tran et al., 2021), Avena sativa for TOgMV (Hassan et al., 2014) and Urochloa maxima for sorghum arundinaceum associated virus (SAAV) (Claverie et al., 2019). For the other viruses, several host plants were identified. Transmission pathways were reported for 9 viral species (21%): potential natural vectors have been identified for wheat dwarf india virus (Psammotettix sp.) and wheat stripe mosaic virus (Polymyxa graminis) (Kumar et al., 2012; Valente et al., 2019). poaceae Liege nepovirus A was successfully transmitted through seed (Maclot et al., 2021). In addition, soil transmission of wheat virus Q was successful although the exact pathway was not identified (Kondo et al., 2021). Alopecurus myosuroides partitivirus 1 and 2 were mentioned to be transmitted by pollen (Sabbadin et al., 2017). Only 3 and 10 publications completed the discovery with local or large-scale survey, respectively.

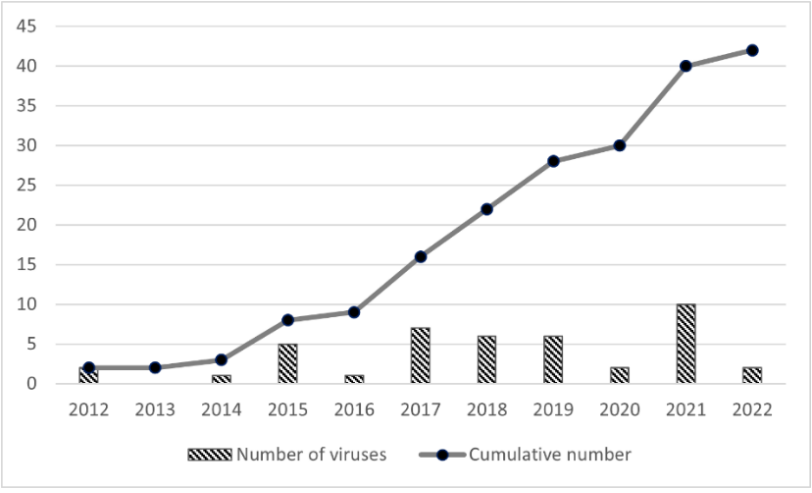


Figure 3-4. Evolution of the number of novel viruses infecting *Poaceae* discovered by HTS since 2012.

Table 3-1. Studies done for each newly identified *Poaceae* viruses for each characterization category, as defined by Hou et al. (2020). Boxes in blue represent actions taken and boxes in white actions not taken.

	Publication	Year	Virus	Complete genome	Primer design	Association with symptoms	Genome diversity	Large-scale survey	Co-infection	Gene diversity	Host range	Latent infection	Transmission	Infectivity	Inoculation in greenhouse	Local survey of prevalence	Herbaceous indicator	Electron microscopy
1	Tran et al	2021	Stenotaphrum nepovirus (SteNV)	■	■	■	■	■	■		■					■		■
2	Gaafar et al	2021	Festuca stripe-associated virus (FSaV)	■	■	■								■	■			■
3	Richet et al	2018	Stipagrostis associated virus (SaV)	■	■													
4	Richet et al	2018	Panicum ecklonii associated virus (PeaV)	■	■													
5	Richet et al	2018	Lolium perenne associated virus (LpaV)	■	■													
6	Richet et al	2018	Holcus lanatus associated virus (HlaV)	■	■													
7	Maclot et al	2021	Poaceae Liege virus 1 (PoLV1)	■	■						■	■	■			■		
8	Maclot et al	2021	Poaceae Liege nepovirus A (PolNVA)	■	■						■	■	■			■		
9	Fu et al	2021	Wheat yellow stunt-associated betaflexivirus (WYSaBV)	■		■	■		■	■				■	■		■	■
10	Kondo et al	2021	Wheat virus Q (WVQ)	■	■	■	■		■	■	■	■	■	■	■			
11	Kraberger et al	2017	Rice latent virus 1 (RLV1)	■	■		■	■				■						
12	Kraberger et al	2017	Rice latent virus 2 (RLV2)	■	■		■	■				■						
13	Sabbadin et al	2017	Alopecurus myosuroides partitivirus 1 (AMPV1)	■	■	■		■	■		■	■	■					
14	Sabbadin et al	2017	Alopecurus myosuroides partitivirus 2 (AMPV2)	■	■	■		■	■		■	■	■					
15	Sabbadin et al	2017	Alopecurus myosuroides varisacovirus (AMVV1)	■	■	■		■	■		■	■	■					
16	Boukari et al	2017	Sugarcane striate virus (SCStV)	■	■	■	■	■										
17	Chen et al	2016	Maize-associated totivirus (MATV)	■	■	■												
18	Claverie et al	2019	Eleusina indica associated virus (EIAV)	■	■		■			■	■							
19	Claverie et al	2019	Sorghum arundinaceum associated virus (SAAV)	■	■		■			■	■							
20	Claverie et al	2019	Melinis repens associated virus (MeRAV)	■	■		■			■	■							
21	Claverie et al	2020	Sorghum mastrevirus-associated alphasatellite (SMasA)	■	■		■		■			■						
22	Somera et al	2021	Barley yellow dwarf virus OYV (BYDV-OYV)	■	■		■		■	■								
23	Kumar et al	2012	Wheat dwarf India virus (WDIV)	■	■	■							■	■	■			
24	Massawe et al	2018	Mayze yellow dwarf virus (MYDV)-like polerovirus	■	■			■	■									
25	Pande et al	2012	Maize streak Réunion virus (MSRV)	■		■	■			■								
26	Read et al	2019	Maize-associated pteridovirus (MaPV)	■	■				■									
27	Read et al	2019	Morogoro maize-associated virus (MMaV)	■	■			■	■									
28	Valente et al	2019	Wheat stripe mosaic virus (WhSMV)	■	■	■	■	■		■			■					
29	Hassan et al	2014	Tall oatgrass mosaic virus (TOgMV)	■	■	■		■			■			■	■		■	■
30	Kraberger et al	2015	Bromus-associated circular DNA virus 1 (BasCV1)	■	■		■	■		■								
31	Kraberger et al	2015	Bromus-associated circular DNA virus 2 (BasCV2)	■	■		■	■		■								
32	Kraberger et al	2015	Bromus-associated circular DNA virus 3 (BasCV3)	■	■		■	■		■								
33	Kraberger et al	2015	Bromus-associated circular DNA virus 4 (BasCV4)	■	■		■	■		■								
34	Kraberger et al	2015	Trifolium-associated circular DNA virus 1 (TasCV-1)	■	■		■	■		■								
35	Tahir et al	2021	Sugarcane umbra-like virus (SULV)	■	■	■			■									
36	Costa et al	2022	Silvergrass cryptic virus 1 (SgCV-1)	■	■							■				■		
37	Redila et al	2021	Wheat umbra-like virus (WULV)	■	■				■									
38	Redila et al	2021	Wheat-associated vipovirus(WaVPV)	■	■													
39	Zhang et al	2017	Wheat leaf yellowing associated virus (WLYaV)	■	■	■								■	■		■	
40	Liu et al	2018	Wheat yellow striate virus (WYSV)	■	■	■							■	■	■			■
41	Guo et al	2022	Wheat yellow dwarf virus (WYDV)	■	■	■		■	■									
42	Bolus et al	2020	Miscanthus yellow fleck virus (MYFV)	■	■	■								■	■		■	■
			Percentage	100%	95%	43%	43%	40%	33%	31%	26%	24%	21%	19%	19%	10%	10%	10%

References

Bolus, S., Malapi-Wight, M., Grinstead, S.C., Fuentes-Bueno, I., Hendrickson, L., Hammond, R.W., Mollov, D., 2020. Identification and characterization of Miscanthus yellow fleck virus, a new polerovirus infecting Miscanthus sinensis. PLOS ONE 15, e0239199. https://doi.org/10.1371/journal.pone.0239199

Boukari, W., Alcalá-Briseño, R.I., Kraberger, S., Fernandez, E., Filloux, D., Daugrois, J.-H., Comstock, J.C., Lett, J.-M., Martin, D.P., Varsani, A., Roumagnac, P., Polston, J.E., Rott, P.C., 2017. Occurrence of a novel mastrevirus in sugarcane germplasm collections in Florida, Guadeloupe and Réunion. Virol. J. 14, 146. https://doi.org/10.1186/s12985-017-0810-9

Chen, S., Cao, L., Huang, Q., Qian, Y., Zhou, X., 2016. The complete genome sequence of a novel maize-associated totivirus. Arch. Virol. 161, 487–490. https://doi.org/10.1007/s00705-015-2657-y

Claverie, S., Ouattara, A., Hoareau, M., Filloux, D., Varsani, A., Roumagnac, P., Martin, D.P., Lett, J.-M., Lefeuvre, P., 2019. Exploring the diversity of Poaceae-infecting mastreviruses on Reunion Island using a viral metagenomics-based approach. Sci. Rep. 9, 12716. https://doi.org/10.1038/s41598-019-49134-9

Claverie, S., Varsani, A., Hoareau, M., Filloux, D., Roumagnac, P., Martin, D.P., Lefeuvre, P., Lett, J.-M., 2020. Sorghum mastrevirus-associated alphasatellites: new geminialphasatellites associated with an African streak mastrevirus infecting wild Poaceae plants on Reunion Island. Arch. Virol. 165, 1925–1928. https://doi.org/10.1007/s00705-020-04685-5

Costa, L.C., Hu, X., Malapi-Wight, M., O'Connell, M., Hendrickson, L.M., Turner, R.S., McFarland, C., Foster, J., Hurtado-Gonzales, O.P., 2022. Genomic characterization of silvergrass cryptic virus 1, a novel partitivirus infecting Miscanthus sinensis. Arch. Virol. 167, 261–265. https://doi.org/10.1007/s00705-021-05294-6

Fu, S., Zhang, T., He, M., Sun, B., Zhou, X., Wu, J., 2021. Molecular characterization of a novel wheat-infecting virus of the family Betaflexiviridae. Arch. Virol. 166, 2875–2879. https://doi.org/10.1007/s00705-021-05175-y

Gaafar, Y.Z.A., Rabenstein, F., Zia, A., Gaafar, A.-R.Z.A., Ziebell, H., 2021. Molecular characterisation of a new tenuivirus from Festuca sp. Virus Res. 304, 198509. https://doi.org/10.1016/j.virusres.2021.198509

Guo, M., Yuan, X., Wu, N., Liu, Y., Wang, X., 2022. Complete genome sequence of a novel wheat-infecting polerovirus associated with yellowing dwarf disease in China. Arch. Virol. 167, 983–987. https://doi.org/10.1007/s00705-022-05360-7

Hassan, M., Širlová, L., Vacke, J., 2014. Tall oatgrass mosaic virus (TOgMV): a novel member of the genus Tritimovirus infecting Arrhenatherum elatius. Arch. Virol. 159, 1585–1592. https://doi.org/10.1007/s00705-013-1905-2

Hou, W., Li, S., Massart, S., 2020. Is There a "Biological Desert" With the Discovery of New Plant Viruses? A Retrospective Analysis for New Fruit Tree Viruses. Front. Microbiol. 11, 2953. https://doi.org/10.3389/fmicb.2020.592816

Kondo, H., Yoshida, N., Fujita, M., Maruyama, K., Hyodo, K., Hisano, H., Tamada, T., Andika, I.B., Suzuki, N., 2021. Identification of a Novel Quinvirus in the Family Betaflexiviridae That Infects Winter Wheat. Front. Microbiol. 12, 2219. https://doi.org/10.3389/fmicb.2021.715545

Kraberger, S., Farkas, K., Bernardo, P., Booker, C., Argüello-Astorga, G.R., Mesléard, F., Martin, D.P., Roumagnac, P., Varsani, A., 2015. Identification of novel Bromus- and

Trifolium-associated circular DNA viruses. Arch. Virol. 160, 1303–1311. https://doi.org/10.1007/s00705-015-2358-6

Kraberger, S., Geering, A.D.W., Walters, M., Martin, D.P., Varsani, A., 2017. Novel mastreviruses identified in Australian wild rice. Virus Res. 238, 193–197. https://doi.org/10.1016/j.virusres.2017.07.003

Kumar, Jitendra, Singh, S.P., Kumar, Jitesh, Tuli, R., 2012. A novel mastrevirus infecting wheat in India. Arch. Virol. 157, 2031–2034. https://doi.org/10.1007/s00705-012-1359-y

Liu, Y., Du, Z., Wang, H., Zhang, S., Cao, M., Wang, X., 2018. Identification and Characterization of Wheat Yellow Striate Virus, a Novel Leafhopper-Transmitted Nucleorhabdovirus Infecting Wheat. Front. Microbiol. 9.

Maclot, F.J., Debue, V., Blouin, A.G., Fontdevila-Pareta, N., Tamisier, L., Filloux, D., Massart, S., 2021. Identification, molecular and biological characterization of two novel secovirids in wild grass species in Belgium. Virus Res. 198397. https://doi.org/10.1016/j.virusres.2021.198397

Massawe, D.P., Stewart, L.R., Kamatenesi, J., Asiimwe, T., Redinbaugh, M.G., 2018. Complete sequence and diversity of a maize-associated Polerovirus in East Africa. Virus Genes 54, 432–437. https://doi.org/10.1007/s11262-018-1560-5

Pande, D., Kraberger, S., Lefeuvre, P., Lett, J.-M., Shepherd, D.N., Varsani, A., Martin, D.P., 2012. A novel maize-infecting mastrevirus from La Réunion Island. Arch. Virol. 157, 1617–1621. https://doi.org/10.1007/s00705-012-1314-y

Read, D.A., Featherston, J., Rees, D.J.G., Thompson, G.D., Roberts, R., Flett, B.C., Mashingaidze, K., Pietersen, G., Kiula, B., Kullaya, A., Mbega, E., 2019a. Characterization and detection of maize-associated pteridovirus (MaPV), infecting maize (Zea mays) in the Arusha region of Tanzania. Eur. J. Plant Pathol. 154, 1165–1170. https://doi.org/10.1007/s10658-019-01703-4

Read, D.A., Featherston, J., Rees, D.J.G., Thompson, G.D., Roberts, R., Flett, B.C., Mashingaidze, K., Pietersen, G., Kiula, B., Kullaya, A., Mbega, E.R., 2019b. Molecular characterization of Morogoro maize-associated virus, a nucleorhabdovirus detected in maize (Zea mays) in Tanzania. Arch. Virol. 164, 1711–1715. https://doi.org/10.1007/s00705-019-04212-1

Redila, C.D., Prakash, V., Nouri, S., 2021. Metagenomics Analysis of the Wheat Virome Identifies Novel Plant and Fungal-Associated Viral Sequences. Viruses 13, 2457. https://doi.org/10.3390/v13122457

Richet, C., Kraberger, S., Filloux, D., Bernardo, P., Harkins, G.W., Martin, D.P., Roumagnac, P., Varsani, A., 2019. Novel circular DNA viruses associated with Apiaceae and Poaceae from South Africa and New Zealand. Arch. Virol. 164, 237–242. https://doi.org/10.1007/s00705-018-4031-3

Sabbadin, F., Glover, R., Stafford, R., Rozado-Aguirre, Z., Boonham, N., Adams, I., Mumford, R., Edwards, R., 2017. Transcriptome sequencing identifies novel persistent viruses in herbicide resistant wild-grasses. Sci. Rep. 7, 41987. https://doi.org/10.1038/srep41987

Sõmera, M., Massart, S., Tamisier, L., Soováli, P., Sathees, K., Kvarnheden, A., 2021. A Survey Using High-Throughput Sequencing Suggests That the Diversity of Cereal and Barley Yellow Dwarf Viruses Is Underestimated. Front. Microbiol. 12, 673218. https://doi.org/10.3389/fmicb.2021.673218

Tahir, M.N., Bolus, S., Grinstead, S.C., McFarlane, S.A., Mollov, D., 2021. A new virus of the family Tombusviridae infecting sugarcane. Arch. Virol. 166, 961–965. https://doi.org/10.1007/s00705-020-04908-9

Tran, N.T., Teo, A.C., Crew, K.S., Campbell, P.R., Thomas, J.E., Geering, A.D.W., 2021. Genome sequence and geographic distribution of a new nepovirus infecting Stenotaphrum secundatum in Australia. Virus Res. 305, 198554. https://doi.org/10.1016/j.virusres.2021.198554

Valente, J.B., Pereira, F.S., Stempkowski, L.A., Farias, M., Kuhnem, P., Lau, D., Fajardo, T.V.M., Nhani Junior, A., Casa, R.T., Bogo, A., da Silva, F.N., 2019. A novel putative member of the family Benyviridae is associated with soilborne wheat mosaic disease in Brazil. Plant Pathol. 68, 588–600. https://doi.org/10.1111/ppa.12970

Zhang, P., Liu, Y., Liu, W., Cao, M., Massart, S., Wang, X., 2017. Identification, Characterization and Full-Length Sequence Analysis of a Novel Polerovirus Associated with Wheat Leaf Yellowing Disease. Front. Microbiol. 8.

Supplementary Material 2

Physostegia chlorotic mottle virus (PhCMoV) is an alphanucleorhabdovirus discovered by high throughput sequencing on Lamiaceae (Menzel et al., 2018). Subsequently, the virus was detected in nine European countries on nine different host plants belonging to seven plant families. Its presence was associated with severe symptoms on cultivated tomato plants (Temple et al., 2022).

In the frame of its characterization, Serratus (Edgar et al., 2022) was used to scan for the presence of PhCMoV sequences in public RNASeq SRAs. Briefly, a palmID derived from the virus L protein (RNA dependent RNA polymerase, RdRp) was used to search the Serratus palmID database. More than 2660 hits were obtained ranging from 93% palm-identity (E-value=3.5e-79) to 37% palm-identity (4.7e-13). To confirm the presence of PhCMoV, SRA datasets with the best palmID hits (above 90% identity) were downloaded and their reads mapped on the PhCMoV reference genome (NC_055466) using Bowtie2 (Langmead and Salzberg, 2012; Langmead et al., 2009) on Galaxy (Galaxy Version 2.4.5) with default parameters. Such a confirmation of Serratus results should always be performed and the filters (mapping or serratus PalmID) should be adapted according to the diversity in the family of the virus under consideration.

The presence of PhCMoV was detected in five SRA datasets belonging to two bioprojects (PRJNA636634 and PRJNA449559). Associated metadata provided information as to the identity of the sequenced samples, and where and when these samples had been collected and sequenced. Interestingly, these “detections” added considerable knowledge on PhCMoV by expanding its distribution range to a new continent (China, Asia). A high number of reads (> 94939 reads) of four biosamples mapped on the PhCMoV genome with a high genome coverage (>99.4%) (see Table 3-2). In addition, the virus was found associated with two new plant species (Polemonium pulcherrimum and Lavandula angustifolia). The detection of PhCMoV in P. pulcherrimum (biosample: SAMN15153850: 305133 PhCMoV reads, Table 3-2) was important because it could represent an expansion of the virus host range to a new family (Polemoniaceae), while L. angustifolia does not represent an expansion of the host range because it belongs to the family Lamiaceae (already known). The P. pulcherrimum sample was collected in a botanical garden in Germany. Furthermore, to better understand the evolution and distribution of the virus, the recovered PhCMoV genomes could be included in an updated PhCMoV phylogenetic tree.

Nevertheless, such findings should be taken with caution, especially in instances where the discovery of the virus of interest in an SRA dataset is unexpected. It is thus recommended to contact the researchers responsible for the bioproject and confirm the information regarding the origin of the plant material and sample context, including potential presence of symptoms. In addition, testing by RT-PCR the original biological material, if any is remaining, would allow to confirm the presence of the virus in a new host or new geographic location.

Without the Serratus interface, it would be very cumbersome to scan the huge number of SRA datasets publicly available. Such an approach allows the serendipitous identification of novel host-virus combinations as it was unlikely that P. pulcherrimum would be considered as a potential plant host for PhCMoV during the bibliographical research (Step 2.1). But as always when important diagnostic results are at stake, care should be taken when trying to interpret such results.

Table 3-2. Number of reads that map to the reference of PhCMoV (NC_055466) for each SRA dataset and their associated metadata.

Bioproject (NCBI)	Run_id (NCBI)	Biosample_id (NCBI)	Palm_id	Plant Family	Host plant	Country of origin	Number of mapped reads	% identity NC_055466	horizontal Coverage
PRJNA636634	SRR12002078	SAMN15153850	u3491	Polemoniaceae	Polemonium pulcherrimum	Germany	305193	97%	100%
PRJNA449559	SRR6980628	SAMN08716985	u3491	Lamiaceae	Lavandula angustifolia	China	94939	99%	99.6%
PRJNA449559	SRR6980626	SAMN08717030	u3491	Lamiaceae	Lavandula angustifolia	China	103126	99%	99.5%
PRJNA449559	SRR6980629	SAMN08716984	u3491	Lamiaceae	Lavandula angustifolia	China	154723	99%	99.6%
PRJNA449559	SRR6980627	SAMN08717026	u3491	Lamiaceae	Lavandula angustifolia	China	103673	99%	99.4%

References

Edgar, R.C., Taylor, J., Lin, V., Altman, T., Barbera, P., Meleshko, D., Lohr, D., Novakovsky, G., Buchfink, B., Al-Shayeb, B., Banfield, J.F., de la Peña, M., Korobeynikov, A., Chikhi, R., Babaian, A. (2022). Petabase-scale sequence alignment catalyses viral discovery. Nature, 602: 142–147.

Langmead, B., Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. Nature Methods, 9: 357–359.

Langmead, B., Trapnell, C., Pop, M., Salzberg, S.L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biology, 10: R25.

Menzel, W., Richert-Pöggeler, K., Winter, S., Knierim, D. (2018). Characterization of a nucleorhabdovirus from Physostegia. Acta Horticulturae, 1193: 29–38.

Temple, C., Blouin, A.G., De Jonghe, K., Foucart, Y., Botermans, M., Westenberg, M., Schoen, R., Gentit, P., Visage, M., Verdin, E., Wipf-Scheibel, C., Ziebell, H., Gaafar, Y.Z.A., Kutnjak, D., Vučurović, A., Rivarez, M.P.S., Richert-Pöggeler, K.R., Ulrich, R., Zia, A., Yan, X.-H., Massart, S. (2022). Biological and genetic characterization of Physostegia chlorotic mottle virus in Europe based on host range, location, and time. Plant Disease.

Chapter 4

Virome scanning of apple and pear germplasm collections by HTS

Synopsis

This chapter is dedicated to the study of the viruses present in the apple and pear collections preserved at the Walloon Agricultural Research Centre (CRA-W), as well as pear collections from Switzerland, preserved at Agroscope Changins, and Slovenia, preserved at Kozjanski Park.

The first part of Chapter 4 is based on the Disease Note, “First reports of Apple luteovirus 1, Apple rubodvirus 1 and Apple hammerhead viroid infecting apples in Belgium”, and Article 2, “Virome scanning of pear germplasm collections identifies a new Velarivirus and extends the geographical spread of three other pear viruses”. The disease note showcases the preliminary, or pilot, survey that was carried out on apple trees at the CRA-W to test and set up the methodology for the more extensive survey and study done as part of Article 2. During the pilot study, two viruses and one viroid were detected for the first time in apple trees in Belgium.

The chapter’s second part and main body are based on Article 3 and extensively describe the results of larger-scale virome scanning using a methodology adapted from the double-stranded RNA (dsRNA) extraction protocol from Marais et al. (2018). In addition, a cross-contamination ratio and threshold were defined and used to assess the crosstalk between the pools of samples during the various manipulations of plant material (i.e., extraction, library preparation, or sequencing) and, therefore, to distinguish between true positives and likely false positives. A new virus species was discovered, and its preliminary characterization started according to the framework presented in Chapter 3.

Chapter 4 – part 1:

First reports of Apple luteovirus 1, Apple rubodvirus 1 and Apple hammerhead viroid infecting apples in Belgium

Adapted from the article 2: Fontdevila et al. (2022). New Disease Reports. DOI: 10.1002/ndr2.12076 List and affiliation of co-authors:

Núria Fontdevila Pareta[1], Marc Lateur[2], Stéphan Steyer[3], Arnaud G. Blouin[1,4], Sébastien Massart[1]

[1] *University of Liège, Gembloux Agro-Bio Tech, Plant Pathology Laboratory, 5030, Gembloux, Belgium*
[2] *Centre Wallon de Recherches Agronomiques (CRA-W), Plant & Forest Biodiversity & Breeding, 5030, Gembloux, Belgium*
[3] *Centre Wallon de Recherches Agronomiques (CRA-W), Crops & Forest Health, 5030, Gembloux, Belgium*
[4] *Agroscope, Plant Protection Department, 1260, Nyon, Switzerland*

For a pilot study on germplasm collections, leaves were collected from six apple trees (Malus domestica) numbered Q9, Q27, Q35, Q37, Q39 and Q41 in a Belgian experimental orchard (CRA-W) in June 2019 and June 2020. At the time of sampling and during subsequent visits, no viral symptoms were observed on the trees. However, Apple luteovirus 1 (ALV-1) has been identified in trees with Rapid apple decline, Apple rubodvirus 1 (ARWV-1) is associated with apple rubbery wood disease, and Apple hammerhead viroid (AHVd) infection presents variable symptoms including swelling or limb flattening.

