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Identification and biological characterization of new viral pathogens affecting fruit trees

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Promotors: Prof. Sébastien Massart
Prof. Shifang Li

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Identification and biological characterization of new viral pathogens affecting fruit trees

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Abstract

Wanying Hou (2023). Identification et caractérisation biologique de nouveaux pathogènes viraux affectant les arbres fruitiers (thése de doctrat). Belgique, Gembloux Agro-Bio Tech, Université de Liège.

Résumé - Les arbres fruitiers sont des cultures de grande valeur et peuvent être infectés par des virus / viroïdes qui ont tendance à s'accumuler en raison de la multiplication végétative et de la greffe des plantes. Certains virus provoquent des symptômes similaires nécessitant l'application de protocoles de laboratoire pour une identification spécifique, tandis que la cause d'autres maladies reste inconnue. L'identification des espèces virales pathogènes et leurs caractéristiques biologiques sont des informations clés pour contrôler les épidémies liées aux maladies virales. Avec l'avènement des technologies de séquençage à haut débit (HTS), le rythme de découverte de nouvelles espèces virales chez les plantes symptomatiques s'est accéléré. Au cours de la dernière décennie, plus de 100 virus / viroïdes ont été signalés à partir d'arbres fruitiers. Ces virus ont été identifiés à partir d'échantillons présentant des symptômes étiologiques non identifiés, ainsi que d'échantillons symptomatiques d'autres virus végétaux connus pour avoir été testés négatifs. La technologie HTS permettra la reconstruction partielle ou complète du génome viral sans aucune information sur la biologie virale. La caractérisation biologique des virus est en effet un processus long et complexe. Ces nouvelles découvertes virales, qui ne correspondent parfois qu'à l'identification d'un nouveau génome élargissent notre compréhension de la biologie virale. Les deux principaux objectifs de cette thèse sont (i) de passer en revue de manière critique les virus d'arbres fruitiers nouvellement découverts afin de répondre à la question : comment la caractérisation biologiques des nouveaux virus suit le rythme actuel de leur découverte ? ainsi que d'effectuer des recommandations sur la priorisation d'expériences complémentaires après la découverte de nouveaux phytovirus. Le deuxième objectif est (ii) de signaler l'identification et la caractérisation génomique d'un nouveau virus : le virus de la mosaïque nécrosante des pommes (apnmv), sur une nouvelle plante hôte : aubépine (*Crataegus spp*) présentant des symptômes de mosaïque sur les feuilles.

(1) Analyse rétrospective de nouveaux virus dans les arbres fruitiers

La revue a analysé les