Double-stranded RNA (dsRNA) was extracted from all trees except Q9, reverse-transcribed and amplified (RT-PCR) for high-throughput sequencing following the protocol described by Marais et al. (2018). Total RNA was extracted from tree Q9 using the RNeasy Plant Mini Kit (Qiagen, Germany). Library preparation was performed at GIGA (University of Liege, Belgium) with the TruSeq Total RNA Library Preparation Kit (Illumina, USA) for the total RNA sample, and with the NEBNext Ultra II DNA library prep kit (New England BioLabs, USA) for the dsRNA samples. The Illumina Novaseq platform (2 × 150 nt) was used for sequencing, reads were assembled using SPAdes, and viral contigs were identified by BLASTn against the NCBI database. In Q9, three contigs of 7,210 nt (GenBank Accession No. OK398019), 1,606 nt (OK398020) and 1,004 nt (OK398021) showed high identity with the three genome segments of ARWV-1: 98.8%, 98.2%, and 98.2% identity; and 99.9%, 99.6%, and 99.5% coverage with isolates 982-11 segment L (NC_055390), 4342–5 segment M (MF062137), and 1148-13 segment S (MF062132), respectively. Four contigs of 2,629 nt (OK424912), 717 nt (OK424913), 407 nt, and 243 nt showed high identity to ALV-1 isolate PA8 (96.8%; 96.5%; 97.7%; and 93.2% identity with NC_040680, covering 67% of the genome) in Q39. In Q39, another contig (OK398018) of 433 nt was identified as AHVd with 94.7% identity and 100% coverage of isolate SD17_3-3 (MK188692). AHVd was also detected in all other samples. To confirm these detections, RNA extracted from original trees and eight surrounding trees of Q9 and Q39 were tested by RT-PCR, with MangoTaqTM DNA Polymerase (Bioline, Meridian Bioscience, UK) and primers ARWaV-1L3639F - ARWaV-1L4058R for ARWV-1 (Rott et al., 2018), ALuDetF6-ALuDetR6 for ALV-1 (Liu et al., 2018), and AHVd-88F-AHVd-331R for AHVd (Serra et al., 2018). Q9 tested positive for ARWV-1 and Q39 tested positive for ALV-1. All original trees were positive for AHVd by RT-PCR. PCR products of ARWV-1 (Q9), ALV-1 (Q39), and AHVd (Q37) were sequenced by Sanger sequencing at Macrogen Europe, confirming the presence of ARWV-1 (OK216005, 98.4% nt identity to NC_055390), ALV-1 (OK216004, 96.9% nt identity NC_040680), and AHVd (OK216006, 96.3% nt identity to MK188692).

In conclusion, this is the first report of ARWV-1, ALV-1, and AHVd in Belgium. The trees showed no visible symptoms of viral infection, suggesting that the symptoms associated with these viruses and viroid are likely to be variable or latent across different cultivars and environments.

References

Liu, H., Wu, L., Nikolaeva, E., Peter, K., Liu, Z., Mollov, D. et al. (2018) Characterization of a new apple luteovirus identified by high-throughput sequencing. Virology Journal, 15, 85. https://doi.org/10.1186/s12985-018-0998-3

Marais, A., Faure, C., Bergey, B. & Candresse, T. (2018) Viral double-stranded RNAs (dsRNAs) from plants: Alternative nucleic acid substrates for high-throughput sequencing. In: Pantaleo, V. & Chiumenti, M. (eds) Viral Metagenomics: Methods and Protocols. Methods in Molecular Biology. New York, NY: Springer, pp. 45–53. https://doi.org/10.1007/978-1-4939-7683-6_4

Rott, M.E., Kesanakurti, P., Berwarth, C., Rast, H., Boyes, I., Phelan, J. et al. (2018) Discovery of negative-sense RNA viruses in trees infected with apple rubbery wood disease by next-generation sequencing. Plant Disease, 102, 1254–1263. https://doi.org/10.1094/PDIS-06-17-0851-RE

Serra, P., Messmer, A., Sanderson, D., James, D. & Flores, R. (2018) Apple hammerhead viroid-like RNA is a bona fide viroid: Autonomous replication and structural features support its inclusion as a new member in the genus Pelamoviroid. Virus Research, 249, 8–15. https://doi.org/10.1016/j.virusres.2018.03.001

Chapter 4 – part 2:

Virome scanning of pear germplasm collections identifies a new Velarivirus and extends the geographical spread of three other pear viruses

Adapted from the article 3: Fontdevila et al. (2024). Phytobiomes Journal.
List and affiliation of co-authors:

Núria Fontdevila Pareta[1], Carole Gailly[1], Arnaud G. Blouin[2], Beatrix Buchmann[3], Markus Buenter[3], Thierry Candresse[4], Nathalie Dubuis[2], Denis Kutnjak[5], Marc Lateur[6], Anja Pecman[5], Stéphan Steyer[7], Sébastien Massart[1]

[1] University of Liège, Gembloux Agro-Bio Tech, Plant Pathology Laboratory, 5030, Gembloux, Belgium
[2] Agroscope, Plant Protection Department, 1260, Nyon, Switzerland
[3] Agroscope,, Plant Health Service, 8820, Wädenswil, Switzerland
[4] Univ. Bordeaux, INRAE, UMR 1332 BFP, 33882, Villenave d'Ornon cedex, France
[5] National Institute of Biology, Department of Biotechnology and Systems Biology, 1000, Ljubljana, Slovenia
[6] Centre Wallon de Recherches Agronomiques (CRA-W), Plant & Forest Biodiversity & Breeding, 5030, Gembloux, Belgium
[7] Centre Wallon de Recherches Agronomiques (CRA-W), Crops & Forest Health, 5030, Gembloux, Belgium

Abstract

In this study, an extensive virome investigation was performed on a germplasm collection of apple and pear trees from CRA-W (Gembloux, Belgium). In total, six apple trees and 128 pear trees were analyzed as pools using high-throughput sequencing (HTS) techniques, and/or tested individually for targeted viruses by RT-PCR. During the virome survey, a novel velarivirus was identified in several asymptomatic trees while four known viruses were detected. High-throughput sequencing (HTS) techniques and bioinformatics tools were used to assemble the genome of the new virus. The pear germplasm collection from Kozjanski Park (Slovenia) and a viral collection from Agroscope (Nyon, Switzerland) were also surveyed for the new pear virus and for three known viruses (CiVA, ARWV-1, and ARWV-2) to study their prevalence and geographic distribution. In Belgium, the new velarivirus was detected by RT-PCR in six of the 99 sampled trees (6%) and citrus virus A (CiVA) in 49 (49%) of them; in Slovenia four of the six trees sampled (67%) were positive for CiVA; and in Switzerland four of the nine trees sampled (44%) were positive for CiVA and 1 (11%) for apple rubbery wood virus 1 and 2 (ARWV-1 and -2). This study, combined pooled HTS analyses to maximize the number of germplasm tested and targeted RT-PCR tests on individual samples for accurate detection. It reports and describes a new velarivirus discovered in pear trees and first detections of CiVA in Belgium, Switzerland and Slovenia, and ARWV-1 and -2 in Switzerland.

Keywords: Virology, Plant pathology, Metagenomics, Molecular biology

1. Introduction

Pear cultivation is a vital sector of the global horticultural industry, contributing to both domestic and international markets. Belgium is one of the top 10 global pear-producing countries, ranking just behind Turkey as the top producer in Europe in 2021. Worldwide, the area under pear cultivation decreased in 2015, but it has been increasing since then (FAO, 2024). In comparison, in the European Union the pear cultivation area has diminished, except in the Netherlands and Belgium. In these two countries, the most produced pear variety is 'Conference', representing 53% of the total pear production. The high production of only a few specific cultivars results in a narrow genetic diversity of cultivated crops, which can threaten the resilience of future production in the face of changes in biotic and abiotic stresses (Shahzad et al., 2021). Planting and using certified plant material in commercial orchards decrease the risk of having severe yield losses due to diseases of viral origin. Still, it should not be neglected that with changes in the environment, emerging viruses and diseases might become more common (Trebicki, 2020).

To tackle this issue, it is essential to better characterize the viruses that can infect pear trees and understand if environmental changes might affect their impact on pear cultivars. Beyond commercial cultivars, genetic diversity is preserved in germplasm collections. Germplasm collections are vital for conserving plant genetic diversity and as a source of genetic material and new traits for breeding, to ensure and sustain the future adaptability of food production. This is why it is necessary to characterize these resources, including their viral status. The Walloon Agricultural Research Centre (CRA-W) owns and develops an extensive collection of plant genetic resources, specializing in old cultivars, which are the basis of research to study their nutritional and biological properties and to promote their use in breeding programs. For pear, the CRA-W fruit tree collection includes more than 500 accessions conserved in ex-situ, and in-situ orchards.

Viral infections in fruit trees can be difficult to observe as symptoms vary greatly depending on the plant variety and the viral isolate (Katsiani et al., 2018; Maliogka et al., 2018). In addition, viruses in fruit trees are transmitted via vegetative propagation such as grafting, ensuring their transmission to the next generation, and raising the risk of accumulating multiple infections over time in a single plant. Hence, detecting plant viruses is essential, though challenging, for the safe propagation and cultivation of fruit trees. Molecular, serological, and biological assays are used to detect plant viruses, and each has its set of advantages and disadvantages (Boonham et al., 2014). High-throughput sequencing (HTS) technologies present great opportunities for virus discovery, detection, identification, and characterization in fruit trees, as they can potentially pinpoint every putative viral agent present in a sample without any prior knowledge of plant origin or symptomatology (Massart et al., 2014).

Pear vein yellows is the most common viral disease of pear. It is caused by apple stem pitting virus (ASPV) infection, without significant effects on growth and yield

when ASPV is present alone (Jelkmann and Paunovic, 2011). However, when ASPV is present in a co-infection with some other viruses or phytoplasmas, significant growth reduction may occur (Yanase et al., 1989). Other viruses commonly infecting pear trees are apple stem grooving virus (ASGV) and apple chlorotic leaf spot virus (ACLSV) (Massart et al., 2011; Yaegashi et al., 2011).

In pear trees, a recent review showed that from 2011 to 2020 only two novel viruses have been discovered using HTS technologies (Hou et al., 2020). In addition, a new robigovirus was identified by HTS in pear trees, and tentatively named pomes virus Greece (PVGR) (Costa et al., 2022). This number is very low compared to apple trees, for which 15 new viruses have been discovered by HTS during the same period (Hou et al., 2020).

The family *Closteroviridae* includes viruses with long filamentous virions of 650 to 2,200 nanometers (nm), and large positive-sense single-stranded RNA genome (up to 19.3 kb). Their current taxonomy is based on the evolutionary histories of the three proteins used for their classification: heat shock protein 70 homolog (HSP70h), RNA-dependent RNA polymerase (RdRp), and the coat protein (CP). Additionally, their genomes encode a duplicated, but divergent, copy of the capsid protein called the minor capsid protein (CPm) (Fuchs et al., 2020). Currently, species belonging to the family *Closteroviridae* (n=57) are classified in seven genera: *Ampelovirus*, *Bluvavirus*, *Closterovirus*, *Crinivirus*, *Menthavirus*, *Olivavirus*, and *Velarivirus*. Velariviruses infect primarily woody hosts and, in most cases, do not induce any apparent symptoms. Mechanical or seed transmission has not been reported for any virus from this genus. On the other hand, a vector has been identified for a single species: areca palm velarivirus 1 (ArPV1) was recently shown to be associated with the yellow leaf disease of Betel palm (*Areca catechu*) and to be transmitted by two mealybugs of the *Pseudococcideae* family (*Ferrisia virgata* and *Pseudococcus cryptus*) (Zhang et al., 2022).

The present study aimed to use HTS technologies for a better characterization of the viruses present in the CRA-W pear germplasm collection.An analysis of the diversity of viral infections in a small selection from two additional collections, in Switzerland and Slovenia, was also performed. The characterization of the virome of the selected collections led to the identification of a novel virus and the report and molecular detection of recently described viruses in new geographical locations. The newly described virus identified during the study was characterized following the optimized scientific and regulatory framework for the characterization and risk analysis of newly discovered plant viruses and viroids (Fontdevila Pareta et al., 2023).

2. Materials and methods

2.1. Plant material origin and outline of the tests conducted

The sampling strategy was designed to take into account the possible heterogenous distribution of plant viruses within a pear tree, following a previously published strategy (Kummert et al., 2004). For each tree, one leaf at each cardinal point at two different heights of the canopy was collected. Therefore, a sample was comprised of eight leaves from one tree. Before extraction using the double-stranded RNA (dsRNA) protocol (see section 2.2.1), generally, four samples (0.75 g each) were mixed into a pool. Then, before sequencing, pools were tagged and mixed into libraries (see section 2.2.1). The collections screened are in the open air without protective nets. The collection at the Centre Wallon de Recherches Agronomiques (CRA-W) in Gembloux (Belgium) is distributed across various orchards, although for this study all samples were collected from the same orchard. In total, 128 pear trees from the CRA-W collection were sampled and analyzed, including 65 pear trees sampled and analyzed by HTS, in a total of 17 pools gathered in seven libraries, and, for the field survey, 99 pear trees were sampled and analyzed by reverse transcription PCR (RT-PCR) (Supplementary Table 4-4). Notably, the trees showed no symptoms of viral infection at the time of sampling. Sampling in the collection of the CRA-W was done in June 2021 for samples that were analyzed with HTS and in May 2023 for samples analyzed by RT-PCR. To validate key detections by HTS, total RNAs (see section 2.2.2) from samples collected in June 2021 were also re-extracted and tested by RT-PCR two years later. Additionally, during spring 2023, the distribution of the newly discovered virus, tentatively named Pyrus virus A (PyVA) within a tree was studied by testing phloem, flowers, and leaves from the four cardinal points of a positive tree. From the collection at Kozjanski Park in Slovenia, 6 pear trees were sampled in May 2021 and tested by reverse transcription polymerase chain reaction (RT-PCR). From the collection at Agroscope in Switzerland, 9 pear trees were sampled in October 2022 and tested by RT-PCR.

2.2. Alien control strategy

An external alien control was used to monitor cross-contamination between samples and to aid in the differentiation between false and true positives, as proposed by Rong et al. (2023) for *Musa* spp. and in the guidelines for the use of HTS in the detection of plant pathogens and pests (Massart et al., 2022). As described by Massart et al. (2022), in plant virus diagnostics, an external alien control corresponds to a plant sample containing one or several viruses (called alien viruses) that are not expected to be present in the tested samples. Thus, the detection of reads from an alien virus in a sample can be considered as a contamination from the alien control. In this study, leaves of a bean plant infected with three endornaviruses (Phaseolus vulgaris virus 1 (PvEV-1), 2 (PvEV-2), and 3 (PvEV-3)) were used as external alien control because their host range is restricted to *Phaseolus vulgaris*.

2.3. Extraction protocols and sequencing

2.3.1. Double-stranded RNA extraction and sequencing

Double-stranded RNA (dsRNA) was extracted from leaf tissue, reverse-transcribed, and amplified to be submitted to HTS, adapting the protocol described by Marais et al. (2018). The extraction protocol was adapted to upscale the extraction buffer and reagent volumes fourfold, so a total weight of 3 g of frozen sample was used for the extraction. In those cases where the number of samples per pool varied, the weight of each sample within the pool was adapted accordingly. Once the starting plant material was ground with liquid nitrogen, the powder was transferred to a 15 ml tube (Greiner bio-one International GmbH) containing the extraction buffer (4 ml of 2x STE, 280 µl of 20% SDS, 160 µl of sodium bentonite, and 5.7 ml of phenol-TE saturated). After adding the powdered sample to the extraction buffer, the tubes were agitated for 30 min on a horizontal shaker and centrifuged for 15 min at 3,000 g. Then, the aqueous phase was transferred to a 1.5 ml Eppendorf tube and centrifuged at 10,000 g for 20 min. After centrifugation, 1 ml of the aqueous phase was transferred to a new 1.5 ml tube, and the rest was kept at -80ºC. Then, dsRNAs were purified by two series of cellulose chromatography, between which a nuclease and proteinase K treatment was performed, as described in Marais et al. (2018).

Complementary DNA (cDNA) synthesis was performed for each sample by denaturing 5 µl of purified dsRNAs and 4.5 µl of diethyl pyrocarbonate (DEPC) treated water at 99ºC for 5 min and then keeping the samples on ice for 1 min. Then, 2 µM of dodeca linkers (François et al., 2018) and DEPC-treated water were added for a total volume of 10.5 µl, and the samples were incubated at 95ºC for 5 min, followed by 1 min on ice. The next mix was composed of 1 mM of dNTPs, 1x reaction buffer, 10 U Superscript III Reverse Transcriptase (Invitrogen), 1 U/µl RNaseOUT Recombinant RNase Inhibitor (Invitrogen), and 10 mM DTT in a 20 µl volume reaction, which was added to each tube. After incubating at 25ºC for 10 min and at 42ºC for 60 min, the RT was inactivated at 70ºC for 5 min, followed by 2 min on ice. The cDNA was purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA), following the manufacturer's instructions. For the amplification step, 5 µl of cDNA were mixed with 1 µM of the multiplex identifier (MID) (François et al., 2018), 1x reaction buffer, 0.50 µl dNTPs, and 1.25 U of DreamTaq polymerase (Thermo Fisher Scientific) for a total 50 µl volume reaction. The tubes were heated at 94ºC for 1 min, at 65ºC for 0 s, 72ºC for 45 s, with a slope of 5ºC per second, followed by 40 cycles of 94ºC for 0 s, 45ºC for 0 s, 72ºC for 5 min (same slope); 72ºC for 5 min, and 37ºC for 5 min. The PCR products were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel), following the manufacturer's instructions.

Library preparation to add the Illumina adapters and prepare the samples for sequencing was performed at the Center of Biomedical Research of Liège University (GIGA, Liège, Belgium) using the TruSeq PCR-free library preparation kit and

sequenced with the Illumina Novaseq sequencing platform, with a read length of 150 base pairs (bp) paired-end.

2.3.2. Total RNA extraction and sequencing

Total RNAs were extracted using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA), following the manufacturer's instructions. Before sequencing, purified RNAs were then treated with amplification grade DNase I (Invitrogen Life Technologies) by adding 1 µl DNase I (1 U/ µl) and 1 µl 10x DNase I reaction buffer for 1 µg of RNA sample in a 10 µl reaction. The samples were incubated for 15 min at room temperature, and the DNase I was inactivated by adding 1 µl of 25mM EDTA solution and incubating at 65ºC for 10 min. Library preparation was performed at the Interdisciplinary Center of Biomedical Research of Liège University (GIGA, Liège, Belgium) with the TruSeq Stranded Total RNA Library Prep Plant (Illumina). After quantification and quality control, the prepared libraries were sequenced with the Illumina NextSeq 500 sequencing platform, with a read length of 150 base pairs (bp) paired-end.

2.4. HTS data analyses

After demultiplexing (Lebas et al., 2022), the sequencing reads' quality was checked using FastQC in Galaxy Europe (https://usegalaxy.eu) (The Galaxy Community, 2022). Then, the following analyses were done on Geneious Prime 2022 (Biomatters Ltd, Auckland, New Zealand). Reads were merged and cleaned by trimming the bases with quality below 30 and removing reads with length below 35 nucleotides, using BBDuk Adapter/Quality Trimming (Kechin et al., 2017) version 38.84. Duplicated reads were removed using Dedupe Duplicate Read Remover (Bushnell et al., 2017) version 38.84 with k-mer seed length set to 31. Following the quality control, trimming, and read cleaning steps, a *de novo* assembly of reads into contigs was performed using RNA SPAdes v. 3.15.5, with default parameters. Contigs of potential viral origin were annotated using tBLASTx against the viral RefSeq database from NCBI (nt) downloaded in April 2023 (release number 216). Mapping to reference genomes from NCBI or reconstructed genomes from *de novo* assembly was done using Geneious Prime 2022, allowing for 10 % mismatches for Citrus virus A (CiVA, *Coguvirus eburi*) (RNA 1: OR825541, RNA 2: OR825542) and apple rubbery wood virus 1 (ARWV-1, *Rubodvirus mali*) (segment L: OK398019, segment M: OK398020, segment S: OK398021); and 20% mismatches for apple stem pitting virus (ASPV) (NC_003462), apple chlorotic leaf spot virus (ACLSV) (NC_001409), phaseolus vulgaris endornavirus 1 (PvEV-1) (NC_039217), phaseolus vulgaris endornavirus 2 (PvEV-2) (NC_038422), phaseolus vulgaris endornavirus 3 (PvEV-3) (NC_040558), and putative virus Pyrus virus A (PyVA) (OR887735), to take into genetic variability within each virus species. The conserved protein domains and the protein functional analysis were predicted using InterProScan (Paysan-Lafosse et al., 2023).

To set a threshold for cross-contamination, the mapped reads per kilobase per million (RPKM) were calculated according to Mortazavi et al. (2008), allowing the normalization and comparison of the viruses' detection from each pool (Figure 4-1a). Based on RPKM values of the alien viruses, the cross-contamination ratios between each sample (from 1 to n samples) and the alien control (ratio alien, $RA_{a\ 1\rightarrow n}$) was obtained by dividing the RPKM of the alien virus in the sample (true contamination / false positive; $RPKM_{a\ 1\rightarrow n}$) by the RPKM of the alien virus in the external alien control library (true positive; $RPKM_{a\ max}$) (Figure 4-1b). The ratios were calculated independently for the 3 alien viruses: PvEV-1, 2, and 3 (3n ratios in total). For each detected virus, a cross-contamination ratio was also calculated for each sample (ratio virus, $RV_{x\ 1\rightarrow n}$) by dividing the RPKM of the virus in the sample ($RPKM_{x\ 1\rightarrow n}$) by the maximal RPKM of the virus among the samples ($RPKM_{x\ max}$) (Figure 4-1c), which was considered as the likely source of contamination. Viruses with $RV_{x\ 1\rightarrow n}$ below the threshold for cross-contamination were considered likely false positives (FP) in the pool, and viruses with $RV_{x\ 1\rightarrow n}$ above the threshold were considered true positives (TP).

a) $$RPKM = \frac{number\ of\ mapped\ reads\ on\ the\ virus\ genome}{\frac{virus\ genome\ length}{1{,}000} \times \frac{total\ number\ of\ reads\ for\ the\ sample}{1{,}000{,}000}}$$

b) $$RA_{a\ 1\rightarrow n} = \frac{RPKM_{a\ 1\rightarrow n}}{RPKM_{a\ max}} \times 100$$

c) $$RV_{x\ 1\rightarrow n} = \frac{RPKM_{x\ 1\rightarrow n}}{RPKM_{x\ max}} \times 100$$

Figure 4-1. a) Formula used to calculate the reads per kilobase per million (RPKM) applied for each detected virus in each sample. b) Formula of the cross-contamination ratio of the alien control for each alien virus. "$RA_{a\ 1\rightarrow n}$" is the ratio for each alien virus (PvEV-1, 2, and 3) in the corresponding sample (n samples in total), "$RPKM_{a\ 1\rightarrow n}$" is the RPKM value of the reads mapped to the alien virus reference genome for each of the n samples, and "$RPKM_{a\ max}$" is the RPKM mapped to the alien virus reference genome in the alien control library (fixed for each alien virus). c) Formula of the cross-contamination ratio of the analyzed samples for each virus in each sample. "$RV_{x\ 1\rightarrow n}$" is the ratio for each virus (with x corresponding to the name of the virus), "$RPKM_{x\ 1\rightarrow n}$" is the RPKM value of the reads mapped to mapped to each virus reference genome independently in n samples, and "$RPKM_{x\ max}$" are the highest RPKM mapped to a virus reference genome (variable for each virus).

2.5. Phylogenetic tree reconstruction

Using the heat shock protein 70 homolog (HSP70h) amino acid(aa) sequence of known viruses within the family *Closteroviridae* and that from the reconstructed genome of PyVA a maximum likelihood phylogenetic tree was constructed on the Galaxy Europe server using MAFFT to generate the multiple alignment and IQ-TREE version 2.1.2 with 1,000 bootstrap replicates to reconstruct the tree (Minh et al., 2020).

ModelFinder (Kalyaanamoorthy et al., 2017) was used to determine the best substitution model for the HSP70h amino acid sequences alignment (rtREV+F+I+G4). The tree was visualized using the iTOL v6.8 tool (https://itol.embl.de/) (Letunic and Bork, 2021).

2.6. Molecular detection of viruses by reverse transcription PCR (RT-PCR)

Complementary DNA (cDNA) synthesis was performed for each sample by denaturing 2 µl of extracted total RNA (protocol described in section 2.2.2) and 2.5 µM of random hexamers (Invitrogen) for a reaction volume of 12 µl, at 65ºC during 5 min and then keeping the samples on ice during 1 min. 0.5 mM of dNTPs, 1x reaction buffer, 10 U Superscript III Reverse Transcriptase (Invitrogen), 2 U/µl RNaseOUT Recombinant RNase Inhibitor (Invitrogen), and 5 mM DTT in a 20 µl total reaction volume were added. After incubating at 25ºC for 5 min and 50ºC for 45 min, the RT was inactivated at 70ºC for 15 min, followed by 2 min on ice. Then 2 µl of cDNA, 1X reaction buffer, 0.2 µM forward primer (Table 4-1), 0.2 µM reverse primer (Table 3-1), 0.2 mM dNTPs, 2 mM MgCL2, and 0.1 U/µl Mango Taq DNA Polymerase (Bioline Reagents Ltd.) were added in a total reaction volume of 20 µl. The PCR products were visualized after migration in a 1% agarose gel.

The PCR products of one positive sample of CiVA from Slovenia, four positive samples of PyVA from Belgium, the positive sample of ARWV-1, and one of CiVA from Switzerland were purified using the Nucleospin Gel and PCR clean up (Macherey-Nagel, Germany), following the manufacturer's instructions, and sent to be sequenced by Sanger sequencing at Macrogen Europe BV (The Netherlands). The positive sample of apple rubbery wood virus 2 (ARWV-2, *Rubodvirus prosserense*) from Switzerland was purified from the agarose gel using the Wizard® SV Gel and PCR Clean-Up System (Promega) and sent to be sequenced by Sanger sequencing at Fasteris (Switzerland). More information regarding the samples tested can be found in Supplementary Table 4-4 and Supplementary Table 4-5.

2.7. Grafting assays

To study the host range of PyVA, grafting assays were conducted in the field at the biological indexing facilities of Agroscope (Switzerland). The scions were taken from the tree with a single infection by PyVA (CRA-W accession number 626). Absence of other commonly found viruses, namely ASPV and ACLSV, was determined by HTS and RT-PCR (Table 4-1). In August 2022, these scions were grafted on the following indicators: Virgina crab apple, Lord Lambourne (*Malus domestica*), *Pyronia veitchii* (*Cydonia oblonga* x *Pyrus communis*), Beurré Hardy (*Pyrus communis*), Williams (*Pyrus communis*), A20 (*Pyrus communis*), Jules d'Airoles (*Pyrus communis*), and C7/1 (*Cydonia oblonga*) using four replicates for each variety. In March 2023, buds collected from the same original pear tree were also grafted by

chip-budding on the following indicators: Pyrodwarf (*Pyrus communis*), M9 (*Malus domestica*), St. Julien (*Prunus domestica*), *Cydonia oblonga* and Gisela® 5 (*Prunus cerasus* x *Prunus canescens*). The grafted plants were kept under greenhouse conditions at the CRA-W (Belgium) and tested by RT-PCR for presence or absence of PyVA.

Table 4-1. List of primers used in this study, including primer name, the virus and viral segment they target, their sequence, annealing temperature (Ta) and relevant reference, if any.

Virus	Primer name	Sequence (5' – 3')	Ta	Reference
Apple rubbery wood virus 1 segment L (AWRV-1)	ARWaV-1L3639F ARWaV-1L4058R	AGAACCAGCAATAGCCAC CTATCCTTATCTTTGCCTACTT	55 ºC	Rott et al., 2018
Apple rubbery wood virus 1 segment S (AWRV-1)	ARWaV-1M479F ARWaV-1M1177R	ATCAATCTCTGTTTTCCCTTATGT TACCATACTTTTGAATCTTTGTGC	52 ºC	Rott et al., 2018
Apple rubbery wood virus 2 segment Sa (ARWV-2)	ARWV2-F1 ARWV2-R1	ATGTTGCATCACAGCTATTGGC ATTGTTCCATGCTGCCACAGAA	60 ºC	Minutolo et al., 2023
Citrus virus A RNA 1 (CiVA)	CiVA_1_2586F CiVA_1_1884R	CTAGGCACAAAGCTTGGTCAGAAG GTCTCCTCTTCATCTGACCTACCT	60 ºC	Designed for this study
Citrus virus A RNA 2 (CiVA)	CiVA_2_1F CiVA_2_285R	ATAACTTTTTTGTTAAAAAGC AATCTTGTTCCTTCACTAT	48 ºC	Bester et al., 2021
Pyrus virus A (PyVA)	PyVA-12722F PyVA-13206R	AGCAGCGAATGAATTGACACCAAA CGCCATCTGAGCCGTTTGATTATT	62ºC	Designed for this study
Apple chlorotic leaf spot virus (ACLSV)	ACLSV-A53-F ACLSV-A52-R	GGCAACCCTGGAACAGA CAGACCCTTATTGAAGTCGAA	56ºC	Candresse et al., 1995
Apple stem pitting virus (ASPV)	ASPF1CP ASPR3CP	GGGTGTACTTTGAGGCAGTATT GAGCGGATGCGGTACATCTGTAT	55ºC	Komorowska et al., 2010

2.8. Transmission electron microscopy (TEM)

Purification of the particles of PyVA was performed according to the protocol from Pilotti et al. (1995), with some modifications. Briefly, 40 g of infected pear leaves and petioles (CRA-W accession number 626) were ground into powder using liquid nitrogen and a mixer (Sorvall Omni Mixer 17150 Homogenizer). Then, the powder was mixed with 6 volumes of extraction buffer (0.5 M Tris, pH 8.2, 5% v/v Triton, 4% v/v Polyclar AT, 0.5% w/v bentonite, 0.2% v/v β-mercaptoethanol). After 20 min of homogenization, the suspension was filtered through a double layer of cotton cloth, and the resulting filtrate was centrifuged at 4,000 rpm for 20 min. The supernatant was collected and transferred to an ultracentrifuge tube, and 5 ml of a 20% sucrose cushion (prepared in 0.1 M Tris, pH 8.2) were added. The tube was centrifuged at 40,000 rpm for 1 h and 30 min. The resulting pellet was incubated overnight at 4°C in 4 ml of 10x resuspension buffer (0.02 M Tris, pH 7.0, 1mM MgCl2). Three microliters were mixed with one volume of 0.1% of bovine serum albumin and one volume of 4% phosphotungstic acid (pH 6.0). Purified particles were observed by transmission electron microscopy (TEM) as described by Mahillon et al. (2023), using a Tecnai G2 Spirit microscope (FEI, Eindhoven).

3. Results

3.1. Viruses detected by high-throughput sequencing

After *de novo* assembly, between 29 and 1,537 contigs were obtained per pool for the 17 pools of samples prepared with the dsRNA virus enrichment protocol, with an average of 610 contigs per pool. An average of 191 contigs longer than 1,000 nucleotides was obtained, with an average N50 length of 1,495 (Supplementary Table 4-6). Using tBLASTx, four known and one unknown pear viruses were detected in the analyzed samples: ASPV, ACLSV, ARWV-1, CiVA, and a tentative novel Closteroviridae member which will be referred from this point on as Pyrus virus A (PyVA).

The cross-contamination ratios of the alien control ($RA_{1\ 1\rightarrow n}$, $RA_{2\ 1\rightarrow n}$, and $RA_{3\ 1\rightarrow n}$) ranged between 0 and 0.5%. Moreover, only 1 read (1.5 RPKM, 0.4% $RV_{ACLSV\ alien}$) from a pear virus (ACLSV) was found in the alien control, used in this case as a negative control, thus strengthening the set threshold of 0.5% for likely false positives (FP). Ratios below or equal to the threshold and above 0% ($0\%<RV_{x\ 1\rightarrow n}\leq 0.5\%$) were considered FP, ratios above the threshold were considered TP ($RV_{x\ 1\rightarrow n}>0.5\%$), and ratios of 0% ($RV_{x\ 1\rightarrow n}=0\%$) were considered true negatives (TN). In addition, confirmatory targeted molecular tests using RT-PCR were applied to the sequenced samples, which have been analyzed individually. It is worth mentioning that pool L7-2 had an unexpectedly high RA ratio (56.6%) for only one of the three alien viruses, namely PvEV-1 (Supplementary Table 4-7). This result was considered as aberrant and was discarded for two reasons: (i) no reads of PvEV-2 and PvEV-3 were observed for this pool (while the alien control showed more reads for these two viruses

compared to PvEV-1), and (2) the detected region of PvEV-1 (316 nt representing 2% of the genome) is not covered by any read in the alien control. Out of the 17 pools analyzed, ASPV and ACLSV were detected by mapping in respectively 17 and 14 pools, while ARWV-1 and CiVA were both detected in six pools. PyVA was detected by mapping in two of the 17 pools analyzed (Table 3-2). The positive pools where CiVA and PyVA were detected by HTS contained at least one positive sample of the virus that was tested by RT-PCR during the field survey in 2023, although five pools that were negative for CiVA by HTS contained one or more samples found positive by RT-PCR and one pool that was considered a likely FP for PyVA by HTS contained one sample found positive by RT-PCR (Table 3-2). An extensive list of the sampled germplasm, the designed pools, and their corresponding libraries, as well as the tests performed and the viruses detected in each sample during the field survey, can be found in Supplementary Table 4-4.

To assemble the complete genome of PyVA, total RNA from leaves of samples included in pool L3-2 were extracted and Illumina sequenced (see section 2.2.2). Two genomic sequences of PyVA were reconstructed from cultivar (cv.) Jean Nicolas (tree Z14, accession 224; and tree Z15, accession 621) and showed 99.9% identity (Genbank OR887735-6). In addition, a nearly complete genomic sequence of a Belgian isolate of CiVA was also reconstructed from accession 224, with RNA 1 having a length of 6,663 nt (Genbank OR825541) and RNA 2 a length of 2,721 nt (Genbank OR825542).

Table 4-2. Presence or absence of CiVA and PyVA in the Belgian collection as determined by HTS and RT-PCR, For HTS and according to the threshold described above, pools with a ratio (RVCiVA 1→n, RVPyVA 1→n) below the threshold of 0.5% were considered negative (-), and pools with a ratio above the threshold were considered as positive (+). For CiVA, detection was considered positive if at least one genomic RNA (RNA 1 or RNA 2) was positive. Abbreviations: citrus virus A (CiVA), high-throughput sequencing (HTS), reverse transcription polymerase chain reaction (RT-PCR), the new virus pyrus virus A (PyVA), reads per kilobase per million (RPKM), not analyzed (na). (*) the detection has been carried out after 2 years of storage at -20°C.

Library	Pool	Sample	CiVA		PyVA	
			HTS	RT-PCR	HTS	RT-PCR
			2021	2023/2021*	2021	2023/2021*
L1	L1-1	W20	+	+	-	-
		W18		+/+		-
		W10		+		-
		W13		+		-
	L1-2	W11	+	+	-	-
		W15		+		-
		W16		-		-
		W19		+		-
	L1-3	W2	+	-	-	-
		W4		+/+		-
		W5		-		-
		W7		+/+		-
L2	L2-1	W6	-	na	-	-
		W21		+/+		-
		W24		na		-
		W12		+/-		-
	L2-2	W14	+	+/-	-	-
		Y13		-		-
		Y15		+/+		-
		Y16		+/+		-
	L2-3	Y9	-	-	-	-
		Y11		-		-
		Y20		+/+		-
		Y21		na		-
L3	L3-1	Z1	+	na	-	na
		Z11		na		na
		Z12		+		-
		Z13		na		na

Table 4-2 Continued

	L3-2	Z2	+	-	+	-/-
		Z3		-		-/-
		Z14		+		+/+
		Z15		-		+/+
L4	L4-1	Z4	-	na	-	na
		Z5		na		na
		Z6		na		na
		Z7		na		na
	L4-2	V14	-	na	-	na
		V18		na		na
		V5		na		na
		V15		na		na
L5	L5-1	V6	-	na	-	na
		V7		na		na
		V8		na		na
		V9		na		na
	L5-2	V10	-	na	-	na
		V11		na		na
		V17		na		na
		V16		na		na
	L5-3	V12	-	na	-	na
		V13		na		na
		X5		+/-		-
		X8		na		na
L6	L6-1	X14	-	-	-	-
		X10		-		-
		X12		-		-
		X9		+/+		+/+
	L6-2	X11	-	-	-	-
		X20		-		-
		X22		+/+		-
		X23		na		na
L7	L7-1	X13	-	-	-	-
		X16		-		-
		X19		-		-
	L7-2	X18	-	-	+	+/+
		X21		-		-/-

3.2. A novel velarivirus infecting pear trees

3.2.1. Molecular and genomic characterization

The two assembled PyVA genomes include all coding regions, but, although attempted, the 3' and 5' UTRs could not be fully assembled. The assembled genome of isolate 621-BE has a length of 17,061 nucleotides (nt) and 3,527 of the RNASeq reads map on the genome, representing 0.09% of the total reads, with an average coverage depth of 31X. The assembled genome of isolate 224-BE has a length of 17,142 nt and 1,228 RNASeq reads map on the genome, representing 0.03% of the total reads, with an average coverage depth of 11X. The size difference of the two isolates is due to incomplete sequencing of the 5' and 3' untranslated regions (UTRs). In the two assembled genomes the partial 5' UTR has a length of 49 nt. On the other hand, the partial 3' UTR of isolate 621-BE has a length of 209 nt and that of isolate 224-BE 290 nt. The two assembled genomes encode nine putative open reading frames (ORFs) (Figure 4-2A) and have a genomic organization resembling that of its closest relative, Malus domestica virus A (MdoVA), and of other velariviruses (Figure 4-2A). Similar to MdoVA, more reads were mapped at the 3' end of the genome (Koloniuk et al., 2020). Electron microscopy observation of semi-purified viral particles showed them to have a length of approximately 2000 nm (Figure 4-2B).

Within the complex ORF1a-ORF1b, two replication-associated domains, the methyltransferase (Mtr) (PF01660) and helicase (Hel) (PF01443), were identified in the product of ORF 1a. This ORF is 7,047 nt long (2,214 aa) and encodes a protein that would weigh 255 kDa. In ORF 1b, which is proposed to be translated through a +1 ribosomal frameshift, an RNA-dependent RNA polymerase (RdRp) (PF00978) conserved domain was identified. For most members of the family *Closteroviridae* the proposed frameshift motif for ORF 1b expression is "GUU_stop_C", which is present in all members of the genus *Velarivirus*, including PyVA (isolate 621-BE, nucleotides 7,091-7,097 "GUU_UGA_C"). However, since this potential shifting motif has not been proven experimentally, ORF 1b was annotated as a separate ORF. ORF 1b is 1,515 nt long (504 aa) and encodes a protein with a molecular weight of 58 kDa. Then there is the putative ORF 2 that would encode the small protein p4 of 102 nt (34 aa) with a molecular weight of 4 kDa. ORF 3 is 1,662 nt long and encodes a 524 aa (61 kDa) protein of the viral heat shock protein 70 homolog family (PF00012 HSP70h), and ORF 5 (1,554nt) encodes a protein of the viral heat shock protein 90 homolog family (PF03225, HSP90h) of 56 kDa (478 aa). ORF 4 (219 nt) is predicted to slightly overlap with ORF 3 and encodes the putative protein p9 of 9 kDa (72 aa). ORF 6 (1,029 nt) and ORF 7 (2,106 nt) respectively encode the structural capsid (CP) (PF01785) of 38 kDa (342 aa) and the minor capsid (CPm) protein of 75 kDa (648 aa). The last two ORFs, 8 (693 nt) and 9 (726 nt), encode the putative proteins p25 of 25 kDa (212 aa) and p26 of 26 kDa (225 aa).

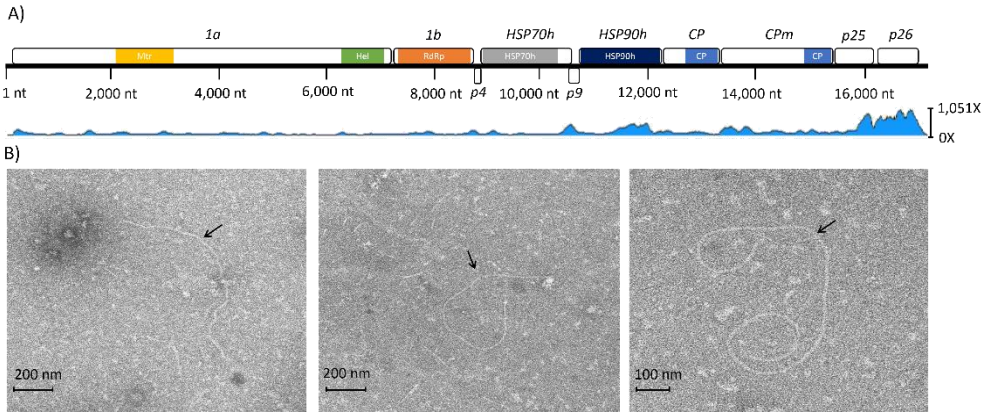


Figure 4-2. A) Graphic representation of the genomic organization of the new virus (PyVA, isolate 621-BE), with each box representing a predicted open reading frame (ORF) and the protein domains highlighted in different colors. The names in italics represent the products of the ORFs, the polyprotein 1a/1b complex, and the hypothetical proteins p4, p9, p25, and p26. The coverage of reads mapped to the genome of PyVA is shown in blue below the genome structure (maximum coverage = 1,051X). Abbreviations: methyltransferase (Mtr), helicase (Hel), RNA-dependent RNA polymerase (RdRp), heat-shock protein 70 homolog (HSP70h), heat-shock protein 90 homolog (HSP90h), capsid protein (CP), and minor capsid protein (CPm). B) Electron micrograph of three viral particles of the PyVA, marked with a black arrow. The particles were purified and observed by TEM, following the staining method described in section 2.6.

3.2.2. Phylogenetic relationship within the family *Closteroviridae*

The newly identified virus fits the demarcation criteria, as well as the distinguishing properties, for viruses belonging to the genus *Velarivirus* and family *Closteroviridae* (Fuchs et al., 2020). The closest relative to PyVA is Malus domestica virus A (MdoVA), with 75% aa identity in the RdRp, 60% in the HSP70h and 41% identity in the CP (Table 3). The two p25 and p26 putative proteins are the most variable within the genus, showing <42% identity for the p25 and <73% for the p26 between all velariviruses (Supplementary Figure 4-4). In addition, a phylogenetic tree using the HSP70h amino acid sequences of known members of Closteroviridae was constructed, which confirmed that PyVA was clustering with strong bootstrap support with other velariviruses (Figure 4-3).

Table 4-3. Percentages of amino acid (aa) identity of 10 proteins between the new virus (PyVA) and other members of the Velarivirus genus, obtained using a multiple sequence alignment tool (MAFFT). The complete name of each sequence used can be found in Figure 4-3. The genomes of cordyline virus 2 (NC_043453), cordyline virus 3 (NC_043107), and cordyline virus 4 (NC_043108) had partial sequences of the ORF 1a, and NC_001836 did not have an annotation for p9. Thus, they were not used for the comparison. A complete comparison of the percentage of identity between the proteins of the accepted velariviruses and PyVA is provided in Supplementary Figure 4-4.

Genbank nº	Virus	1a	1b	p4	HSP70h	p9	HSP90h	CP	CPm	p25	p26
NC_027121	Areca palm velarivirus 1 (APV-1)	22%	56%	26%	40%	13%	23%	23%	13%	15%	11%
NC_038421	Cordyline virus 1 (CoV-1)	22%	55%	36%	43%	14%	28%	30%	14%	15%	12%
NC_043453	Cordyline virus 2 (CoV-2)	-	54%	24%	44%	24%	31%	31%	17%	15%	14%
NC_043107	Cordyline virus 3 (CoV-3)	-	57%	15%	46%	26%	31%	27%	13%	17%	9%
NC_043108	Cordyline virus 4 (CoV-4)	-	56%	16%	44%	25%	26%	30%	15%	21%	12%
NC_016436	Grapevine leafroll-associated virus 7 (GLRaV-7)	23%	55%	18%	42%	20%	28%	26%	11%	14%	10%
NC_001836	Little cherry virus 1 (LChV-1)	30%	60%	21%	48%	-	34%	21%	19%	13%	15%
NC_055599	Malus domestica virus A (MdoVA)	46%	75%	61%	60%	49%	52%	41%	26%	32%	27%

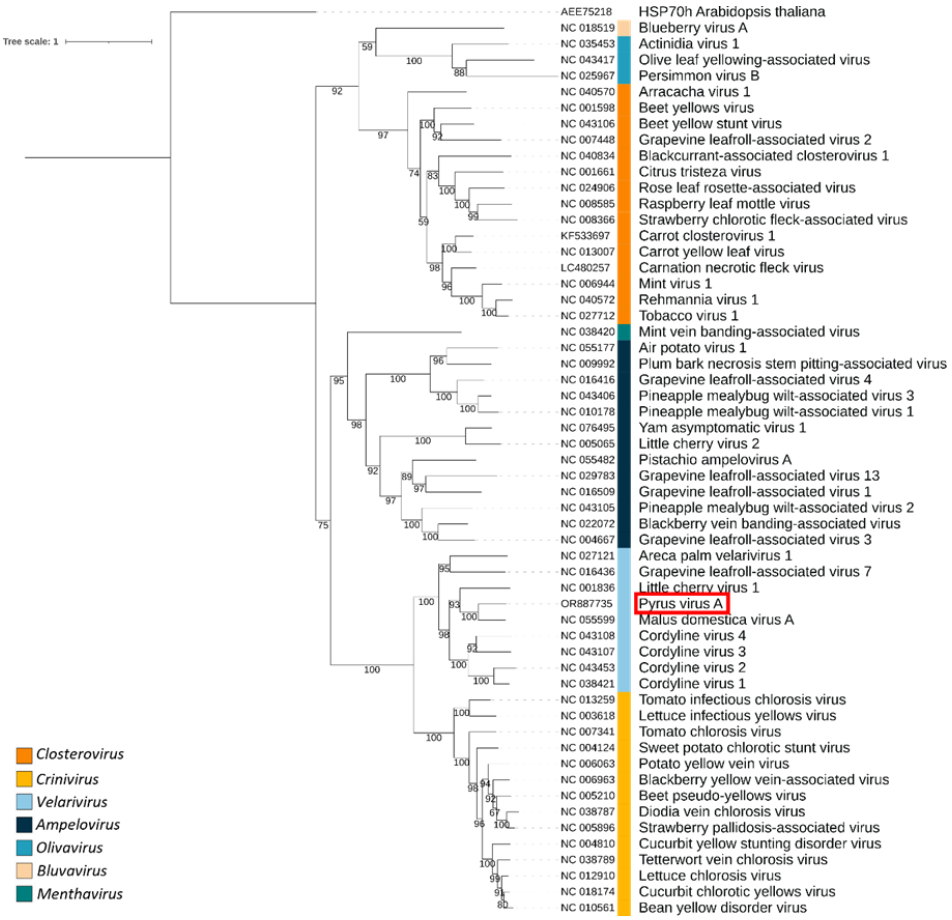


Figure 4-3. Maximum likelihood phylogenetic tree (rtREV+F+I+G4 substitution model, MAFFT alignment, 1000 bootstraps) based on the alignment of HSP70h amino acid (aa) sequences of members of the Closteroviridae family and of PyVA (highlighted in red). The phylogenetic analysis was performed using the Galaxy server and visualized using the iTOL v6.8 tool. The HSP70 sequence of Arabidopsis thaliana was used as outgroup to root the tree. Bootstrap values are shown for each branch, and colored labels represent the genus that each virus belongs to as shown in the legend on the left.

3.2.3. Biological characterization of the new virus Pyrus virus A (PyVA)

To provide some insights in the biology of PyVA, its distribution within a tree, host range and preliminary geographic distribution were studied together with its graft transmissibility to pear trees. PyVA was successfully transmitted by chip budding to healthy plants of *Pyronia veitchii* (*Cydonia oblonga* x *Pyrus communis*) with a transmission rate of 25% (1/4), and to different pear cultivars (Beurré Hardy: 50% (2/4); Williams: 75% (3/4); A20: 75% (3/4); Jules d'Airoles: 50% (2/4)). None of the grafted plants infected with PyVA developed symptoms during their first year of

growth. There was no graft transmission of PyVA to the following indicators: Virginia crab apple, Lord Lambourne (*Malus domestica*), C7/1 (*Cydonia oblonga*), M9 (*Malus domestica*), St. Julien (*Prunus domestica*), and Gisela® 5 (*Prunus cerasus* x *Prunus canescens*). At the same time buds from the positive tree (X18) and from commercial pear varieties Conférence and Doyenné du Comice were grafted by double chip budding to quince (*Cydonia oblonga*) and Pyrodwarf (*Pyrus communis*) rootstocks. There was transmission to the commercial pear varieties Conférence and Doyenné du Comice, and to the Pyrodward rootstocks. Comparatively, there was no transmission of PyVA to the quince rootstocks.

Within the original infected tree, PyVA was detected in all individual flower and leaf samples sampled from tree X18, and in phloem collected from branches facing North, East and West, suggesting that it is quite evenly distributed in that tree. Distribution within the orchard was rather sparse, as only six trees were found to be infected with PyVA, representing five different cultivars. Out of the five trees of cultivar Beau Présent tested, only one was positive (1/5); of the three trees of cultivar Jean Nicolas tested, two were positive (2/3), the unique trees tested for cultivars Camberlain Blanc, Semis Henin, and Poire Grognet were positive as well. Moreover, except for the two trees of cultivar Jean Nicolas, the positives trees were not contiguous in the orchard.

3.3. Field survey by reverse transcription PCR (RT-PCR)

During the bioinformatic analyses of the HTS data, two known and widely distributed viruses (ASPV and ACLSV), one new virus (PyVA), and two recently discovered viruses (CiVA and ARWV-1) were detected in the tested Belgian samples. ARWV-1 had previously been identified in the CRA-W germplasm collection during a preliminary survey (Fontdevila Pareta et al., 2022), thus further efforts in Belgium focused on the distribution and prevalence of CiVA and PyVA. With the observed prevalence of ARWV-1 in the Belgian collection, the samples originating from Slovenia and Switzerland were also tested for ARWV-1. In addition, as one sample tested positive in Switzerland for ARWV-1 which is occasionally present in mixed infections with ARWV-2, it was also tested for ARWV-2.

PyVA was detected in six of the 99 trees sampled (6%) and CiVA in 49 of them (49%). In three trees (3%) there was a mixed infection of CiVA and PyVA, and 47 trees (47%) did not test positive for CiVA or PyVA (Supplementary Table 4-5). From the six pear trees sampled in Slovenia, four were positive for CiVA; and from the nine trees sampled in Switzerland, four were positive for CiVA and one for ARWV-1. Additionally, the sample that tested positive for ARWV-1 also tested positive for ARWV-2.

Specificity of the amplified PCR products of PyVA from Belgium, CiVA from Slovenia, and ARWV-1, -2, and CiVA from Switzerland was confirmed by Sanger sequencing. The partial RdRp sequence of CiVA from Slovenia, isolate KP-SLO-1 (Genbank OR825539), had an amplicon size of 655 nt and 99.1% nucleotide (nt)

identity with RNA 1 of CiVA isolate B175 (Genbank MZ463039). From Switzerland, the partial CP sequence of ARWV-1, isolate 39652-CH (Genbank OR825538), had an amplicon size of 777 nt and 99.6% nt identity to segment S of ARWV-1 isolate BR-Mishima (Genbank MK936225), the partial CP sequence of ARWV-2, isolate 39652-CH (Genbank PP319005), had an amplicon size of 228 nt and 100% nt identity to segment Sa of ARWV-2 isolate CE30 (Genbank OP583932), and the partial RdRp sequence of CiVA, isolate 39653-CH (Genbank OR825540), had an amplicon size of 655 nt and 97.4% nt identity to RNA 1 of CiVA isolate P215/CIVA (Genbank MZ330076). From Belgium, partial CP sequences of PyVA from isolates 615-BE, 626-BE, and 638-BE (Genbank OR936022-4) had a length of 436 nt and 97.7% nt identity to isolate 224-BE (Genbank OR887735); while sequences from isolate 847-BE (Genbank OR936025) showed 98.2% nt identity to isolate 224-BE (Genbank OR887735).

4. Discussion

Scanning the virome of fruit tree germplasm collections has become possible with the evolution of HTS technologies and bioinformatic tools (Adams et al., 2018; Rott et al., 2017). Such an approach is a useful tool to evaluate the viruses present in these germplasms before their potential use in breeding, provided the detection test is reliable.

In this context, an international initiative proposed guidelines for the reliable use of HTS technologies to detect plant pathogens and pests. One of the innovative aspects in these guidelines was the use of an alien control to monitor the levels of cross-contamination to differentiate between true and false positives (Massart et al., 2022). In this study, the dsRNA viral enrichment protocol, based on the binding properties of dsRNA to cellulose, was used to analyze the virome of pear germplasms as it has proven very useful for large scale virome analyses (Marais et al., 2024; Schönegger et al., 2023). Because this protocol relies on numerous steps, there is an increased risk of cross-contamination between samples. Anticipating the potential uncertainty when identifying FP arising from cross-contamination events, an alien control strategy was adopted. To our knowledge, the present study is the first time this specific approach has been used in combination with the dsRNA viral enrichment protocol. To evaluate cross-contamination, a contamination ratio was calculated for the alien viruses ($RA_{1\rightarrow n}$) and the detected pear viruses ($RV_{x\ 1\rightarrow n}$). This ratio was inspired by Cont-ID, a tool designed to evaluate cross-contamination between samples/pools (Rollin et al., 2023), and created by reusing and adapting some of its principles to the dataset without the duplication step. In our case, the maximum cross-contamination background level using the alien viruses was between 0.4 and 0.5%, with the later value used as the cross-contamination threshold to distinguish true from likely false positives. In total, 14 detection events (related to ASPV, ACLSV and PyVA), out of a total of 48, were considered as likely false positive using this threshold.

To reinforce the reliability of detection, a second survey (based on RT-PCR tests) was carried out in 2023. All positive HTS results were confirmed for CiVA and PyVA (n=8) while five and one negative HTS results contained at least one positive sample in the pool for CiVA and PyVA, respectively. Therefore, total RNA was extracted from leaf material of individual trees from 2021 after 2-year storage and tested by RT-PCR for detection confirmation. CiVA was detected in nine of the 12 individual trees, confirming the presence of CiVA in those samples, and PyVA was detected in four of the seven individual trees, thus confirming its presence. Considering these results five pools considered as true negatives for CiVA and one as a likely false positive for PyVA based in HTS results were found to be true positives following RT-PCR analysis of the plants constituting these pools, challenging the diagnostic sensitivity of the HTS test, although no absolute conclusion can be advanced since, for example, the heterogeneity of the virus's titer within tested trees is not known. Notably, dsRNA-based HTS indexing has been shown to have a lower sensitivity for negative-stranded RNA viruses (-ssRNA), such as ARWV-1 and CiVA (Marais et al., 2024; Schönegger et al., 2023), so that reliable detection of such viruses in fruit trees may necessitate the testing of individual trees -Marais et al., 2024) and not of pools of samples as was done here.

The virome study of the pear collection from the CRA-W in Belgium strengthened the assumption that there is a lower number of viruses that infect pear trees compared to other fruits trees, such as apple, given that only five viruses were detected in a total of 65 trees of diverse origins and genetic backgrounds analyzed by HTS. In contrast, a virome study of experimental and commercial apple orchards, of 18 cultivars, in British Columbia detected 21 plant viruses and one plant viroid by HTS (Xiao et al., 2022). Moreover, this study ratified the fact that pooling samples prior to extraction is a powerful approach to reduce the costs when performing large scale virome surveys, as seen by Fowkes et al. (2021) and Nyirakanani et al. (2021), although false negative can occur, underlining the importance of using an alien control to monitor the cross-contamination and accuracy of the test (Massart et al., 2022). For the most important detections, downstream individual testing on trees tested negative or positive by HTS on pooled samples, is recommended for a better virome characterization and an improved reliability of the findings.

This study describes a putative novel velarivirus tentatively named Pyrus virus A (PyVA) identified in pear (*Pyrus communis*) trees and the first report of CiVA in Slovenia, Switzerland, and Belgium. Moreover, it represents the first molecular evidence of presence of ARWV-1 and -2 in Switzerland. As a suggestion to the International Committee on Taxonomy of Viruses (ICTV), the authors tentatively propose the latinized form Velarivirus gembloutense, for its species name, after the region it was detected. Within the genus *Velarivirus*, the assembled sequences of PyVA have less than 75 % aa identity to the RdRp, CP, and HSP70h products, which are the relevant gene products chosen by the ICTV for the species demarcation criteria of the genus (Fuchs et al., 2020). The size of the viral particle, and the genome

structure and organization are similar to other viruses of the genus *Velarivirus* in the family *Closteroviridae*.

Additionally, to our knowledge, this study provides the first TEM of a *Velarivirus*. There is a rather high diversity of genome organization and similarity levels within the genus. The most conserved ORF is ORF 1b, with a percentage of aa identity between 51% and 78% (Supplementary Figure 4-4). In contrast, putative proteins p25 and p26 are the most divergent, with a percentage of aa identity lower than 42% and 73% respectively (Supplementary Figure 4-4). Overall, the two closest viruses within the genus are PyVA and MdoVA, followed by Cordyline virus 1 and Cordyline virus 2, which could be due to their adaptations to similar plant hosts over time or their divergence from a common ancestor infecting the same plant host.

One of the characteristics of the family *Closteroviridae* is the hypothetical expression of the RdRp domain, encoded by ORF 1b, through a +1 ribosomal frameshift. This assumption has not been experimentally demonstrated; hence the identity of the frameshifting site remains speculative. Nevertheless, it has been proposed that in most members of this family the +1 ribosomal frameshift could occur at a conserved "GUU_stop_C" motif that includes the ORF 1a stop codon. This motif would promote a slippage of the ribosome from GUU to UUU (Agranovsky, 2016; Maia et al., 1996). To be cautious, in this case, ORFs 1a and 1b of PyVA were annotated individually but with a note explaining the alternative possibility of the RdRp domain to be encoded through a +1 ribosomal frameshift.

While other studies have shown a high genetic diversity within the *Closteroviridae* family (Liu et al., 2021), the set of sequences obtained in this study is too small and originates from the same location; thus, the sequences are expected to present low genetic variability. Additionally, the available data is not enough to draw any conclusions in this direction.

The characterization of PyVA was performed following the revised framework for the characterization of a novel plant virus or viroid discovered in an HTS dataset (Fontdevila Pareta et al., 2023). First, the first two steps of the framework, consisting of (i) the design of a detection test, confirmation of the detection and obtention of the genome sequence; and (ii) gathering contextual information and first notification and discussion with stakeholders, including the scientific community and plant protection agencies, were carried out. PyVA was suggested as a "no priority" virus since the infected trees in the field did not present symptoms, and there was no apparent rapid spread. To evaluate the association between the presence of the virus and symptoms in the plant host, as part of step three of the framework, a large-scale field survey and transmission assays were conducted. Again, no symptoms were observed on trees infected with PyVA alone or in mixed infection with other viruses (ASPV, ACLSV, and CiVA). Moreover, there were no symptoms observed on the graft inoculated trees with PyVA at Agroscope. It would be possible that symptoms may develop after some years, although it is unlikely as no visible symptoms on leaves and fruits were

observed during sampling campaigns in 2021 and 2023. Monitoring of symptoms was also done during summer 2022, with no symptoms that could be linked to PyVA. The transmission of the virus to different cultivars of *Pyrus communis* was possible, although the transmission rate was not 100%. Moreover, other studies showed that there can be a high variability in the rate of transmission by grafting between fruit tree viruses (Khalili et al., 2023). Nonetheless, it would be possible that the choice of grafting technique may have had an impact on the transmission rate. In addition, other transmission methods aside from grafting could be tested, such as pollen, for example.

In general, the biological characteristics of the new virus and the results obtained during the study argue in favor of a rather low phytosanitary risk of PyVA, indicating that it may not require immediate action from pest risk managers and authorities.

Moreover, the large-scale field survey assisted in the completion of data gaps for CiVA, ARWV-1, and ARWV-2 by providing insight into the prevalence of CiVA and the geographic distribution of CiVA, ARWV-1, and ARWV-2. This study reports the first detection of CiVA in Belgium, Switzerland, and Slovenia, and the first molecular evidence of presence of ARWV-1 and ARWV-2 in Switzerland. In citrus trees, CiVA has been found associated with disease symptoms (Beris et al., 2021; de Bruyn et al., 2022; Park et al., 2022), but its association with symptoms in pear trees has not been proven. CiVA was found with a high prevalence in collections in Belgium, Switzerland, and Slovenia, in trees showing no visible symptom on leaves and/or fruits, although more samples from Switzerland and Slovenia should be tested to strengthen the risk evaluation process. Even though the collections targeted here were selected mainly to prioritize the study of the virome in ancient and local pear cultivars, commercial orchards could be studied to evaluate if a similar trend in the distribution and prevalence of these viruses is observed.

In conclusion, this is the first report of ARWV-1, ALV-1, and AHVd in Belgium. The trees showed no visible symptoms of viral infection, suggesting that the symptoms associated with these viruses and viroid are likely to be variable or latent across different cultivars and environments. Moreover, this study provides further evidence of the interest of virome survey of germplasm collections using HTS approaches while simultaneously showcasing the limitations that still exist. A proper evaluation of the virus infection status of these collections should become a cornerstone before evaluating their genetic potential. Besides detecting known viruses, including some poorly described ones, our study revealed an unknown virus in latent or asymptomatic infections, bringing valuable insights into the diversity and complexity of viral infections in pear trees. The downstream characterization of such new or poorly characterized viruses should accompany any virome survey as such additional studies can provide useful information to the stakeholders to evaluate the potential phytosanitary risk posed by the detected viruses.

References

FAO, 2024. FAOSTAT [WWW Document]. Food Agric. Organ. U. N. URL https://www.fao.org/faostat/en/#data/QCL (accessed 11.5.23).

Adams, I.P., Fox, A., Boonham, N., Massart, S., De Jonghe, K., 2018. The impact of high throughput sequencing on plant health diagnostics. Eur. J. Plant Pathol. 152, 909–919. https://doi.org/10.1007/s10658-018-1570-0

Agranovsky, A.A., 2016. Closteroviruses: Molecular Biology, Evolution and Interactions with Cells, in: Gaur, R.K., Petrov, N.M., Patil, B.L., Stoyanova, M.I. (Eds.), Plant Viruses: Evolution and Management. Springer, Singapore, pp. 231–252. https://doi.org/10.1007/978-981-10-1406-2_14

Beris, D., Ioanna, M., Vassilakos, N., Theologidis, I., Rampou, A., Kektsidou, O., Massart, S., Varveri, C., 2021. Association of Citrus Virus A to Citrus Impietratura Disease Symptoms. Phytopathology 111, 1782–1789. https://doi.org/10.1094/PHYTO-01-21-0027-R

Bester, R., Karaan, M., Cook, G., Maree, H.J., 2021. First report of citrus virus A in citrus in South Africa. J. Citrus Pathol. 8. https://doi.org/10.5070/C481049000

Boonham, N., Kreuze, J., Winter, S., van der Vlugt, R., Bergervoet, J., Tomlinson, J., Mumford, R., 2014. Methods in virus diagnostics: From ELISA to next generation sequencing. Virus Res., Plant Virus Epidemiology Symposium Ecology, evolution and control of plant viruses and their vectors 186, 20–31. https://doi.org/10.1016/j.virusres.2013.12.007

Bushnell, B., Rood, J., Singer, E., 2017. BBMerge – Accurate paired shotgun read merging via overlap. PLoS ONE 12. https://doi.org/10.1371/JOURNAL.PONE.0185056

Candresse, T., Lanneau, M., Revers, F., Macquaire, G., German, S., Dunez, J., Grasseau, N., Malinovsky, T., 1995. An immunicapture PCR assay adapted to the detection and the analysis of molecular variability of apple chlorotic leaf spot virus. Acta Hortic. 136–147. https://doi.org/10.17660/ActaHortic.1995.386.17

Costa, L.C., Hu, X., Malapi-Wight, M., Foster, J., McFarland, C., Hurtado-Gonzales, O.P., 2022. Identification of a novel robigovirus and a Prunus-infecting tepovirus in Pyrus communis and their transmissibility on Malus spp. Eur. J. Plant Pathol. 162, 275–288. https://doi.org/10.1007/s10658-021-02402-9

de Bruyn, R., Bester, R., Cook, G., Steyn, C., Breytenbach, J.H.J., Maree, H.J., 2022. Distribution and Genetic Diversity of Coguvirus eburi in South African Citrus and the Development of a Real-Time RT-PCR Assay for Citrus-Infecting Coguviruses. Plant Dis. 106, 2221–2227. https://doi.org/10.1094/PDIS-11-21-2409-RE

Fontdevila Pareta, N., Khalili, M., Maachi, A., Rivarez, M.P.S., Rollin, J., Salavert, F., Temple, C., Aranda, M.A., Boonham, N., Botermans, M., Candresse, T., Fox, A., Hernando, Y., Kutnjak, D., Marais, A., Petter, F., Ravnikar, M., Selmi, I., Tahzima, R., Trontin, C., Wetzel, T., Massart, S., 2023. Managing the deluge of newly discovered plant viruses and viroids: an optimized scientific and regulatory framework for their characterization and risk analysis. Front. Microbiol. 14.

Fontdevila Pareta, N., Lateur, M., Steyer, S., Blouin, A. g., Massart, S., 2022. First reports of Apple luteovirus 1, Apple rubodvirus 1 and Apple hammerhead viroid infecting apples in Belgium. New Dis. Rep. 45, e12076. https://doi.org/10.1002/ndr2.12076

Fowkes, A.R., McGreig, S., Pufal, H., Duffy, S., Howard, B., Adams, I.P., Macarthur, R., Weekes, R., Fox, A., 2021. Integrating High throughput Sequencing into Survey Design Reveals Turnip Yellows Virus and Soybean Dwarf Virus in Pea (Pisum

Sativum) in the United Kingdom. Viruses 13, 2530. https://doi.org/10.3390/v13122530

François, S., Filloux, D., Fernandez, E., Ogliastro, M., Roumagnac, P., 2018. Viral Metagenomics Approaches for High-Resolution Screening of Multiplexed Arthropod and Plant Viral Communities, in: Pantaleo, V., Chiumenti, M. (Eds.), Viral Metagenomics: Methods and Protocols, Methods in Molecular Biology. Springer, New York, NY, pp. 77–95. https://doi.org/10.1007/978-1-4939-7683-6_7

Fuchs, M., Bar-Joseph, M., Candresse, T., Maree, H.J., Martelli, G.P., Melzer, M.J., Menzel, W., Minafra, A., Sabanadzovic, S., Report Consortium, I., 2020. ICTV Virus Taxonomy Profile: Closteroviridae. J. Gen. Virol. 101, 364–365. https://doi.org/10.1099/jgv.0.001397

Hou, W., Li, S., Massart, S., 2020. Is There a "Biological Desert" With the Discovery of New Plant Viruses? A Retrospective Analysis for New Fruit Tree Viruses. Front. Microbiol. 11, 2953. https://doi.org/10.3389/fmicb.2020.592816

Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., von Haeseler, A., Jermiin, L.S., 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat. Methods 14, 587–589. https://doi.org/10.1038/nmeth.4285

Katsiani, A., Maliogka, V.I., Katis, N., Svanella-Dumas, L., Olmos, A., Ruiz-García, A.B., Marais, A., Faure, C., Theil, S., Lotos, L., Candresse, T., 2018. High-Throughput Sequencing Reveals Further Diversity of Little Cherry Virus 1 with Implications for Diagnostics. Viruses 2018 Vol 10 Page 385 10, 385–385. https://doi.org/10.3390/V10070385

Kechin, A., Boyarskikh, U., Kel, A., Filipenko, M., 2017. CutPrimers: A New Tool for Accurate Cutting of Primers from Reads of Targeted Next Generation Sequencing. J. Comput. Biol. 24, 1138–1143. https://doi.org/10.1089/CMB.2017.0096

Khalili, M., Candresse, T., Koloniuk, I., Safarova, D., Brans, Y., Faure, C., Delmas, M., Massart, S., Aranda, M.A., Caglayan, K., Decroocq, V., Drogoudi, P., Glasa, M., Pantelidis, G., Navratil, M., Latour, F., Spak, J., Pribylova, J., Mihalik, D., Palmisano, F., Saponari, A., Necas, T., Sedlak, J., Marais, A., 2023. The Expanding Menagerie of Prunus-Infecting Luteoviruses. Phytopathology 113, 345–354. https://doi.org/10.1094/PHYTO-06-22-0203-R

Koloniuk, I., Přibylová, J., Fránová, J., Špak, J., 2020. Genomic characterization of Malus domestica virus A (MdoVA), a novel velarivirus infecting apple. Arch. Virol. 165, 479–482. https://doi.org/10.1007/s00705-019-04478-5

Komorowska, B., Malinowski, T., Michalczuk, L., 2010. Evaluation of several RT-PCR primer pairs for the detection of Apple stem pitting virus. J. Virol. Methods 168, 242–247. https://doi.org/10.1016/j.jviromet.2010.04.024

Kummert, J., Malice, M., Marbot, S., Lepoivre, P., Steyer, S., Oger, R., 2004. Sampling protocols and risk of error significance in molecular detection tests for fruit trees certification. Acta Hortic. 541–546. https://doi.org/10.17660/ActaHortic.2004.657.88

Lebas, B., Adams, I., Al Rwahnih, M., Baeyen, S., Bilodeau, G.J., Blouin, A.G., Boonham, N., Candresse, T., Chandelier, A., De Jonghe, K., Fox, A., Gaafar, Y.Z.A., Gentit, P., Haegeman, A., Ho, W., Hurtado-Gonzales, O., Jonkers, W., Kreuze, J., Kutjnak, D., Landa, B., Liu, M., Maclot, F., Malapi-Wight, M., Maree, H.J., Martoni, F., Mehle, N., Minafra, A., Mollov, D., Moreira, A., Nakhla, M., Petter, F., Piper, A.M., Ponchart, J., Rae, R., Remenant, B., Rivera, Y., Rodoni, B., Roenhorst, J.W., Rollin, J., Saldarelli, P., Santala, J., Souza-Richards, R., Spadaro, D., Studholme, D.J.,

Sultmanis, S., van der Vlugt, R., Tamisier, L., Trontin, C., Vazquez-Iglesias, I., Vicente, C.S.L., Vossenberg, B.T.L.H., Wetzel, T., Ziebell, H., Massart, S., 2022. Facilitating the adoption of high-throughput sequencing technologies as a plant pest diagnostic test in laboratories: A step-by-step description. EPPO Bull. 52, 394–418. https://doi.org/10.1111/epp.12863

Letunic, I., Bork, P., 2021. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Res. 49, W293–W296. https://doi.org/10.1093/nar/gkab301

Liu, H., Wu, L., Nikolaeva, E., Peter, K., Liu, Z., Mollov, D. et al. (2018) Characterization of a new apple luteovirus identified by high-throughput sequencing. Virology Journal, 15, 85. https://doi.org/10.1186/s12985-018-0998-3

Liu, Q., Zhang, S., Mei, S., Zhou, Y., Wang, J., Han, G.-Z., Chen, L., Zhou, C., Cao, M., 2021. Viromics unveils extraordinary genetic diversity of the family Closteroviridae in wild citrus. PLOS Pathog. 17, e1009751. https://doi.org/10.1371/journal.ppat.1009751

Mahillon, M., Brodard, J., Kellenberger, I., Blouin, A.G., Schumpp, O., 2023. A novel weevil-transmitted tymovirus found in mixed infection on hollyhock. Virol. J. 20, 17. https://doi.org/10.1186/s12985-023-01976-6

Maia, I.G., Séron, K., Haenni, A.-L., Bernardi, F., 1996. Gene expression from viral RNA genomes. Plant Mol. Biol. 32, 367–391. https://doi.org/10.1007/BF00039391

Maliogka, V.I., Minafra, A., Saldarelli, P., Ruiz-García, A.B., Glasa, M., Katis, N., Olmos, A., 2018. Recent Advances on Detection and Characterization of Fruit Tree Viruses Using High-Throughput Sequencing Technologies. Viruses 10, 436. https://doi.org/10.3390/v10080436

Marais, A., Faure, C., Bergey, B., Candresse, T., 2018. Viral Double-Stranded RNAs (dsRNAs) from Plants: Alternative Nucleic Acid Substrates for High-Throughput Sequencing, in: Pantaleo, V., Chiumenti, M. (Eds.), Viral Metagenomics: Methods and Protocols, Methods in Molecular Biology. Springer, New York, NY, pp. 45–53. https://doi.org/10.1007/978-1-4939-7683-6_4

Marais, A., Gentit, P., Brans, Y., Renvoisé, J.P., Faure, C., Saison, A., Cousseau, P., Castaing, J., Chambon, F., Pion, A., Calado, G., Lefebvre, M., Garnier, S., Latour, F., Bresson, K., Grasseau, N., Candresse, T., 2024. Comparative performance evaluation of double-stranded RNA high-throughput sequencing for the detection of viral infection in temperate fruit crops. Phytopathology. https://doi.org/10.1094/PHYTO-12-23-0480-R

Massart, S., Adams, I., Al Rwahnih, M., Baeyen, S., Bilodeau, G.J., Blouin, A.G., Boonham, N., Candresse, T., Chandellier, A., De Jonghe, K., Fox, A., Gaafar, Y.Z.A., Gentit, P., Haegeman, A., Ho, W., Hurtado-Gonzales, O., Jonkers, W., Kreuze, J., Kutjnak, D., Landa, B.B., Liu, M., Maclot, F., Malapi-Wight, M., Maree, H.J., Martoni, F., Mehle, N., Minafra, A., Mollov, D., Moreira, A.G., Nakhla, M., Petter, F., Piper, A.M., Ponchart, J.P., Rae, R., Remenant, B., Rivera, Y., Rodoni, B., Botermans, M., Roenhorst, J.W., Rollin, J., Saldarelli, P., Santala, J., Souza-Richards, R., Spadaro, D., Studholme, D.J., Sultmanis, S., van der Vlugt, R., Tamisier, L., Trontin, C., Vazquez-Iglesias, I., Vicente, C.S.L., van de Vossenberg, B.T.L.H., Westenberg, M., Wetzel, T., Ziebell, H., Lebas, B.S.M., 2022. Guidelines for the reliable use of high throughput sequencing technologies to detect plant pathogens and pests. Peer Community J. 2. https://doi.org/10.24072/pcjournal.181

Massart, S., Olmos, A., Jijakli, H., Candresse, T., 2014. Current impact and future directions of high throughput sequencing in plant virus diagnostics. Virus Res. 188, 90–96. https://doi.org/10.1016/j.virusres.2014.03.029

Minh, B.Q., Schmidt, H.A., Chernomor, O., Schrempf, D., Woodhams, M.D., von Haeseler, A., Lanfear, R., 2020. IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. Mol. Biol. Evol. 37, 1530–1534. https://doi.org/10.1093/molbev/msaa015

Minutolo, M., Cinque, M., Di Serio, F., Navarro, B., Alioto, D., 2023. Occurrence of apple rubbery wood virus 1 and apple rubbery wood virus 2 in pear and apple in Campania (southern Italy) and development of degenerate primers for the rapid detection of rubodviruses. J. Plant Pathol. 105, 567–572. https://doi.org/10.1007/s42161-023-01316-1

Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L., Wold, B., 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods 5, 621–628. https://doi.org/10.1038/nmeth.1226

Nyirakanani, C., Bizimana, J.P., Kwibuka, Y., Nduwumuremyi, A., Bigirimana, V. de P., Bucagu, C., Lassois, L., Malice, E., Gengler, N., Massart, S., Bragard, C., Habtu, M., Brostaux, Y., Thonar, C., Vanderschuren, H., 2021. Farmer and Field Survey in Cassava-Growing Districts of Rwanda Reveals Key Factors Associated With Cassava Brown Streak Disease Incidence and Cassava Productivity. Front. Sustain. Food Syst. 5.

Park, J.-W., da Graça, J.V., Gonzalez, M., Louzada, E.S., Alabi, O.J., Kunta, M., 2022. First Report of Citrus Virus A in Texas Associated with Oak Leaf Patterns in Citrus sinensis. Plant Dis. 106, 2005. https://doi.org/10.1094/PDIS-03-21-0628-PDN

Paysan-Lafosse, T., Blum, M., Chuguransky, S., Grego, T., Pinto, B.L., Salazar, G.A., Bileschi, M.L., Bork, P., Bridge, A., Colwell, L., Gough, J., Haft, D.H., Letunić, I., Marchler-Bauer, A., Mi, H., Natale, D.A., Orengo, C.A., Pandurangan, A.P., Rivoire, C., Sigrist, C.J.A., Sillitoe, I., Thanki, N., Thomas, P.D., Tosatto, S.C.E., Wu, C.H., Bateman, A., 2023. InterPro in 2022. Nucleic Acids Res. 51, D418–D427. https://doi.org/10.1093/nar/gkac993

Pilotti, M., Faggioli, F., Barba, M., 1995. Characterization of Italian isolates of pear vein yellows virus. Acta Hortic. 148–154. https://doi.org/10.17660/ActaHortic.1995.386.18

Rollin, J., Rong, W., Massart, S., 2023. Cont-ID: detection of sample cross-contamination in viral metagenomic data. BMC Biol. 21, 217. https://doi.org/10.1186/s12915-023-01708-w

Rong, W., Rollin, J., Hanafi, M., Roux, N., Massart, S., 2023. Validation of high throughput sequencing as virus indexing test for Musa germplasm: performance criteria evaluation and contamination monitoring using an alien control. PhytoFrontiersTM. https://doi.org/10.1094/PHYTOFR-03-22-0030-FI

Rott, M., Xiang, Y., Boyes, I., Belton, M., Saeed, H., Kesanakurti, P., Hayes, S., Lawrence, T., Birch, C., Rast, H., 2017. Application of Next Generation Sequencing for Diagnostic Testing of Tree Fruit Viruses and Viroids. https://doi.org/10.1094/PDIS-03-17-0306-RE

Rott, M.E., Kesanakurti, P., Berwarth, C., Rast, H., Boyes, I., Phelan, J., Jelkmann, W., 2018. Discovery of Negative-Sense RNA Viruses in Trees Infected with Apple Rubbery Wood Disease by Next-Generation Sequencing. Plant Dis. 102, 1254–1263. https://doi.org/10.1094/PDIS-06-17-0851-RE

Schönegger, D., Moubset, O., Margaria, P., Menzel, W., Winter, S., Roumagnac, P., Marais, A., Candresse, T., 2023. Benchmarking of virome metagenomic analysis approaches using a large, 60+ members, viral synthetic community. J. Virol. 97, e01300-23. https://doi.org/10.1128/jvi.01300-23

Serra, P., Messmer, A., Sanderson, D., James, D. & Flores, R. (2018) Apple hammerhead viroid-like RNA is a bona fide viroid: Autonomous replication and structural features support its inclusion as a new member in the genus Pelamoviroid. Virus Research, 249, 8–15. https://doi.org/10.1016/j.virusres.2018.03.001

Shahzad, A., Ullah, S., Dar, A.A., Sardar, M.F., Mehmood, T., Tufail, M.A., Shakoor, A., Haris, M., 2021. Nexus on climate change: agriculture and possible solution to cope future climate change stresses. Environ. Sci. Pollut. Res. 28, 14211–14232. https://doi.org/10.1007/s11356-021-12649-8

The Galaxy Community, 2022. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2022 update. Nucleic Acids Res. 50, W345–W351. https://doi.org/10.1093/nar/gkac247

Trebicki, P., 2020. Climate change and plant virus epidemiology. Virus Res. 286, 198059. https://doi.org/10.1016/j.virusres.2020.198059

Xiao, H., Hao, W., Storoschuk, G., MacDonald, J.L., Sanfaçon, H., 2022. Characterizing the Virome of Apple Orchards Affected by Rapid Decline in the Okanagan and Similkameen Valleys of British Columbia (Canada). Pathogens 11, 1231. https://doi.org/10.3390/pathogens11111231

Zhang, Z., Qi, S., Tang, N., Zhang, X., Chen, S., Zhu, P., Ma, L., Cheng, J., Xu, Y., Lu, M., Wang, H., Ding, S.-W., Li, S., Wu, Q., 2014. Discovery of Replicating Circular RNAs by RNA-Seq and Computational Algorithms. PLOS Pathogens 10, e1004553. https://doi.org/10.1371/journal.ppat.1004553

Zhang, H., Zhao, X., Cao, X., Khan, L.U., Zhao, R., Wang, H., Huang, X., 2022. Transmission of Areca Palm Velarivirus 1 by Mealybugs Causes Yellow Leaf Disease in Betel Palm (Areca catechu). Httpsdoiorg101094PHYTO-06-21-0261-R 112, 700–707. https://doi.org/10.1094/PHYTO-06-21-0261-R

Supplementary Table 4-4. List of samples that were analyzed by high-throughput sequencing (HTS), either using double-stranded (dsRNA) or total RNA extraction protocols, or by reverse transcription polymerase chain reaction (RT-PCR) for the field survey. The library number and pool identification number for each sample are provided in separate columns. Tree ID identifies the location in the orchard (line and tree) from each collection.

Country	Institution	Tree ID	Acc. nº	Cultivar	ID Total RNA	Library dsRNA	Pool (dsRNA)
Belgium	CRA-W	W2	599	Poire d'Abbe	-	L1	L1-3
Belgium	CRA-W	W4	670	Poire d'Abbe	-	L1	L1-3
Belgium	CRA-W	W5	643	Pwèr d'Aoust	-	L1	L1-3
Belgium	CRA-W	W6	602	Belle de Bruxelles	-	L2	L2-1
Belgium	CRA-W	W7	169	Poire de Notre Dame	-	L1	L1-3
Belgium	CRA-W	W10	646	Poire de Notre Dame	-	L1	L1-1
Belgium	CRA-W	W11	461	Poire de Gros	-	L1	L1-2
Belgium	CRA-W	W12	462	Poire de Gros	-	L2	L2-1
Belgium	CRA-W	W13	490	Poire de Gros	-	L1	L1-1
Belgium	CRA-W	W14	491	Poire de Gros	-	L2	L2-2
Belgium	CRA-W	W15	672	Pwar di Pétia	-	L1	L1-2
Belgium	CRA-W	W16	608	Poire de Rondia	-	L1	L1-2
Belgium	CRA-W	W18	603	Poire de Tutia	-	L1	L1-1
Belgium	CRA-W	W19	582	Pomme Poire	-	L1	L1-2
Belgium	CRA-W	W20	583	Pomme Poire	-	L1	L1-1
Belgium	CRA-W	W21	600	Pomme Poire	-	L2	L2-1
Belgium	CRA-W	W23	669	Pomme Poire	-	-	-
Belgium	CRA-W	W24	678	Gadelette	-	L2	L2-1
Belgium	CRA-W	W25	485	Jules d'Airoles	-	-	-
Belgium	CRA-W	W27	854	Belle de Blégny	-	-	-
Belgium	CRA-W	W29	855	Bec d'Oie	-	-	-
Belgium	CRA-W	W32	P218	Comice	-	-	-

Supplementary Table 4-4 continued

Belgium	CRA-W	W34	512	Beurré Hardy	-	-	-
Belgium	CRA-W	W36	856	Jules d'Airoles	-	-	-
Belgium	CRA-W	W39	533	Poirier Gooris 2	-	-	-
Belgium	CRA-W	W41	535	Franc de Rixensart	-	-	-
Belgium	CRA-W	W43	1016	Carisi / PBB	-	-	-
Belgium	CRA-W	W45	594	Bergamotte Hostelart	-	-	-
Belgium	CRA-W	W47	617	Poire a Sirop Schyns	-	-	-
Belgium	CRA-W	W49	115	Jargonelle - Beurré d'Amanlis	-	-	-
Belgium	CRA-W	W51	137	Zure Brederode	-	-	-
Belgium	CRA-W	W56	41	Philippe Couvreur	-	-	-
Belgium	CRA-W	W59	1271	Saint Mathieu Lubrent	-	-	-
Belgium	CRA-W	Y2	519	Clermontoise	-	-	-
Belgium	CRA-W	Y3	227	Poire d'Argile	-	-	-
Belgium	CRA-W	Y9	908	Poire de Malades falso	-	L2	L2-3
Belgium	CRA-W	Y11	910	Poire de Malades	-	L2	L2-3
Belgium	CRA-W	Y13	923	Poire de Malades	-	L2	L2-2
Belgium	CRA-W	Y15	627	Comburland	-	L2	L2-2
Belgium	CRA-W	Y16	231	Poire de Mouxhy	-	L2	L2-2
Belgium	CRA-W	Y17	522	Poire de Mouxhy	-	-	-
Belgium	CRA-W	Y20	238	Poire de Pâques Rouge	-	L2	L2-3
Belgium	CRA-W	Y21	372	Poire de Pâques Rouge	-	L2	L2-3

Supplementary Table 4-4 continued

Belgium	CRA-W	Y22	528	Poire de Pâques Rouge	-	-	-
Belgium	CRA-W	Y24	530	Grisette	-	-	-
Belgium	CRA-W	Y25	653	Beurré de Mérode	-	-	-
Belgium	CRA-W	Y27	659	Poire de Dailly	-	-	-
Belgium	CRA-W	Y28	661	Poire de Stembert	-	-	-
Belgium	CRA-W	Y30	666	Semis du Plit	-	-	-
Belgium	CRA-W	Y38	613	Poirier Sauvage Gemmenich	-	-	-
Belgium	CRA-W	Y40	845	Bec d'Oie	-	-	-
Belgium	CRA-W	Y42	850	Légipont Gris	-	-	-
Belgium	CRA-W	Y44	P13	Ananas de Courtrai	-	-	-
Belgium	CRA-W	Y45	P16	Beurré de Naghin	-	-	-
Belgium	CRA-W	Y48	P200	Docteur Lentier	-	-	-
Belgium	CRA-W	Y49	137	Zure Brederode	-	-	-
Belgium	CRA-W	Y56	99	Beurré d'Amanlis	-	-	-
Belgium	CRA-W	Y58	866	Beurré d'Hardenpont	-	-	-
Belgium	CRA-W	Y60	863	Margueritte Maria falso	-	-	-
Belgium	CRA-W	Z1	232	Poire de Tranche	-	L3	L3-1
Belgium	CRA-W	Z2	523	Poire de Tranche falso	T1	L3	L3-2
Belgium	CRA-W	Z3	220	Poire d'Espece Blanche	T2	L3	L3-2
Belgium	CRA-W	Z4	664	Poire d'Espèce Grise	-	L4	L4-1
Belgium	CRA-W	Z5	221	Poire d'Espèce Grise	-	L4	L4-1

Supplementary Table 4-4 continued

Belgium	CRA-W	Z6	244	Marguerite Marillat	-	L4	L4-1
Belgium	CRA-W	Z7	525	Poire d'Espèce Grise	-	L4	L4-1
Belgium	CRA-W	Z11	223	Gertrude	-	L3	L3-1
Belgium	CRA-W	Z12	521	Beurré Hardy	-	L3	L3-1
Belgium	CRA-W	Z13	240	Immortelle	-	L3	L3-1
Belgium	CRA-W	Z14	224	Jean Nicolas	T1	L3	L3-2
Belgium	CRA-W	Z15	621	Jean Nicolas	T2	L3	L3-2
Belgium	CRA-W	Z31	833	Beurré d'Etaille	-	-	-
Belgium	CRA-W	Z33	835	Joséphine de Malines	-	-	-
Belgium	CRA-W	Z35	837	Poire Tardive	-	-	-
Belgium	CRA-W	Z37	839	Poire Istasse	-	-	-
Belgium	CRA-W	Z39	840	Beurré de Naghin	-	-	-
Belgium	CRA-W	Z41	842	Beurré d'Hardenpont	-	-	-
Belgium	CRA-W	Z43	845	Bec d'Oie	-	-	-
Belgium	CRA-W	Z45	846	Saint Michel	-	-	-
Belgium	CRA-W	Z47	847	Poire Grognet	-	-	-
Belgium	CRA-W	Z49	887	Beurré Lebrun	-	-	-
Belgium	CRA-W	Z51	889	Légipont d'Hiver 889	-	-	-
Belgium	CRA-W	Z53	884	Poire Cuisse Madame	-	-	-
Belgium	CRA-W	Z55	885	Jeanne d'Arc	-	-	-
Belgium	CRA-W	Z57	748	Beurré Chaboceau	-	-	-
Belgium	CRA-W	Z59	224	Jean Nicolas	-	-	-
Belgium	CRA-W	V5	581	Poire de Brûlee	-	L4	L4-2
Belgium	CRA-W	V6	497	Poire de Gauniau	-	L5	L5-1
Belgium	CRA-W	V7	593	Poire de Gauniau	-	L5	L5-1
Belgium	CRA-W	V8	658	Poire de Gauniau	-	L5	L5-1

Supplementary Table 4-4 continued

Belgium	CRA-W	V9	592	Poire de Madeleine	-	L5	L5-1
Belgium	CRA-W	V10	171	Poire de Notre Dame	-	L5	L5-2
Belgium	CRA-W	V11	470	Poire de Notre Dame	-	L5	L5-2
Belgium	CRA-W	V12	470	Poire de Notre Dame	-	L5	L5-3
Belgium	CRA-W	V13	575	Poire de Notre Dame	-	L5	L5-3
Belgium	CRA-W	V14	576	Poire de Notre Dame falso	-	L4	L4-2
Belgium	CRA-W	V15	595	Poire de Notre Dame	-	L4	L4-2
Belgium	CRA-W	V16	596	Poire de Notre Dame	-	L5	L5-2
Belgium	CRA-W	V17	647	Poire de Notre Dame	-	L5	L5-2
Belgium	CRA-W	V18	650	Poire de Re	-	L4	L4-2
Belgium	CRA-W	X1	637	Poire de Cloche	-	-	-
Belgium	CRA-W	X4	428	Poire de Thisnes	-	-	-
Belgium	CRA-W	X5	429	Poire de Thisnes	-	L5	L5-3
Belgium	CRA-W	X8	452	Beau Présent	-	L5	L5-3
Belgium	CRA-W	X9	615	Beau Présent	-	L6	L6-1
Belgium	CRA-W	X10	616	Beau Présent	-	L6	L6-1
Belgium	CRA-W	X11	636	Beau Présent falso	-	L6	L6-2
Belgium	CRA-W	X12	662	Beau Présent	-	L6	L6-1
Belgium	CRA-W	X13	620	Bec d'Oie	-	L7	L7-1
Belgium	CRA-W	X14	216	Belle de Bruxelles	-	L6	L6-1
Belgium	CRA-W	X16	527	Bouton d'Or	-	L7	L7-1
Belgium	CRA-W	X18	626	Camberlain Blanc	-	L7	L7-2
Belgium	CRA-W	X19	217	Camberlain Rouge	-	L7	L7-1
Belgium	CRA-W	X20	243	Camberlain Rouge	-	L6	L6-2
Belgium	CRA-W	X21	188	Cardinal	-	L7	L7-2
Belgium	CRA-W	X22	219	Camberlain Rouge	-	L6	L6-2

Supplementary Table 4-4 continued

Belgium	CRA-W	X23	524	Cardinal	-	L6	L6-2
Belgium	CRA-W	X24	463	Grisette	-	-	-
Belgium	CRA-W	X27	584	Poire de Notre Dame	-	-	-
Belgium	CRA-W	X28	638	Semis Henin	-	-	-
Belgium	CRA-W	X29	614	Semis Légipont	-	-	-
Belgium	CRA-W	X31	571	Poire 1835	-	-	-
Belgium	CRA-W	X33	574	Petite Grise	-	-	-
Belgium	CRA-W	X34	586	Poire d'Hiver	-	-	-
Belgium	CRA-W	X53	873	Poirette Petite Chapelle	-	-	-
Belgium	CRA-W	X55	874	Poire d'Avoine	-	-	-
Belgium	CRA-W	X57	880	Poire Brune d'Aubel	-	-	-
Belgium	CRA-W	X59	884	Poire Cuisse Madame	-	-	-
Slovenia	Kozjanski Park	SLO-1	-	Anglumenka	-	-	-
Slovenia	Kozjanski Park	SLO-2	-	Avranška - Dobra Lujza	-	-	-
Slovenia	Kozjanski Park	SLO-3	-	Hardijeva	-	-	-
Slovenia	Kozjanski Park	SLO-4	-	Krasanka	-	-	-
Slovenia	Kozjanski Park	SLO-5	-	Viljamovka	-	-	-
Slovenia	Kozjanski Park	SLO-6	-	Zimska Dekanka	-	-	-
Switzerland	Agroscope	5-1	39649	Comice	-	-	-
Switzerland	Agroscope	5-3	39651	Packam's Triumph	-	-	-
Switzerland	Agroscope	5-4	39652	Abbé Fettel	-	-	-
Switzerland	Agroscope	5-5	39653	Sept en Gueule	-	-	-
Switzerland	Agroscope	5-6	39654	Culotte suisse	-	-	-
Switzerland	Agroscope	5-7	39655	Culotte suisse	-	-	-
Switzerland	Agroscope	5-8	39656	Epine d'hiver	-	-	-
Switzerland	Agroscope	5-9	39657	Packam's Triumph	-	-	-

Supplementary Table 4-4 continued

Switzerland	Agroscope	5-10	39658	Packam's Triumph	-	-	-

Supplementary Table 4-5. Continuation of the samples that were analyzed by HTS, either using dsRNA or total RNA extraction protocol, or by RT-PCR for the field survey. The results of the field survey by RT-PCR are shown as well: samples that were positive are colored in blue and samples that were negative in light grey. Samples not analyzed with that specific method are left in white.

Tree ID	HTS		RT-PCR	Sanger sequencing	Field survey			
	dsRNA	Total RNA			PyVA	CiVA	ARWV-1	ARWV-2
W2	Yes	No	Yes	No				
W4	Yes	No	Yes	No				
W5	Yes	No	Yes	No				
W6	Yes	No	No	No				
W7	Yes	No	Yes	No				
W10	Yes	No	Yes	No				
W11	Yes	No	Yes	No				
W12	Yes	No	Yes	No				
W13	Yes	No	Yes	No				
W14	Yes	No	Yes	No				
W15	Yes	No	Yes	No				
W16	Yes	No	Yes	No				
W18	Yes	No	Yes	No				
W19	Yes	No	Yes	No				
W20	Yes	No	Yes	No				
W21	Yes	No	Yes	No				
W23	No	No	Yes	No				
W24	Yes	No	No	No				
W25	No	No	Yes	No				
W27	No	No	Yes	No				
W29	No	No	Yes	No				
W32	No	No	Yes	No				
W34	No	No	Yes	No				
W36	No	No	Yes	No				
W39	No	No	Yes	No				
W41	No	No	Yes	No				
W43	No	No	Yes	No				
W45	No	No	Yes	No				
W47	No	No	Yes	No				
W49	No	No	Yes	No				
W51	No	No	Yes	No				
W56	No	No	Yes	No				
W59	No	No	Yes	No				
Y2	No	No	Yes	No				
Y3	No	No	Yes	No				
Y9	Yes	No	Yes	No				

Supplementary Table 4-5 continued

Y11	Yes	No	Yes	No	
Y13	Yes	No	Yes	No	
Y15	Yes	No	Yes	No	
Y16	Yes	No	Yes	No	
Y17	No	No	Yes	No	
Y20	Yes	No	Yes	No	
Y21	Yes	No	No	No	
Y22	No	No	Yes	No	
Y24	No	No	Yes	No	
Y25	No	No	Yes	No	
Y27	No	No	Yes	No	
Y28	No	No	Yes	No	
Y30	No	No	Yes	No	
Y38	No	No	Yes	No	
Y40	No	No	Yes	No	
Y42	No	No	Yes	No	
Y44	No	No	Yes	No	
Y45	No	No	Yes	No	
Y48	No	No	Yes	No	
Y49	No	No	Yes	No	
Y56	No	No	Yes	No	
Y58	No	No	Yes	No	
Y60	No	No	Yes	No	
Z1	Yes	No	No	No	
Z2	Yes	Yes	No	No	
Z3	Yes	Yes	Yes	No	
Z4	Yes	No	No	No	
Z5	Yes	No	No	No	
Z6	Yes	No	No	No	
Z7	Yes	No	No	No	
Z11	Yes	No	No	No	
Z12	Yes	No	Yes	No	
Z13	Yes	No	No	No	
Z14	Yes	Yes	Yes	No	
Z15	Yes	Yes	No	No	
Z31	No	No	Yes	No	
Z33	No	No	Yes	No	
Z35	No	No	Yes	No	
Z37	No	No	Yes	No	
Z39	No	No	Yes	No	
Z41	No	No	Yes	No	
Z43	No	No	Yes	No	
Z45	No	No	Yes	No	

Supplementary Table 4-5 continued

Z47	No	No	Yes	No	
Z49	No	No	Yes	No	
Z51	No	No	Yes	No	
Z53	No	No	Yes	No	
Z55	No	No	Yes	No	
Z57	No	No	Yes	No	
Z59	No	No	Yes	No	
V5	Yes	No	No	No	
V6	Yes	No	No	No	
V7	Yes	No	No	No	
V8	Yes	No	No	No	
V9	Yes	No	No	No	
V10	Yes	No	No	No	
V11	Yes	No	No	No	
V12	Yes	No	No	No	
V13	Yes	No	No	No	
V14	Yes	No	No	No	
V15	Yes	No	No	No	
V16	Yes	No	No	No	
V17	Yes	No	No	No	
V18	Yes	No	No	No	
X1	No	No	Yes	No	
X4	No	No	Yes	No	
X5	Yes	No	Yes	No	
X8	Yes	No	No	No	
X9	Yes	No	Yes	No	
X10	Yes	No	No	No	
X11	Yes	No	Yes	No	
X12	Yes	No	No	No	
X13	Yes	No	Yes	No	
X14	Yes	No	No	No	
X16	Yes	No	Yes	No	
X18	Yes	No	Yes	No	
X19	Yes	No	Yes	No	
X20	Yes	No	Yes	No	
X21	Yes	No	No	No	
X22	Yes	No	Yes	No	
X23	Yes	No	No	No	
X24	No	No	Yes	No	
X27	No	No	Yes	No	
X28	No	No	Yes	No	
X29	No	No	Yes	No	
X31	No	No	Yes	No	

Supplementary Table 4-5 continued

X33	No	No	Yes	No
X34	No	No	Yes	No
X53	No	No	Yes	No
X55	No	No	Yes	No
X57	No	No	Yes	No
X59	No	No	Yes	No
SLO-1	No	No	Yes	No
SLO-2	No	No	Yes	No
SLO-3	No	No	Yes	No
SLO-4	No	No	Yes	No
SLO-5	No	No	Yes	No
SLO-6	No	No	Yes	CiVA
5-1	No	No	Yes	No
5-3	No	No	Yes	No
5-4	No	No	Yes	ARWV-1 and 2
5-5	No	No	Yes	CiVA
5-6	No	No	Yes	No
5-7	No	No	Yes	No
5-8	No	No	Yes	No
5-9	No	No	Yes	No
5-10	No	No	Yes	No

Supplementary Table 4-6. Stats of the cleaned reads and contigs produced for each pool, analyzed either with the dsRNA or total RNA extraction protocol.

Extraction protocol	Library	Pool	Trees included in the pool	Total nº contigs	Contigs >=1000 bp	N50 Length (bp)	Total nº reads
dsRNA	L1	L1-1	W20, W18, W10, W13	1413	555	4209	1648404
		L1-2	W11, W15, W16, W19	1537	640	2927	789890
		L1-3	W2, W4, W5, W7	1197	454	2196	1341260
	L2	L2-1	W6, W21, W24, W12	408	126	1634	575729
		L2-2	W14, Y13, Y15, Y16	882	281	1200	998836
		L2-3	Y9, Y11, Y20, Y21	765	248	1192	889113
	L3	L3-1	Z1, Z11, Z12, Z13	474	127	1076	351510
		L3-2	Z2, Z3, Z14, Z15	362	116	2699	276198
	L4	L4-1	Z4, Z5, Z6, Z7	1123	271	1062	979069
		L4-2	V14, V18, V5, V15	950	150	760	1097530
	L5	L5-1	V6, V7, V8, V9	310	45	668	608901
		L5-2	V10, V11, V17, V16	290	64	957	280057
		L5-3	V12, V13, X5, X8	243	29	696	272520
	L6	L6-1	X14, X10, X12, X9	108	59	1692	314217
		L6-2	X11, X20, X22, X23	49	1	503	262464
	L7	L7-1	X13, X16, X19	70	28	1446	134289
		L7-2	X18, X21	66	9	625	174318
	Alien control (ac)		Bean	29	6	871	86604
Total RNA	T1		Z2, Z14	17051	6873	1283	4765901
	T2		Z3, Z15	19415	8414	1360	4047580

Supplementary Table 4-7. Results of the mapped reads in each pool for each virus. The columns refer to the number of reads mapped, the reads per kilo base per million (RPKM), and the column called RVx/RAx shows the percentage of RPKM present in one sample compared to the sample with the highest RPKM for each virus (cross-contamination ratio=100%). The column called coverage shows the horizontal coverage of the mapping, thus how much of the genome is covered by the mapped reads. Rows colored in blue are considered true positives (TP), rows colored in orange are considered likely false positives (FP) because they are below the positivity threshold, and rows colored in white are considered true negatives (TN). Rows colored in light grey represent cross-contamination events between the alien control and the pools analyzed and are thus true negatives (TN).

Pool	ASPV				ACLSV				PyVA			
	Mapping	RPKM	RV_{ASPV}	Horizontal coverage	Mapping	RPKM	RV_{ACLSV}	Horizontal coverage	Mapping	RPKM	RV_{PyVA}	Horizontal coverage
L1-1	116018	7542,0	30,5%	100,0%	3124	250,8	60,4%	76,9%	0	0,0	0,0%	0,0%
L1-2	182175	24714,2	100,0%	100,0%	2478	415,2	100,0%	76,8%	0	0,0	0,0%	0,0%
L1-3	88196	7046,3	28,5%	100,0%	17	1,7	0,4%	15,4%	0	0,0	0,0%	0,0%
L2-1	493	91,8	0,4%	62,0%	291	66,9	16,1%	36,4%	0	0,0	0,0%	0,0%
L2-2	9688	1039,4	4,2%	100,0%	480	63,6	15,3%	65,3%	0	0,0	0,0%	0,0%
L2-3	6098	734,9	3,0%	94,4%	86	12,8	3,1%	31,4%	0	0,0	0,0%	0,0%
L3-1	4819	1469,1	5,9%	97,0%	254	95,6	23,0%	37,6%	2	0,3	0,0%	1,2%
L3-2	40838	15844,2	64,1%	98,7%	636	304,8	73,4%	53,9%	26299	5554,7	100,0%	99,9%
L4-1	83725	9163,6	37,1%	99,8%	44	5,9	1,4%	20,0%	0	0,0	0,0%	0,0%
L4-2	23957	2339,1	9,5%	94,7%	1	0,1	0,0%	2,7%	0	0,0	0,0%	0,0%
L5-1	37086	6526,6	26,4%	97,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L5-2	14507	5550,8	22,5%	93,8%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L5-3	10192	4007,6	16,2%	90,2%	2	1,0	0,2%	2,3%	0	0,0	0,0%	0,0%
L6-1	48	16,4	0,1%	30,0%	0	0,0	0,0%	0,0%	7	1,3	0,0%	2,2%
L6-2	56	22,9	0,1%	26,7%	2	1,0	0,2%	2,1%	0	0,0	0,0%	0,0%
L7-1	3	2,4	0,0%	3,2%	1	1,0	0,2%	1,7%	23	10,0	0,2%	11,7%
L7-2	6	3,7	0,0%	6,8%	1	0,8	0,2%	1,7%	13557	4536,9	81,7%	34,5%
Alien	0	0,0	0,0%	0,0%	1	1,5	0,4%	1,7%	0	0,0	0,0%	0,0%
T1	1901	42,7	na	99,4%	534	14,8	na	86,4%	1228	15	na	100%
T2	4	0,1	na	6,5%	38	1,2	na	36,3%	3526	51	na	100%

Supplementary Table 4-7 continued (horizontally)

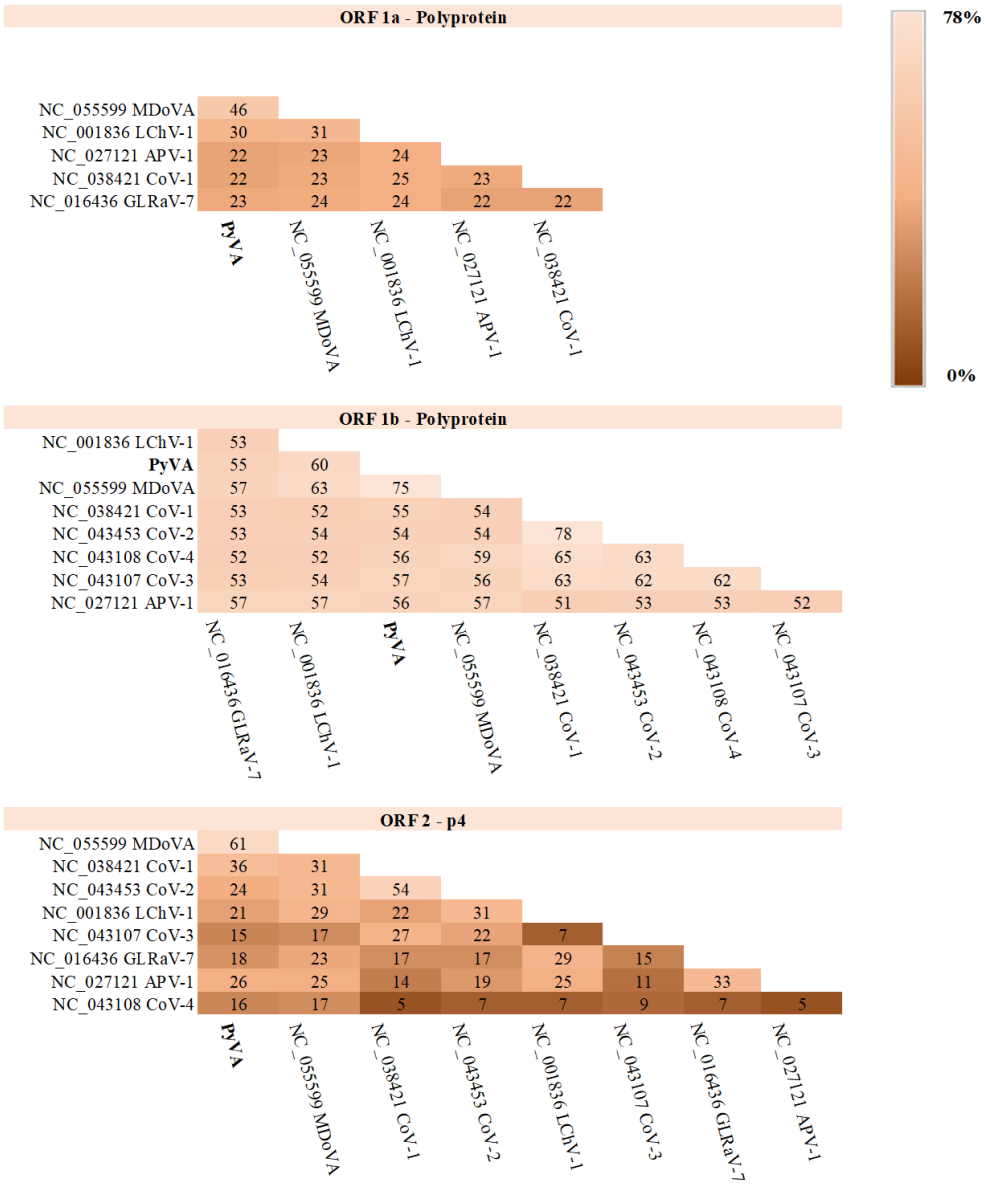
Pool	ARWV-1											
	Segment L				Segment M				Segment S			
	Mapping	RPKM	$RV_{ARWV-1(L)}$	Horizontal coverage	Mapping	RPKM	$RV_{ARWV-1(M)}$	Horizontal coverage	Mapping	RPKM	$RV_{ARWV-1(S)}$	Horizontal coverage
L1-1	2	0,2	39,6%	3,8%	19	7,2	42,6%	29,9%	0	0,0	0,0%	0,0%
L1-2	0	0,0	0,0%	0,0%	8	6,3	37,5%	36,9%	0	0,0	0,0%	0,0%
L1-3	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L2-1	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L2-2	0	0,0	0,0%	0,0%	27	16,8	100,0%	63,0%	2	1,5	44,5%	16,7%
L2-3	0	0,0	0,0%	0,0%	15	10,5	62,4%	36,1%	4	3,3	100,0%	24,7%
L3-1	0	0,0	0,0%	0,0%	3	5,3	31,6%	12,5%	0	0,0	0,0%	0,0%
L3-2	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L4-1	3	0,4	100,0%	2,3%	2	1,3	7,6%	17,2%	0	0,0	0,0%	0,0%
L4-2	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L5-1	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L5-2	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L5-3	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L6-1	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L6-2	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L7-1	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L7-2	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
Alien	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
T1	0	0,0	na	0,0%	13	1,7	na	64,2%	32	5,0	na	86,1%
T2	1	0,0	na	2,1%	20	3,1	na	79,8%	5	0,9	na	37,4%

Supplementary Table 4-7 continued (horizontally)

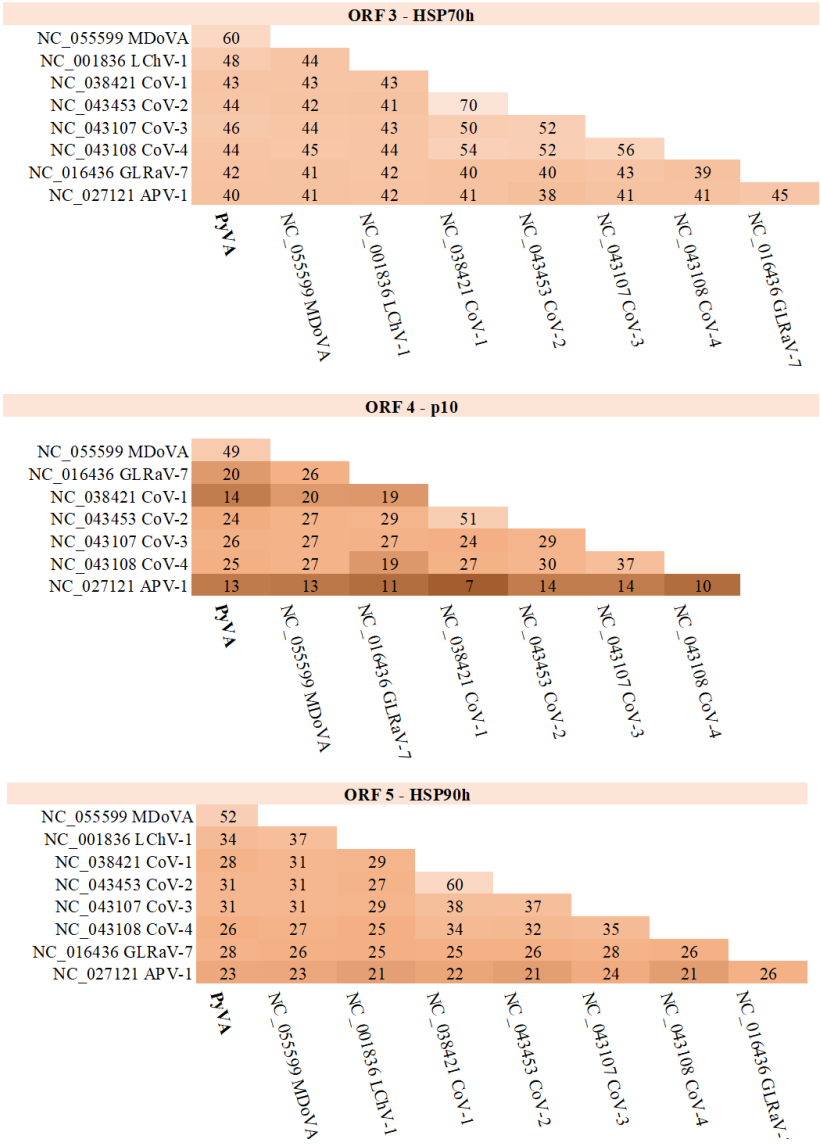
Pool	CiVA							
	RNA 1				RNA 2			
	Mapping	RPKM	$RV_{CiVA(1)}$	Horizontal coverage	Mapping	RPKM	$RV_{CiVA(2)}$	Horizontal coverage
L1-1	19	1,7	21,2%	19,9%	83	18,5	61,0%	62,6%
L1-2	36	6,8	83,9%	27,1%	45	20,9	69,1%	60,3%
L1-3	1	0,1	1,4%	1,9%	16	4,4	14,5%	37,4%
L2-1	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L2-2	14	2,1	25,8%	15,9%	16	5,9	19,4%	30,2%
L2-3	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L3-1	12	5,1	62,9%	12,2%	29	30,2	100,0%	42,7%
L3-2	15	8,2	100,0%	9,1%	2	2,7	8,8%	9,0%
L4-1	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L4-2	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L5-1	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L5-2	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L5-3	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L6-1	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L6-2	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L7-1	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L7-2	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
Alien	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
T1	206	6,5	na	99,4%	679	52	na	100%
T2	0	0,0	na	0,0%	0	0	na	0%

Supplementary Table 4-7 continued (horizontally)

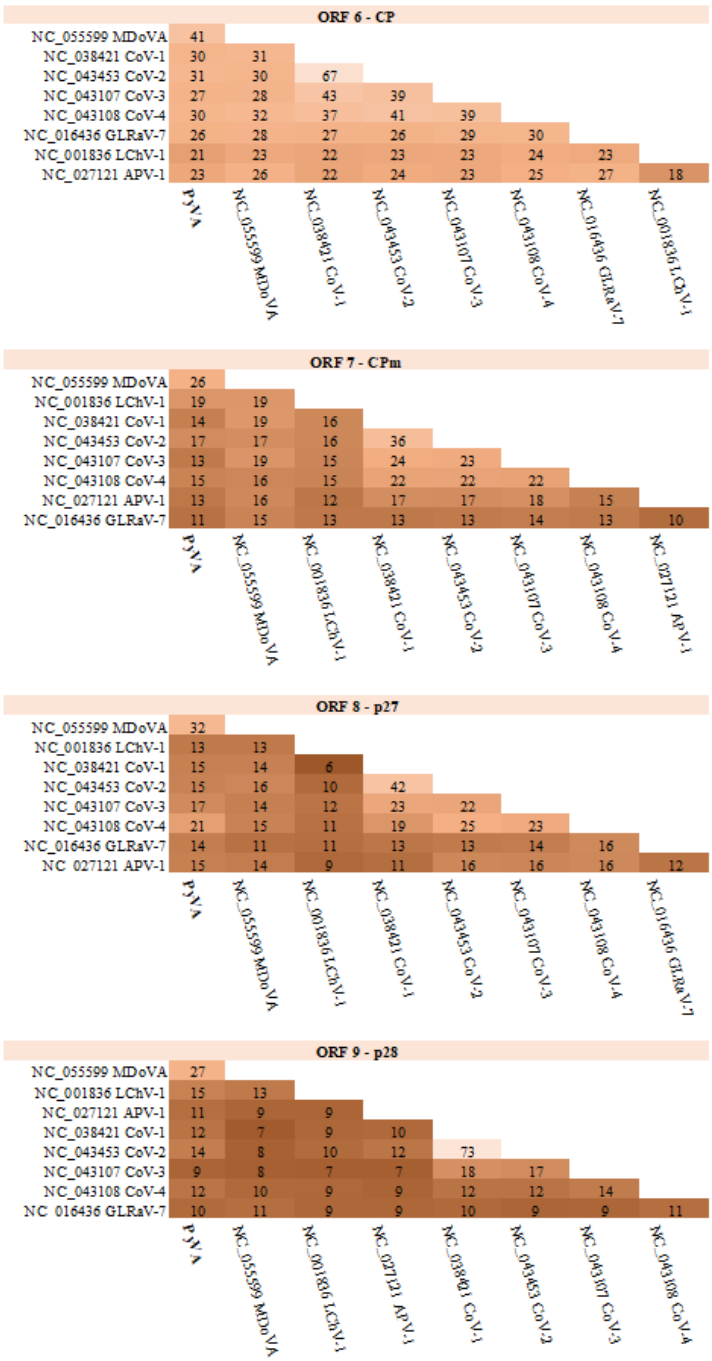
Pool	PvEV-1				PvEV-2				PvEV-3			
	Mapping	RPKM	RAa_1	Horizontal coverage	Mapping	RPKM	RAa_2	Horizontal coverage	Mapping	RPKM	RAa_3	Horizontal coverage
L1-1	0	0,0	0,0%	0,0%	3	0,1	0,0%	1,6%	0	0,0	0,0%	0,0%
L1-2	0	0,0	0,0%	0,0%	1	0,1	0,0%	0,9%	0	0,0	0,0%	0,0%
L1-3	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L2-1	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L2-2	0	0,0	0,0%	0,0%	2	0,1	0,0%	1,5%	0	0,0	0,0%	0,0%
L2-3	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L3-1	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L3-2	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L4-1	0	0,0	0,0%	0,0%	2	0,1	0,0%	1,0%	0	0,0	0,0%	0,0%
L4-2	2	0,1	0,0%	0,9%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L5-1	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L5-2	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L5-3	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L6-1	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L6-2	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L7-1	5	2,6	0,5%	2,1%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
L7-2	739	301,3	56,6%	2,2%	0	0,0	0,0%	0,0%	0	0,0	0,0%	0,0%
Alien	649	532,5	100,0%	13,2%	45410	35387,8	100,0%	60,7%	12054	9153,9	100,0%	17,2%
T1	na	na	na	na	na	na	na	na	na	na	na	na
T2	na	na	na	na	na	na	na	na	na	na	na	na



Supplementary Figure 4-4. Percentage of identity at aminoacid level between the proteins with identified functional domains (polyprotein 1a-1b, HSP70h, HSP90h, CP, CPm) and the putative proteins (p4, p10, p27, p28) of the new virus and the other velariviruses. The complete name of each sequence can be found in Figure 2. The genomes of NC_043453, NC_043107, and NC_043108 had partial sequences of the ORF 1a. Genome NC_001836 did not have an annotation for p10.



Supplementary Figure 4-4 continued



Supplementary Figure 4-4 continued

Chapter 5

Discussion

As explained in the introduction, viruses in pome fruits tend to accumulate due to the vegetative propagation and grafting of fruit trees, potentially leading to yield losses and reduced life span of the trees. Therefore, their identification, detection, and characterization are crucial. Traditional diagnostic techniques such as PCR or ELISA are mostly limited to the detection of known viruses. In the case of PCR, this technique is also limited by the target's available genomic information, which means that the correct detection of a known virus by PCR can be problematic due to its usually high genome variability. On the contrary, the untargeted nature of HTS allows the identification of distant variants of known viruses and facilitates the discovery of novel viruses. In recent years, the use of HTS in plant virology has identified hundreds of new plant viruses, generated thousands of new genomes for known viruses, and contributed to the host range extension of many known viruses. These discoveries can have regulatory impacts, and their current pace challenges the traditional phytosanitary risk evaluation.

This thesis represents a significant advancement in the field by delving into managing the phytosanitary risk analysis, considering the abundance of novel viruses discovered, particularly by HTS. It also explores the diversity of viruses affecting apple and pear trees. A key highlight is the characterization of a novel velarivirus, a unique contribution to the field, identified in pear trees and tentatively named Pyrus virus A.

1. Creation of a comprehensive framework designed to guide the biological characterization of novel plant viruses and viroids

In 2017, a framework to evaluate the impact of plant viruses and viroids identified by HTS was proposed by researchers as a response to the significant number of novel viruses published (Massart et al., 2017). This framework was widely used and cited but became outdated as it did not fully accommodate the fast pace of virus discovery and the feasibility of post-discovery virus characterization. Moreover, since then, two publications that reviewed the discovery of novel viruses and the characterization efforts carried out post-discovery found that there was little follow-up after the initial publication of a novel virus, and even in this initial publication, little information was provided about its characteristics and properties (Hou et al., 2020; Rivarez et al., 2021). For instance, Hou et al. (2020) found that a local survey of prevalence, which provides information regarding local epidemiology, was done in 30% of the publications presenting a new fruit tree virus and in zero publications citing the first discovery reports. Similarly, a survey at a large scale that provides information about global epidemiology was done in almost 50% of the publications of a new fruit tree virus. In this case, however, some citing publications provide further information regarding global epidemiology studies, which were finally carried out for 61% of the newly discovered viruses (Hou et al., 2020).

Within the project INEXTVIR (Innovative Network for Next Generation Training and Sequencing of Virome), encompassing my individual Ph.D. project, a group of international researchers, doctoral students, and risk managers revised the previously published framework. Following this revision, it was clear that creating a new framework better suited to the current rate of virus discovery was needed. The new framework was created and designed to take into account the findings from the two reviews about the discovery of novel viruses by HTS and the characterization efforts following said discoveries and to incorporate the recent reconsideration of the application of Koch's postulates to demonstrate causal association in plant virology from Fox (2020).

The novelties of the newly developed scientific and regulatory framework to assist in the characterization and risk analysis of novel plant viruses and viroid discovered by HTS include an extension of the data-gathering step (see Chapter 3, section 2.2) and an earlier evaluation of the causal association to support a quicker response by stakeholders (researchers, risk managers, policymakers, etc.) to control and manage novel viruses that pose a phytosanitary risk (see Chapter 3, section 2.3). Numerous technical advances have allowed the creation of algorithms to assist researchers in analyzing and treating bioinformatic data. To create a framework incorporating these innovative tools and approaches, we suggested additional analyses be performed during the data-gathering step.

Such analyses include the screening and data mining of public databases, prompted by the publication of Serratus, a user-friendly web-based interface (Edgar et al., 2022), and a more transversal approach that predicts the function of genomic sequences based on their structural features (Tahzima et al., 2021). Although Serratus revolutionized how scientists scan publicly available datasets for known or unknown viruses, data mining was already a regular practice. For example, data mining was used to study the genetic diversity of two trichoviruses infecting grapevine worldwide, which allowed the study of their evolutionary history (Hily et al., 2020). Another example is the discovery of novel viruses through data mining publicly available datasets (Bejerman et al., 2023; Khalili et al., 2023). However, such an approach may pose risks for the trade of plant material and lead to an economic impact because of new reports of viruses. The ethical issues associated with this strategy need to be taken into consideration, and confirmatory tests and notification with the NPPO of the country where the samples originated from are recommended, as stated in Chapter 3.

Moreover, the framework was designed to accommodate the different types of new viruses. For instance, there will not be the same reaction and prioritization to an unknown virus being discovered in an economically important (staple) crop as to an unknown virus being discovered in a wild plant. On the other hand, the appearance of symptoms linked to the virus adds a layer of concern, particularly in staple crops and wild plants that could act as reservoirs and inoculum sources for crops grown in fields nearby. Hence, the causal association evaluation is recommended early on to have information about the association of the presence or absence of the virus with

symptoms. At this stage, information about the host range and transmission modes can be obtained via field surveys and greenhouse assays done to study the causal association. Nonetheless, it is worth mentioning that a limitation of this approach is that the detection of a virus in a sample does not necessarily imply that this virus has or does not have pathogenic potential and is associated with symptoms. Further analysis needs to be done, for example, to take into consideration the relative abundance of the virus, or viruses if there is a mixed infection, in the host(s) and its genetic variability, and how these may impact symptom emergence.

The divergence in the dedicated efforts to characterizing a novel virus depending on the threat they pose is evidenced by Rivarez et al. (2021), which showcases the rapid characterization of tomato brown rugose fruit virus (ToBRFV), an emerging virus of significant economic importance, compared to other viruses discovered during the last decade (Rivarez et al., 2021). Another case is the characterization of Physostegia chlorotic mottle virus (PhCMoV), an emerging virus characterized by international collaborations (Temple et al., 2022), whose characterization was completed five years after its discovery.

In general, when comparing the information provided by original publications following the discovery of a novel fruit tree viruses, we can observe that the framework created in this thesis is more adapted to what is feasible and realistic for laboratories in terms of resources and time available to employ on the characterization and further study of novel viruses and viroids because the steps of the new framework are completed in sequentially in a decreasing manner (Figure 5-1).

Notably, recently published articles announcing novel viruses or describing the biological characterization of known viruses followed a similar structure to what is suggested in the newly created framework, strengthening the consideration that it is well adapted. For instance, there are publications describing newly discovered viruses that were detected in symptomatic plants/samples which provided information until step 3 of the created framework, including the reconstructed genome, provisional taxonomic assignation, phylogenetic analysis with viruses of the same genus or family, and field surveys and/or transmission assays to study the symptomatology, host range, and mode of transmission (Chabi-Jesus et al., 2023; García-Rodríguez et al., 2023; Neeraganti et al., 2023; Uehara-Ichiki et al., 2022). Comparatively, publications describing novel viruses identified in plants that are not as commercially significant mainly focused on steps 1 and 2, with research centered on the molecular characterization and phylogenetic relationship with other viruses (Peng et al., 2023; Zhou et al., 2023).

The framework was designed to be applicable and adapted by different users regardless of the type of virus discovered, its biological properties, and the risk it might pose, which means that the end goal was not to create a standalone document but to create something that would be adapted by different organisms and stakeholders involved in the field of virus discovery, detection, and/or diagnostics. The fact that

recent publications describing newly discovered viruses presented their results similarly to what was proposed in the framework indicates that it has the potential to be well followed and adopted by researchers. It can be brought and adapted by regulatory authorities, plant protection organizations (NPPOs), and policymakers. Each institution or organism may adapt the created framework to their current validated and adopted protocols to form a more fluid communication between the involved stakeholders, including, for example, researchers, NPPOs, or grower/farmer associations.

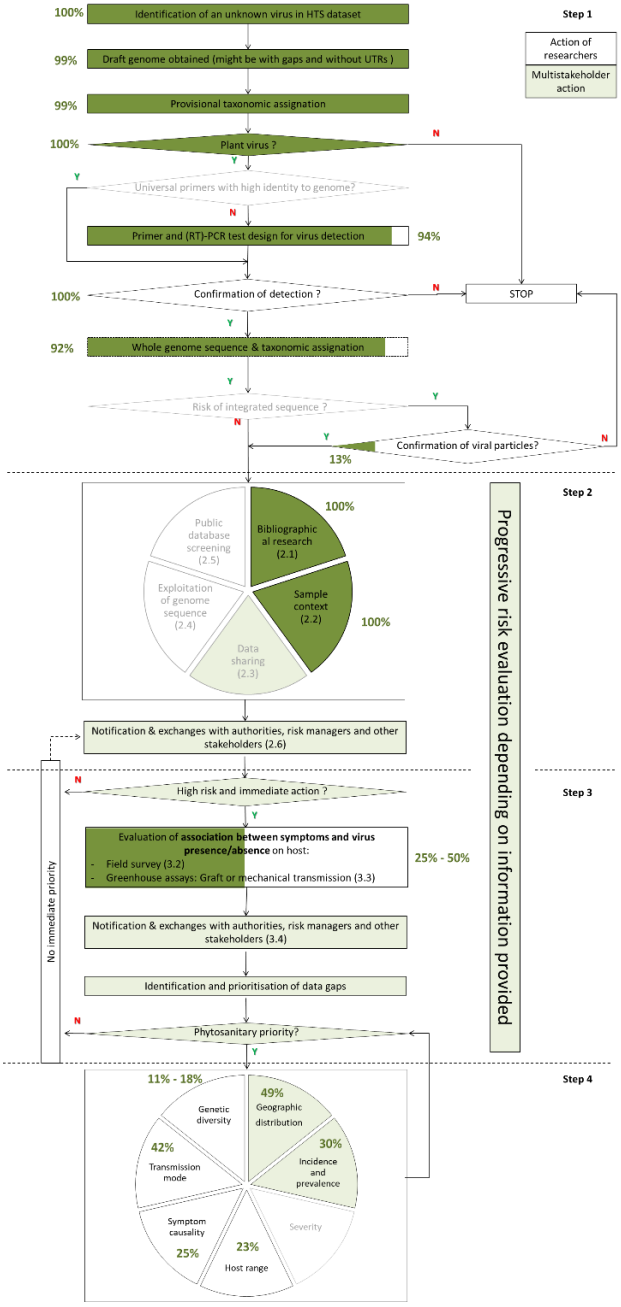


Figure 5-1. This diagram showcases the newly created framework for characterizing a novel virus or viroid, as is described in detail in Chapter 3. Boxes and percentages in dark green represent the fraction of original publications providing information for the new fruit tree virus, as seen in Hou et al. (2020). Boxes in light grey represent information not described in the previous framework. Figure modified and adapted from Fontdevila Pareta et al. (2023) under the Creative Commons Attribution License (CC BY).

2. The study of the apple and pear virome

In recent years, we have seen the limitations of the paradigm established by Koch and Hill's postulates of "one pathogen - one disease" where a disease is caused by a single pathogen in a specific host. For that reason, there have been several attempts to improve and revisit Koch's postulate to adapt it to the reality of plant virology (Fox, 2020). Moreover, this paradigm is shifting towards a broader approach to the concept of pathogenesis, referred to as the pathobiome, to integrate the high biological diversity of microorganisms and their interactions with other microorganisms, the environment, hosts, and vectors (Vayssier-Taussat et al., 2014).

Along these lines, the plant viral metagenome, or plant virome, refers to viruses infecting the plant itself and those infecting organisms associated with the plant, such as fungi, bacteria, and other microorganisms. This concept encompasses the diversity of viruses within the plant ecosystem, thus providing insight into the complex interactions between viruses, their hosts, and associated organisms (Vainio et al., 2024). Therefore, studying the plant's virome is critical to understanding the factors driving the spread of plant viruses and the possible emergence of plant viral diseases.

The second objective of this thesis was to study the virome of apple and pear from the germplasm collection from the Walloon Agricultural Research Centre (CRA-W) in Gembloux (Belgium). This collection was selected because it hosts many pome and stone fruit trees, including local and ancient cultivars. HTS has been widely used to study the virome of various plants and crops, such as carrot (Schönegger et al., 2023a), tomato (Rivarez et al., 2021; Temple et al., 2023), and *Prunus* spp. (Jo et al., 2018; Khalili et al., 2023), or alfalfa (Nemchinov et al., 2022); so, the viruses infecting apple and pear trees from the aforementioned collection in Belgium were scanned using the double-stranded RNA approach to enrich the viruses in the trees. This approach has been widely used to study fruit tree viruses, providing a more complete view of a complex virome mainly constituting RNA viruses (Schönegger et al., 2023b). This study also integrated a pooling approach before nucleic acid isolation and purification to increase the number of samples processed. This cost-effective pooling approach has proven effective in studying SNP frequencies to characterize and study the geographic distribution of different viral haplotypes (Nyirakanani et al., 2023) and to study the virome composition of *Poaceae* communities in Belgium with different anthropogenic management methods (Maclot et al., 2023).

Although latent and mixed viral infections are common in pome fruit trees and might impact yield or productivity under specific circumstances, most publications studying the virome of apples and pears focused on diseased and symptomatic trees to study the disease's etiology and possible causal agent(s). For instance, a recent publication studied the virome of diseased apple trees, showing necrosis and mosaic-like symptoms compared to healthy-looking trees in India. Globally, they detected five viruses, including apple necrotic mosaic virus (ApNMV), apple mosaic virus (ApMV), apple stem grooving virus (ASGV), apple stem pitting virus (ASPV), and

apple chlorotic leaf spot virus (ACLSV); and one viroid, apple hammerhead viroid (AHVd) (Nabi et al., 2022). In another article, 21 plant viruses and one viroid were detected in apple orchards affected by rapid apple decline (RAD) disease in Canada, with most trees infected with more than two viruses. The most prevalent viruses (>50% positive samples) were apple ilarvirus 2 (AIV2), Citrus concave gum associated virus (CCGaV), ASPV, and ACLSV. ASGV was detected in 41.9% of the samples, and the remaining viruses and viroid were detected in less than 30% (Xiao et al., 2022).

These results aligned with what was found in the collection in Belgium, where ASPV, ACLSV, and ASGV were the most prevalent viruses (unpublished data), usually found in mixed infections also with viruses reported for the first time in Belgium, such as apple rubbery wood virus 1 (ARWV-1), apple luteovirus 1 (ALV1) and apple hammerhead viroid 1 (Fontdevila Pareta et al., 2022). Similarly, ASGV and ASPV are also highly prevalent in pear trees and present in mixed infections with, for example, ACLSV, ApMV, Citrus virus A (CiVA), and ARWV-1 (Khan et al., 2024).

ARWV-1 is a relatively newly discovered virus, and since its first description, it has been detected in many countries, including China, South Korea, and Brazil (Hu et al., 2021; Lim et al., 2019; Nickel et al., 2020). Noteworthy, it is associated with apple rubbery wood disease (ARWD) (Rott et al., 2018), which is a disease that has been known for decades and is widely spread (Jakovljevic et al., 2017). Therefore, it is essential to be careful of the wording when reporting this virus and apple rubbery wood virus 2 (ARWV-2) versus their first molecular detection. These viruses are associated with the disease, and if the disease was already reported in the country, it could be assumed that the viruses were there, too.

CiVA was first described in nonsymptomatic sweet orange trees in 2018 (Navarro et al., 2018). Since then, it has been detected in an expanding number of countries, thus showcasing a large geographic distribution. CiVA has been reported in France (Svanella-Dumas et al., 2019), South Africa (Bester et al., 2021b), Australia (Donovan et al., 2022), China (Yang et al., 2023), the United States (Park et al., 2022), Greece (Beris et al., 2021), India (Khan et al., 2024), Belgium, Slovenia, and Switzerland (see Chapter 4). Although its geographic distribution has been extended, CiVA does not show a high prevalence in commercial orchards (Diaz-Lara et al., 2022; Wang et al., 2022). In comparison, in the germplasm collection from the CRA-W, CiVA was found with a high prevalence (49%). However, no visible symptoms of a viral infection were observed during the sampling campaigns and subsequent visits. Nonetheless, this high prevalence does not imply that this virus poses a high risk as it is not associated with any symptoms in pear trees, and no vector is known.

The virome study in the Belgian germplasm collection supported the completion of data gaps of newly discovered viruses (CiVA and ARWV-1) and helped identify a novel velarivirus. During the 2010s, it was typical for virome surveys done with HTS to uncover novel viruses or viroids, such as the novel AIV2 discovered during the

survey in Canadian apple orchards affected with RAD disease (Xiao et al., 2022). Nowadays, following the "gold rush" of virus discovery, we are seeing a decline in the number of novel viruses being reported. Particularly in pome and stone fruit trees, this loss in momentum may imply that we are arriving at the plateau phase of virus discovery. Figure 5-2 illustrates the novel viruses discovered in pome and stone fruit trees, in which we can observe an apparent exponential increase in the number of viruses discovered between 2011 and 2020 following the advent of HTS. Moreover, another element to consider aside from the number of viruses discovered is the efforts in terms of sequencing depth and volume of samples tested. It is possible that the decrease in the speed of discovery of novel viruses is linked to the need to sequence more samples to discover a new virus (the most prevalent being discovered first) or the decrease in the sequencing and sampling efforts.

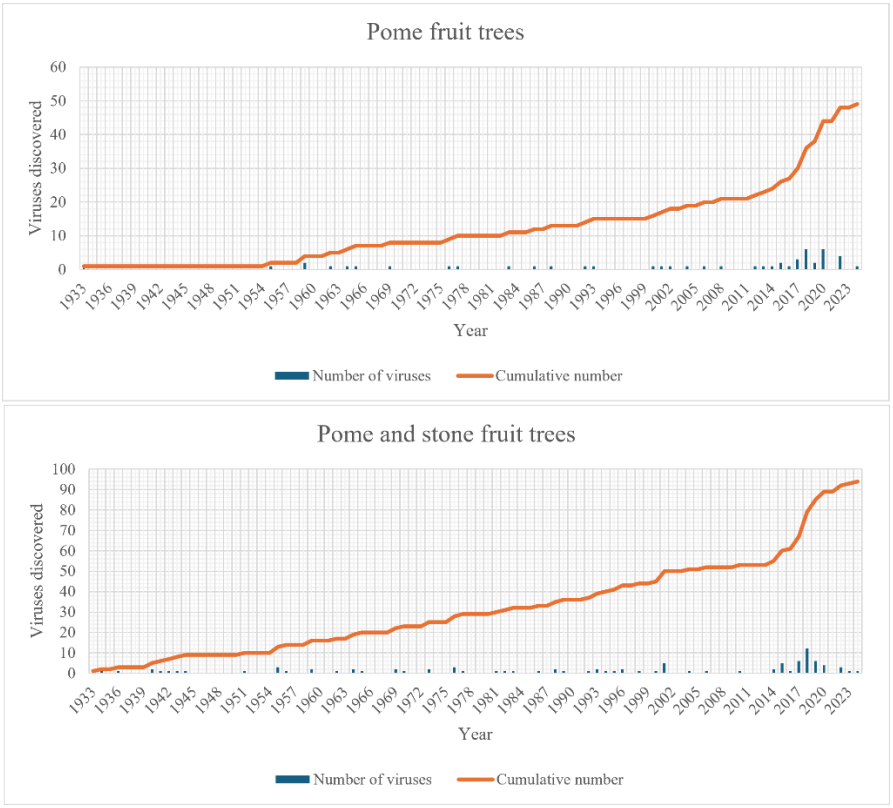


Figure 5-2. Evolution of the number and cumulative number of viruses discovered in pome fruit trees (image above) and pome and stone fruit trees combined (image below). The accumulation of viruses in both cases goes at a steady pace until 2011 for pome fruits and until 2013 for pome and stone fruits, where it grows exponentially until 2021, when the speed of virus discovery decelerates, thus reaching a plateau.

In the study of the virome of fruit trees, the primary concern regarding the pooling strategy was that pome fruit viruses' distribution, replication, and concentration may vary and be heterogeneous within a tree. Therefore, a balance between detection sensitivity and cost should be found through an appropriate number of samples pooled that would still allow the detection of potentially all the viruses infecting one tree. The study of the virome conducted in this thesis showed the potential of dsRNA in combination with pooling and HTS not only for research purposes but also in plant virus diagnostics, given that it correctly identified positive and negative sense RNA (+/- ssRNA) viruses such as ASPV or CiVA in pooled samples.

Viral enrichment techniques would allow for higher sequencing depth and coverage of viruses compared to currently used approaches, such as total RNA or small RNA (sRNA) sequencing. For instance, total RNA sequences the genetic material of the plant's pathogens and the plant's genetic material, resulting in a significantly smaller proportion of viral reads in the dataset. Additionally, in sRNA sequencing, a weaker defense response generated by the plant to viral infection can potentially affect the detectability of the viruses (Bester et al., 2021a). An innate variation is associated with using HTS to detect viruses associated with each approach, ranging from sample collection to interpretation of the results. Therefore, an extensive evaluation process is required before using the dsRNA protocol and pooling strategy in routine diagnostics.

In citrus trees, there was a comprehensive and thorough evaluation of the application of HTS for routine plant virus and viroid detection in quarantine and certification schemes, which compared various extraction protocols and sequencing platforms (Bester et al., 2021a). Moreover, an HTS test, based on the total RNA sequencing, was successfully used in virus indexing of *Musa* germplasm and presented a higher analytical sensitivity than RT-PCR (Rong et al., 2023). This study also incorporated an alien control to monitor cross-contamination and index hopping, or crosstalk, from the samples (individual, pooled, and/or multiplexed) towards the control and vice versa. This alien control can help validate the results and ensure the accurate detection of the targets. In comparison, other studies that use dsRNA use a negative extraction control to monitor contamination from the samples towards the control (Schönegger et al., 2022). The use of controls is highly recommended, if not required, to validate the results and the viruses detected by HTS, as described in the guidelines to assist in adopting HTS technologies as a plant pest diagnostic test (Lebas et al., 2022), as well as in the EPPO Standard on Diagnostics PM 7/151 (1) regarding the considerations for the use of HTS in plant health diagnostics. Although in this study we used an external alien control to monitor crosstalk, other methodologies might emerge in the future, like the use of sets of internal alien controls in each sample or the use of more complex (multiple infection) external alien controls.

A similar evaluation approach to what has been done for grapevine, Citrus, and temperate fruit trees can be used to validate the strategy proposed in this study (Bester et al., 2021a; Marais et al., 2024; Soltani et al., 2021). For example, a complex of

known viruses and viroids can be inoculated by grafting to healthy trees (i.e., apple or pear trees), which will enable the evaluation of performance criteria, such as the analytical sensitivity and specificity or the limit of detection of the target(s). The extraction and library preparation protocols' performance criteria can be evaluated and compared to the results obtained with validated molecular tests (RT-PCR) and HTS approaches, such as total RNA sequencing.

3. Virus discovery: characterization of a novel velarivirus identified in pear trees

As a result of the virome scanning at the collection from the CRA-W, a putative novel velarivirus tentatively named Pyrus virus A (PyVA) was identified in pear trees (*Pyrus communis*). This novel virus is suggested as a novel addition to the genus *Velarivirus* within the family *Closteroviridae*. Globally, the viruses within the genus *Velarivirus* are relatively divergent, as shown in Supplementary Figure 4-4, although there are high similarities between some of them. For example, with PyVA being a novel member of the genus, the two closest viruses within the genus are PyVA and Malus domestica virus A (MdoVA). Moreover, the comparison between the two reconstructed genomes of PyVA and the partial sequences acquired through Sanger sequencing revealed a remarkable similarity, which may suggest a probable low genetic variability. However, the fact that the two reconstructed genomes that were virtually identical were isolated from contiguous trees may also be an indication of horizontal transmission, at a low rate, of the virus within the orchard. Nonetheless, this dataset lacks sufficient samples to conclude anything in this regard.

A comprehensive study, comprising large-scale field surveys and transmission assays, was conducted to study the transmissibility by grafting, the host range, and to assess the association between the presence of the virus and symptomatic expression in the plant host, as per the third step of the created framework (see Chapter 3, section 2.3). There were no viral symptoms observed on the trees that were positive with PyVA, either in single or mixed infection, and on the inoculated rootstocks and plants used for the transmission assays performed at the CRA-W (in a greenhouse) and Agroscope (in the field). While it remains plausible that symptoms would manifest over time, the absence of visible leaf symptoms during the sampling campaigns in 2021 and 2023 and subsequent monitoring in summer 2022 suggests otherwise. It would be possible for symptomless infections to lead to yield losses or to lasting effects and phenological changes on the infected trees (Valentova et al., 2022). Noteworthy, the full virome of the trees and rootstocks was not considered. It would be possible for the rootstocks, which had a plant passport, to be infected with other viruses aside from the regulated ones that would, in turn, interact with PyVA and affect its transmissibility. Moreover, lack of transmission could also occur due to heterogeneity of the virus within the tree. Hence, if the plant material that was grafted did not contain the virus, there would be no transmission to the rootstock.

Further analysis could be done to study the plant immune system and host-virus interactions to better understand how the virus may interact and compete with other viruses potentially present in the sample and how this can affect its virulence and the emergence and appearance of symptoms on the host.

Regarding host range, the novel virus was successfully transmitted only to different cultivars of pear, albeit the transmission rate was not 100%. Given that (i) the current host range of PyVA is limited to pear, (ii) there are no symptoms associated with this viral infection, and (iii) it has only been detected in Belgium with low prevalence, PyVA could be considered as a virus with relatively low phytosanitary risk.

Plant pathologists have been traditionally trained to identify and manage plant viruses that harm their hosts. In contrast, the negative connotation associated with plant viruses has shifted in recent years. There are many examples of viruses that are not harmful to plants, as well as viruses that may have a positive effect. For instance, *Peach latent mosaic virus* (PLMVd) has been associated with bloom delay (which may be beneficial to avoid spring frost-related injuries on flowers), reduced tree vigor, or higher fruiting efficiency (Gibson et al., 2008). Keeping this in mind, it would be possible that during the propagation of pome fruit trees, breeders have unknowingly selected plants infected by graft-transmissible agents, such as viruses, that produce the desirable traits. Eventually, continuing to study and monitor the evolution of plants infected with PyVA, in single and mixed infections, may provide valuable insight into the potential beneficial effects of this novel virus for the plant's survival and fruit production.

4. The effects of the current virus discovery rate

The publication of a novel viral species' sequence(s) no longer requires providing a complete genome sequence with complete 5' and 3' UTRs (Simmonds et al., 2017). Moreover, it is no longer recommended by ICTV if the complete set of ORFs is detected. However, there is a difference, and a wide range of different cases, between a partial genome with the complete set of ORFs and short partial sequences that miss protein-coding regions. While it is true that obtaining complete or nearly complete genomes can be time-consuming and, in some instances, not cost-effective, given that the new virus might not be a priority or pose a risk to plant health, there is a risk of oversaturating such repositories with products of sequencing artifacts or chimeras or even sequences that are misassigned to the incorrect organism or taxonomic group.

Aside from human error, misassignment of viruses, although uncommon, can happen when comparing a virus discovered prior to HTS for which no sequence is available and a novel virus. Scanning and testing virus collections and herbarium samples to study older isolates of known or uncharacterized viruses can provide insight into their host range and evolutionary history and enhance the characterization and identification of phantom agents (Alvarez-Quinto et al., 2023; Fowkes et al., 2022). For instance, *Plantain virus X* was an accepted species by the ICTV the

sequence of which was not available until later efforts to sequence the original isolates following a proposal to the ICTV to abolish the species. After obtaining the complete genome of the original isolates from conserved samples, researchers realized that this virus was equivalent to the newer *Actinidia virus X* (Hammond et al., 2021).

Other cases include multiple names for the same species, such as cherry green ring mottle virus (CGRMV), which has the synonym names of cherry green ring mottle foveavirus and Sour cherry green mottle virus, or publication of a new viral species that is then integrated as an isolate of a known virus (accepted by the ICTV), such as apple green crinkle associated virus (AGCaV) which can be considered as a distant variant of ASPV (Li et al., 2020). Furthermore, species names are renamed to comply with the binomial species format recently adopted by the ICTV. For example, the species name of ASPV is now *Foveavirus mali*, which is how it can be found on the ICTV. However, the change was not made in other platforms and repositories such as NCBI Taxonomy or EPPO Global Database. While the adopted binomial Latinized form of naming viral species was a much-needed change, its adaptation still poses some problems, particularly for novel viruses where the original publication may propose a tentative name that ICTV may not accept.

In contrast, legislation evolves slowly and conservatively; thus, keeping them up to date with the rate of virus discovery after the advent of HTS is challenging. Risk assessors and risk managers face a lot of uncertainties when evaluating whether to add a pathogen to a list, as risk avoidance is a tempting strategy that can have a commercial benefit by restricting the trade of infected plant material. However, adding a virus to a list also imposes additional constraints and more costs, and means that for viruses with high prevalence there is an increase in the number of cultivars that must be cleaned. Meanwhile, disease agents are also present in lists of regulated plant pathogens that have been identified. An example is apple rubbery wood viruses 1 and 2, first discovered in trees showing symptoms of apple rubbery wood and flat limb disease. However, they have also been identified in mixed and single infections healthy-looking and diseased trees. With this idea in mind, hypothetically speaking, excluding such agents that do not negatively affect their host could potentially reduce the costs and time of testing for the presence of the viruses. Nonetheless, it would also mean that this negligible effect of the virus would have to be tested under all possible conditions, such as plant varieties including all relevant hosts, viral isolates, environmental/cultural conditions, or possible co-infection scenarios to accept the pathogen removal from the list. Moreover, the idea behind regulating viruses is to exclude them from orchards and crop production systems to ensure profitable and sustainable production. But, is excluding all viruses the best way to control disease?

For a disease caused by a pathogen to appear, there has to be a virulent pathogen, the appropriate environmental conditions that are favorable for the disease to develop, and a susceptible host. Moreover, viruses and their hosts have co-evolved towards milder symptoms in order to maintain the ecological fitness of the host. Additionally, there are viruses known to induce host tolerance or resistance against abiotic stresses.

For instance, tomato yellow leaf curl virus (TYLCV) has been found to improve drought tolerance in tomato plants (Botto et al., 2023). In other cases, viruses can also be part of control measures against aggressive strains or even other pathogens. For example, mild strains of a virus can be used to protect plants from a disease that is caused by a severe strain of the same virus, such as the use of mild strains of pepino mosaic virus (PepMV) in tomato plants to protect them from aggressive strains (Hernando and Aranda, 2024). Another example would be the use of virus-based biological control agents to control other pathogens and pests, such as the use of mycovirus Cryphonectria hypovirus 1 (CHV1) to control and fight against chestnut blight disease (Wagemans et al., 2022).

Globally, HTS does not categorically facilitate or complexify plant viruses' study, detection, and classification, but its role and influence are more subtle. HTS has signified a massive step forward in identifying and characterizing plant viruses, providing a more complete and nuanced understanding of the plant's pathobiome and the interactions and relationships between the organisms within an ecosystem. By discovering new viruses from old and current plant material, we can also fill in some of the blanks in virus evolution and classification. Meanwhile, in the short term, although it may potentially be reaching the plateau, the current rate of virus discovery makes it complex to keep up and organize the new findings. In contrast, it might enable a faster virus classification and taxonomic assignation in the long term, thanks to the higher availability of meticulously validated and curated reference genomes.

5. Conclusions and future perspectives

This thesis has focused on the study of plant viruses infecting apple and pear trees from the CRA-W collection using high-throughput sequencing (HTS) technologies. Additionally, it has contributed to the development of a new scientific and regulatory framework for the characterization of novel viruses and viroids discovered through HTS. Since its publication, this newly established framework has proven effective for the characterization of the emerging Physostegia chlorotic mottle virus (PhCMoV) (Temple et al., 2024).

Future improvements to the framework should address the economic impact of novel viruses and viroids on primary plant hosts, facilitating the risk assessment process (Rao and Reddy, 2020). Assessing the impact of a novel virus on yield and estimating economic losses can be achieved through severity studies conducted in greenhouse conditions (Temple et al., 2024). Nevertheless, this is challenging, as productivity and yield impacts are influenced not only by biotic factors, including interactions between microorganisms and host plants, but also by abiotic factors and the surrounding environment, which may alter pathogen population densities. Another valuable approach involves incorporating field impact assessments through farmer interviews and considering sociodemographic, economic, and agronomic variables (Nyirakanani et al., 2021; Temple et al., 2023).

The HTS study conducted on the CRA-W collection has filled data gaps regarding the geographic distribution of recently discovered viruses, such as ARWV-1 and CiVA. Additionally, a novel velarivirus was identified in pear trees, which was further characterized using the developed framework. This study also introduced the use of a cross-contamination ratio to monitor crosstalk between the pools of samples and the alien control, by establishing a threshold to distinguish true positives from likely false positives. Future research should involve the use of multiple internal and external controls to monitor crosstalk and optimize HTS application in regulatory settings and certification schemes. The next steps in the characterization of the novel velarivirus, PyVA, should focus on understanding plant-virus interactions and the virus's behavior in mixed infections and how they affect the plant host. It is also conceivable that the novel virus might confer beneficial traits to the plant, such as resistance or tolerance to other pathogens and pests.

In summary, this research has laid a solid foundation for the characterization and understanding of plant viruses and viroids using HTS. Continuous refinement of the framework and further studies on the novel virus, as well as its interaction with other viruses and the host plant, can help to improve our knowledge and capabilities in managing plant health, ultimately benefiting agricultural productivity and biosecurity.

References

Alvarez-Quinto, R., Amao, M., Muller, G., Fuentes, S., Grinstead, S., Fuentes-Bueno, I., Roenhorst, A., Westenberg, M., Botermans, M., Kreuze, J., Mollov, D., 2023. Evidence that an Unnamed Isometric Virus Associated with Potato Rugose Disease in Peru Is a New Species of Genus Torradovirus. Phytopathology® 113, 1716–1728. https://doi.org/10.1094/PHYTO-11-22-0449-V

Bejerman, N., Dietzgen, R., Debat, H., 2023. Novel Tri-Segmented Rhabdoviruses: A Data Mining Expedition Unveils the Cryptic Diversity of Cytorhabdoviruses. Viruses 15, 2402. https://doi.org/10.3390/v15122402

Beris, D., Ioanna, M., Vassilakos, N., Theologidis, I., Rampou, A., Kektsidou, O., Massart, S., Varveri, C., 2021. Association of Citrus Virus A to Citrus Impietratura Disease Symptoms. Phytopathology 111, 1782–1789. https://doi.org/10.1094/PHYTO-01-21-0027-R

Bester, R., Cook, G., Breytenbach, J.H.J., Steyn, C., De Bruyn, R., Maree, H.J., 2021a. Towards the validation of high-throughput sequencing (HTS) for routine plant virus diagnostics: measurement of variation linked to HTS detection of citrus viruses and viroids. Virology Journal 18, 61. https://doi.org/10.1186/s12985-021-01523-1

Bester, R., Karaan, M., Cook, G., Maree, H.J., 2021b. First report of citrus virus A in Citrus in South Africa. Journal of Citrus Pathology 8. https://doi.org/10.5070/C481049000

Sacco Botto, Camilla, Slavica Matić, Amedeo Moine, Walter Chitarra, Luca Nerva, Chiara D'Errico, Chiara Pagliarani, and Emanuela Noris. 2023. "Tomato Yellow Leaf Curl Sardinia Virus Increases Drought Tolerance of Tomato" *International Journal of Molecular Sciences* 24, no. 3: 2893. https://doi.org/10.3390/ijms24032893

Chabi-Jesus, C., Ramos-González, P.L., Tassi, A.D., Rossetto Pereira, L., Bastianel, M., Lau, D., Canale, M.C., Harakava, R., Novelli, V.M., Kitajima, E.W., Freitas-Astúa, J., 2023. Citrus Bright Spot Virus: A New Dichorhavirus, Transmitted by Brevipalpus azores, Causing Citrus Leprosis Disease in Brazil. Plants 12, 1371. https://doi.org/10.3390/plants12061371

Diaz-Lara, A., Wunderlich, L., Nouri, M.T., Golino, D., Al Rwahnih, M., 2022. Incidence and detection of negative-stranded RNA viruses infecting apple and pear trees in California. Journal of Phytopathology 170, 15–20. https://doi.org/10.1111/jph.13051

Donovan, N., Chambers, G., Englezou, A., Forbes, W., Dando, A., Holford, P., 2022. First report of citrus virus A in Australia. Journal of Citrus Pathology 9. https://doi.org/10.5070/C49157292

Fontdevila Pareta, N., Lateur, M., Steyer, S., Blouin, A. g., Massart, S., 2022. First reports of Apple luteovirus 1, Apple rubodvirus 1 and Apple hammerhead viroid infecting apples in Belgium. New Disease Reports 45, e12076. https://doi.org/10.1002/ndr2.12076

Fowkes, A., Adams, I.P., Jones, R.A.C., Fox, A., McGreig, S., Boonham, N., 2022. Historical and recent tomato black ring virus and beet ringspot virus isolate genomes reveal interspecies recombination and plant health regulation inconsistencies. Plant Pathology 71, 729–740. https://doi.org/10.1111/ppa.13507

Fox, A., 2020. Reconsidering causal association in plant virology. Plant Pathology 69, 956–961. https://doi.org/10.1111/ppa.13199

García-Rodríguez, D.A., Partida-Palacios, B.L., Regla-Márquez, C.F., Centeno-Leija, S., Serrano-Posada, H., Bañuelos-Hernández, B., Cárdenas-Conejo, Y., 2023. Sida chlorotic leaf virus: a new recombinant begomovirus found in non-cultivated plants and Cucumis sativus L. PeerJ 11, e15047. https://doi.org/10.7717/peerj.15047

Gibson, P.G., Reighard, G.L., Scott, S.W., Marini, D., 2008. Phenotypical variation in peach trees inoculated with defined mixtures of viruses and peach latent mosaic viroid. Acta Hortic. 781, 541–546. https://doi.org/10.17660/ActaHortic.2008.781.79

Hammond, J., Adams, I.P., Fowkes, A.R., McGreig, S., Botermans, M., van Oorspronk, J.J.A., Westenberg, M., Verbeek, M., Dullemans, A.M., Stijger, C.C.M.M., Blouin, A.G., Massart, S., De Jonghe, K., Heyneman, M., Walsh, J.A., Fox, A., 2021. Sequence analysis of 43-year old samples of Plantago lanceolata show that Plantain virus X is synonymous with Actinidia virus X and is widely distributed. Plant Pathology 70, 249–258. https://doi.org/10.1111/ppa.13310

Hernando, Y., & Aranda, M. A. (2024). Cross-protection against pepino mosaic virus, more than a decade of efficient disease control. Annals of Applied Biology, 184(2), 174–182. https://doi.org/10.1111/aab.12884

Hily, J.-M., Poulicard, N., Candresse, T., Vigne, E., Beuve, M., Renault, L., Velt, A., Spilmont, A.-S., Lemaire, O., 2020. Datamining, Genetic Diversity Analyses, and Phylogeographic Reconstructions Redefine the Worldwide Evolutionary History of Grapevine Pinot gris virus and Grapevine berry inner necrosis virus. Phytobiomes Journal 4, 165–177. https://doi.org/10.1094/PBIOMES-10-19-0061-R

Hou, W., Li, S., Massart, S., 2020. Is There a "Biological Desert" With the Discovery of New Plant Viruses? A Retrospective Analysis for New Fruit Tree Viruses. Frontiers in Microbiology 11, 2953. https://doi.org/10.3389/fmicb.2020.592816

Hu, G.J., Dong, Y.F., Zhang, Z.P., Fan, X.D., Ren, F., Lu, X.K., 2021. First Report of Apple Rubbery Wood Virus 1 in Apple in China. Plant Disease 105, 3770. https://doi.org/10.1094/PDIS-01-21-0175-PDN

Jakovljevic, V., Otten, P., Berwarth, C., Jelkmann, W., 2017. Analysis of the apple rubbery wood disease by next generation sequencing of total RNA. Eur J Plant Pathol 148, 637–646. https://doi.org/10.1007/s10658-016-1119-z

Jo, Y., Lian, S., Chu, H., Cho, J.K., Yoo, S.-H., Choi, H., Yoon, J.-Y., Choi, S.-K., Lee, B.C., Cho, W.K., 2018. Peach RNA viromes in six different peach cultivars. Sci Rep 8, 1844. https://doi.org/10.1038/s41598-018-20256-w

Khalili, M., Candresse, T., Koloniuk, I., Safarova, D., Brans, Y., Faure, C., Delmas, M., Massart, S., Aranda, M.A., Caglayan, K., Decroocq, V., Drogoudi, P., Glasa, M., Pantelidis, G., Navratil, M., Latour, F., Spak, J., Pribylova, J., Mihalik, D., Palmisano, F., Saponari, A., Necas, T., Sedlak, J., Marais, A., 2023. The Expanding Menagerie of Prunus-Infecting Luteoviruses. Phytopathology 113, 345–354. https://doi.org/10.1094/PHYTO-06-22-0203-R

Khan, Z.A., Diksha, D., Thapa, P., Mailem, Y.S., Sharma, S.K., Gupta, N., Kishan, G., Watpade, S., Baranwal, V.K., 2024. Genome analysis of viruses of Phenuiviridae, Betaflexiviridae and Bromoviridae, and apple scar skin viroid in pear by high-throughput sequencing revealing host expansion of a rubodvirus and an ilarvirus. Physiological and Molecular Plant Pathology 129, 102196. https://doi.org/10.1016/j.pmpp.2023.102196

Lebas, B., Adams, I., Al Rwahnih, M., Baeyen, S., Bilodeau, G.J., Blouin, A.G., Boonham, N., Candresse, T., Chandelier, A., De Jonghe, K., Fox, A., Gaafar, Y.Z.A., Gentit, P., Haegeman, A., Ho, W., Hurtado-Gonzales, O., Jonkers, W., Kreuze, J., Kutjnak, D., Landa, B., Liu, M., Maclot, F., Malapi-Wight, M., Maree, H.J., Martoni, F., Mehle, N., Minafra, A., Mollov, D., Moreira, A., Nakhla, M., Petter, F., Piper, A.M., Ponchart, J., Rae, R., Remenant, B., Rivera, Y., Rodoni, B., Roenhorst, J.W., Rollin, J., Saldarelli, P., Santala, J., Souza-Richards, R., Spadaro, D., Studholme, D.J., Sultmanis, S., van der Vlugt, R., Tamisier, L., Trontin, C., Vazquez-Iglesias, I., Vicente, C.S.L., Vossenberg, B.T.L.H., Wetzel, T., Ziebell, H., Massart, S., 2022. Facilitating the adoption of high-throughput

sequencing technologies as a plant pest diagnostic test in laboratories: A step-by-step description. EPPO Bulletin 52, 394–418. https://doi.org/10.1111/epp.12863

Li, C., Yaegashi, H., Kishigami, R., Kawakubo, A., Yamagishi, N., Ito, T., Yoshikawa, N., 2020. Apple Russet Ring and Apple Green Crinkle Diseases: Fulfillment of Koch's Postulates by Virome Analysis, Amplification of Full-Length cDNA of Viral Genomes, in vitro Transcription of Infectious Viral RNAs, and Reproduction of Symptoms on Fruits of Apple Trees Inoculated With Viral RNAs. Front. Microbiol. 11. https://doi.org/10.3389/fmicb.2020.01627

Lim, S., Baek, D., Moon, J.S., Cho, I.S., Choi, G.S., Do, Y.S., Lee, D.H., Lee, S.H., 2019. First Report of Apple Luteovirus 1 and Apple Rubbery Wood Virus 1 on Apple Tree Rootstocks in Korea. Plant Disease 103, 591. https://doi.org/10.1094/PDIS-08-18-1351-PDN

Maclot, F., Debue, V., Malmstrom, C.M., Filloux, D., Roumagnac, P., Eck, M., Tamisier, L., Blouin, A.G., Candresse, T., Massart, S., 2023. Long-Term Anthropogenic Management and Associated Loss of Plant Diversity Deeply Impact Virome Richness and Composition of Poaceae Communities. Microbiol Spectr 11, e0485022. https://doi.org/10.1128/spectrum.04850-22

Marais, A., Gentit, P., Brans, Y., Renvoisé, J.P., Faure, C., Saison, A., Cousseau, P., Castaing, J., Chambon, F., Pion, A., Calado, G., Lefebvre, M., Garnier, S., Latour, F., Bresson, K., Grasseau, N., Candresse, T., 2024. Comparative performance evaluation of double-stranded RNA high-throughput sequencing for the detection of viral infection in temperate fruit crops. Phytopathology. https://doi.org/10.1094/PHYTO-12-23-0480-R

Massart, S., Candresse, T., Gil, J., Lacomme, C., Predajna, L., Ravnikar, M., Reynard, J.-S., Rumbou, A., Saldarelli, P., Škorić, D., Vainio, E.J., Valkonen, J.P.T., Vanderschuren, H., Varveri, C., Wetzel, T., 2017. A Framework for the Evaluation of Biosecurity, Commercial, Regulatory, and Scientific Impacts of Plant Viruses and Viroids Identified by NGS Technologies. Frontiers in Microbiology 8, 45. https://doi.org/10.3389/fmicb.2017.00045

Nabi, S.U., Baranwal, V.K., Rao, G.P., Mansoor, S., Vladulescu, C., Raja, W.H., Jan, B.L., Alansi, S., 2022. High-Throughput RNA Sequencing of Mosaic Infected and Non-Infected Apple (Malus × domestica Borkh.) Cultivars: From Detection to the Reconstruction of Whole Genome of Viruses and Viroid. Plants (Basel) 11, 675. https://doi.org/10.3390/plants11050675

Navarro, B., Zicca, S., Minutolo, M., Saponari, M., Alioto, D., Di Serio, F., 2018. A Negative-Stranded RNA Virus Infecting Citrus Trees: The Second Member of a New Genus Within the Order Bunyavirales. Frontiers in Microbiology 9, 2340. https://doi.org/10.3389/fmicb.2018.02340

Neeraganti, D.K., Natra, N.T., Naidu, R.A., Kodetham, G., 2023. Molecular characterization of a novel gammacarmovirus infecting cucurbits in India. Arch Virol 168, 65. https://doi.org/10.1007/s00705-022-05643-z

Nemchinov, L.G., Irish, B.M., Grinstead, S., Shao, J., Vieira, P., 2022. Diversity of the virome associated with alfalfa (Medicago sativa L.) in the U.S. Pacific Northwest. Sci Rep 12, 8726. https://doi.org/10.1038/s41598-022-12802-4

Nickel, O., Fajardo, T.V.M., Candresse, T., 2020. First Report on Detection of Three Bunya-Like Viruses in Apples in Brazil. Plant Disease 104, 3088–3088. https://doi.org/10.1094/PDIS-02-20-0283-PDN

Nyirakanani, C., Bizimana, J.P., Kwibuka, Y., Nduwumuremyi, A., Bigirimana, V. de P., Bucagu, C., Lassois, L., Malice, E., Gengler, N., Massart, S., Bragard, C., Habtu, M., Brostaux, Y., Thonar, C., Vanderschuren, H., 2021. Farmer and Field Survey in Cassava-Growing

Districts of Rwanda Reveals Key Factors Associated With Cassava Brown Streak Disease Incidence and Cassava Productivity. Frontiers in Sustainable Food Systems 5.

Nyirakanani, C., Tamisier, L., Bizimana, J.P., Rollin, J., Nduwumuremyi, A., Bigirimana, V. de P., Selmi, I., Lasois, L., Vanderschuren, H., Massart, S., 2023. Going beyond consensus genome sequences: An innovative SNP-based methodology reconstructs different Ugandan cassava brown streak virus haplotypes at a nationwide scale in Rwanda. Virus Evolution 9, vead053. https://doi.org/10.1093/ve/vead053

Park, J.-W., da Graça, J.V., Gonzalez, M., Louzada, E.S., Alabi, O.J., Kunta, M., 2022. First Report of Citrus Virus A in Texas Associated with Oak Leaf Patterns in Citrus sinensis. Plant Disease 106, 2005. https://doi.org/10.1094/PDIS-03-21-0628-PDN

Peng, Z., Chen, Y., Luo, Z., Peng, J., Zheng, H., Wu, G., Rao, S., Wu, J., Xu, Z., Chen, J., Lu, Y., Guo, F., Yan, F., 2023. Complete genome sequence of a new virus from Allium sativum L in China. Arch Virol 168, 167. https://doi.org/10.1007/s00705-023-05794-7

Rao, G.P., Reddy, M.G., 2020. Overview of yield losses due to plant viruses, in: Awasthi, L.P. (Ed.), Applied Plant Virology. Academic Press, pp. 531–562. https://doi.org/10.1016/B978-0-12-818654-1.00038-4

Rivarez, M.P.S., Vučurović, A., Mehle, N., Ravnikar, M., Kutnjak, D., 2021. Global Advances in Tomato Virome Research: Current Status and the Impact of High-Throughput Sequencing. Frontiers in Microbiology 12, 1064. https://doi.org/10.3389/fmicb.2021.671925

Rong, W., Rollin, J., Hanafi, M., Roux, N., Massart, S., 2023. Validation of High-Throughput Sequencing as Virus Indexing Test for Musa Germplasm: Performance Criteria Evaluation and Contamination Monitoring Using an Alien Control. PhytoFrontiersTM 3, 91–102. https://doi.org/10.1094/PHYTOFR-03-22-0030-FI

Rott, M.E., Kesanakurti, P., Berwarth, C., Rast, H., Boyes, I., Phelan, J., Jelkmann, W., 2018. Discovery of Negative-Sense RNA Viruses in Trees Infected with Apple Rubbery Wood Disease by Next-Generation Sequencing. Plant Disease 102, 1254–1263. https://doi.org/10.1094/PDIS-06-17-0851-RE

Schönegger, D., Babalola, B.M., Marais, A., Faure, C., Candresse, T., 2022. Diversity of polerovirus-associated RNAs in the virome of wild and cultivated carrots. Plant Pathology 71, 1892–1900. https://doi.org/10.1111/ppa.13623

Schönegger, D., Marais, A., Babalola, B.M., Faure, C., Lefebvre, M., Svanella-Dumas, L., Brázdová, S., Candresse, T., 2023a. Carrot populations in France and Spain host a complex virome rich in previously uncharacterized viruses. PLOS ONE 18, e0290108. https://doi.org/10.1371/journal.pone.0290108

Schönegger, D., Moubset, O., Margaria, P., Menzel, W., Winter, S., Roumagnac, P., Marais, A., Candresse, T., 2023b. Benchmarking of virome metagenomic analysis approaches using a large, 60+ members, viral synthetic community. Journal of Virology 97, e01300-23. https://doi.org/10.1128/jvi.01300-23

Simmonds, P., Adams, M.J., Benkő, M., Breitbart, M., Brister, J.R., Carstens, E.B., Davison, A.J., Delwart, E., Gorbalenya, A.E., Harrach, B., Hull, R., King, A.M.Q., Koonin, E.V., Krupovic, M., Kuhn, J.H., Lefkowitz, E.J., Nibert, M.L., Orton, R., Roossinck, M.J., Sabanadzovic, S., Sullivan, M.B., Suttle, C.A., Tesh, R.B., van der Vlugt, R.A., Varsani, A., Zerbini, F.M., 2017. Virus taxonomy in the age of metagenomics. Nat Rev Microbiol 15, 161–168. https://doi.org/10.1038/nrmicro.2016.177

Soltani, N., Stevens, K.A., Klaassen, V., Hwang, M.-S., Golino, D.A., Al Rwahnih, M., 2021. Quality Assessment and Validation of High-Throughput Sequencing for Grapevine Virus Diagnostics. Viruses 13, 1130. https://doi.org/10.3390/v13061130

Svanella-Dumas, L., Marais, A., Depasse, F., Faure, C., Lefebre, M., Brans, Y., Castaing, J., Latour, F., Bechti, A., Candresse, T., 2019. First Report of Citrus Virus A (CiVA) Infecting Pear (Pyrus communis) in France. Plant Disease 103, 2703–2703. https://doi.org/10.1094/PDIS-01-19-0028-PDN

Tahzima, R., Haegeman, A., Massart, S., Hébrard, E., 2021. Flexible spandrels of the global plant virome: Proteomic-wide evolutionary patterns of structural intrinsic protein disorder elucidate modulation at the functional virus–host interplay, in: Uversky, V.N. (Ed.), Progress in Molecular Biology and Translational Science, Dancing Protein Clouds: Intrinsically Disordered Proteins in the Norm and Pathology, Part C. Academic Press, pp. 355–409. https://doi.org/10.1016/bs.pmbts.2021.06.007

Temple, C., Blouin, A.G., Boezen, D., Botermans, M., Durant, L., De Jonghe, K., de Koning, P., Goedefroit, T., Minet, L., Steyer, S., Verdin, E., Zwart, M., Massart, S., 2024. Biological characterization of Physostegia chlorotic mottle virus, an emergent virus infecting vegetables in diversified production systems. Phytopathology®. https://doi.org/10.1094/PHYTO-06-23-0194-R

Temple, C., Blouin, A.G., De Jonghe, K., Foucart, Y., Botermans, M., Westenberg, M., Schoen, R., Gentit, P., Visage, M., Verdin, E., Wipf-Scheibel, C., Ziebell, H., Gaafar, Y.Z.A., Zia, A., Yan, X.-H., Richert-Pöggeler, K.R., Ulrich, R., Rivarez, M.P.S., Kutnjak, D., Vučurović, A., Massart, S., 2022. Biological and Genetic Characterization of Physostegia Chlorotic Mottle Virus in Europe Based on Host Range, Location, and Time. Plant Dis 106, 2797–2807. https://doi.org/10.1094/PDIS-12-21-2800-RE

Temple, C., Blouin, A.G., Tindale, S., Steyer, S., Marechal, K., Massart, S., 2023. High throughput sequencing technologies complemented by growers' perceptions highlight the impact of tomato virome in diversified vegetable farms and a lack of awareness of emerging virus threats. Front. Sustain. Food Syst. 7. https://doi.org/10.3389/fsufs.2023.1139090

Uehara-Ichiki, T., Uke, A., Hanada, K., Hishida, A., Nakazono-Nagaoka, E., Kodaira, E., 2022. Scopolia mild mottle virus: a new tobamovirus isolated from a Scopolia japonica plant in Japan. Arch Virol 167, 947–951. https://doi.org/10.1007/s00705-022-05371-4

Vainio, E.J., Rumbou, A., Diez, J.J., Büttner, C., 2024. Forest Tree Virome as a Source of Tree Diseases and Biological Control Agents. Curr. For. Rep. https://doi.org/10.1007/s40725-024-00214-8

Valentova, L., Rejlova, M., Franova, J., Cmejla, R., 2022. Symptomless infection by strawberry virus 1 (StrV-1) leads to losses in strawberry yields. Plant Pathology 71, 1220–1228. https://doi.org/10.1111/ppa.13548

Vayssier-Taussat, M., Albina, E., Citti, C., Cosson, J.F., Jacques, M.-A., Lebrun, M.-H., Le Loir, Y., Ogliastro, M., Petit, M.-A., Roumagnac, P., Candresse, T., 2014. Shifting the paradigm from pathogens to pathobiome: new concepts in the light of meta-omics. Front. Cell. Infect. Microbiol. 4. https://doi.org/10.3389/fcimb.2014.00029

Wagemans, J., Holtappels, D., Vainio, E., Rabiey, M., Marzachì, C., Herrero, S., Ravanbakhsh, M., Tebbe, C. C., Ogliastro, M., Ayllón, M. A., & Turina, M. (2022). Going Viral: Virus-Based Biological Control Agents for Plant Protection. Annual Review of Phytopathology, 60(Volume 60, 2022), 21-42. https://doi.org/10.1146/annurev-phyto-021621-114208

Wang, Yanxiang, Wang, Ying, Wang, G., Li, Q., Zhang, Z., Li, L., Lv, Y., Yang, Z., Guo, J., Hong, N., 2022. Molecular Characteristics and Incidence of Apple Rubbery Wood Virus 2 and Citrus Virus A Infecting Pear Trees in China. Viruses 14, 576. https://doi.org/10.3390/v14030576

Xiao, H., Hao, W., Storoschuk, G., MacDonald, J.L., Sanfaçon, H., 2022. Characterizing the Virome of Apple Orchards Affected by Rapid Decline in the Okanagan and Similkameen Valleys of

British Columbia (Canada). Pathogens 11, 1231. https://doi.org/10.3390/pathogens11111231

Yang, X., Xu, Q., Liu, Z., Zhou, C., Cao, M., 2023. First Report of Citrus Virus A Infecting Citrus (Citrus reticulata) in China. Plant Disease 107, 2269. https://doi.org/10.1094/PDIS-06-22-1344-PDN

Zhou, J., Hu, X., Vieira, P., Atha, B., McFarland, C., Foster, J.A., Hurtado-Gonzales, O.P., 2023. Molecular characterization of horse nettle virus A, a new member of subgroup B of the genus Nepovirus. Arch Virol 168, 86. https://doi.org/10.1007/s00705-023-05708-7

APPENDIX

Publications and conferences

Publications

Maclot FJ, Debue V, Blouin AG, **Fontdevila Pareta N**, Tamisier L, Filloux D, Massart S. Identification, molecular and biological characterization of two novel secovirids in wild grass species in Belgium. *Virus Res*. 2021 Jun;298:198397. doi: 10.1016/j.virusres.2021.198397. Epub 2021 Mar 18. PMID: 33744338.

Fontdevila Pareta, N., Lateur, M., Steyer, S., Blouin, A.G. & Massart, S. (2022) First reports of Apple Luteovirus 1, Apple rubodvirus 1 and Apple hammerhead viroid infecting apples in Belgium. *New Dis Rep*. 45, e12076. https://doi.org/10.1002/ndr2.12076

Fontdevila Pareta N, Khalili M, Maachi A, Rivarez MPS, Rollin J, Salavert F, Temple C, Aranda MA, Boonham N, Botermans M, Candresse T, Fox A, Hernando Y, Kutnjak D, Marais A, Petter F, Ravnikar M, Selmi I, Tahzima R, Trontin C, Wetzel T, Massart S. Managing the deluge of newly discovered plant viruses and viroids: an optimized scientific and regulatory framework for their characterization and risk analysis. *Front Microbiol*. 2023 May 30;14:1181562. doi: 10.3389/fmicb.2023.1181562. PMID: 37323908; PMCID: PMC10265641.

Temple C, Blouin AG, **Fontdevila N**, Steyer S, Massart S. First report of Melon chlorotic spot virus in cultivated sorrel (Rumex acetosa) in Belgium. *Plant Dis*. 2023 Dec 12. doi: 10.1094/PDIS-06-23-1155-PDN. Epub ahead of print. PMID: 38085240.

Fontdevila Pareta, N., Gailly, C., Blouin, A.G., Buchmann, B., Buenter, M., Candresse, T., Dubuis, N., Kutnjak, D., Lateur, M., Pecman, A., Steyer, S., Massart, S., in publication. Virome scanning of pear germplasm collections identifies a new Velarivirus and extends the geographical spread of three other pear viruses. Phytobiomes Journal. Accepted for publication

Oral presentations and posters at conferences

Fontdevila Pareta, N., Lateur, M., Steyer, S., Blouin, A., Rollin, J., & Massart, S. (September 2021). *Virome characterization of pome fruit European genetic resources for future breeding and risk analysis* [Poster presentation]. 18 ème Rencontres de Virologie Végétale, Aussois, France.

Fontdevila Pareta, N., Khalili, M., Maachi, A., Rivarez, M. P. S., Rollin, J., Salavert, F., Temple-Boyer-Dury, C., Aranda, M. A., Boonham, N., Botermans, M., Candresse, T., Fox, A., Hernando, Y., Kutnjak, D., Marais, A., Petter, F., Ravnikar, M., Selmi, I., Tahzima, R., ... Massart, S. (05 October 2022). *Managing the deluge of newly discovered plant viruses and viroids* [Paper presentation]. International Advances in Plant Virology (IAPV22).

Fontdevila Pareta, N., Lateur, M., Steyer, S., Blouin, A. G., & Massart, S. (January 2023). *Pyrus virus A (PyVA), characterization of a novel velarivirus identified in pear trees* [Poster presentation]. 19 ème Rencontres de Virologie Végétale, Aussois, France.

Fontdevila Pareta, N., Wanying, H., Khalili, M., Maachi, A., Rivarez, M. P. S., Rollin, J., Salavert, F., Temple-Boyer--Dury, C., Aranda, A. M., Boonham, N., Botermans, M., Candresse, T., Fox, A., Hernando, Y., Kutnjak, D., Marais, A., Petter, F., Ravnikar, M., Selmi, I., ... Massart, S. (July 2023). *Managing the deluge of newly discovered plant viruses and viroids: an optimized scientific and regulatory framework for their characterization and risk analysis* [Paper presentation]. 25th International Conference on Virus and other graft transmissible diseases of Fruit crops, Wageningen, Netherlands.

Fontdevila Pareta, N., Gailly, C., Lateur, M., Steyer, S., Dubuis, N., Blouin, A. G., & Massart, S. (July 2023). *Characterization of pyrus virus A (PyVA), a novel velarivirus identified in pear trees* [Poster presentation]. 25th International Conference on Virus and other graft transmissible diseases of Fruit crops, Wageningen, Netherlands.

Fontdevila Pareta, N.*, Tahzima, R.*, Rong, W., Blouin, A. G., Reynard, J.-S., Bobev, S. G., Caglayan, K., Candresse, T., Castaing, J., Foucart, Y., Jelkmann, W., Zikeli, K., Mavrič Pleško, I., Meekes, E., Minafra, A., Rott, M., Shifang, L., Leichtfried, T., Vončina, D., ... De Jonghe, K.*, Massart, S.*. (July 2023). *Uncovering the worldwide diversity of little cherry virus 1 and 2 and the associated viruses in Prunus spp* [Poster presentation]. 25th International Conference on Virus and other graft transmissible diseases of Fruit crops, Wageningen, Netherlands.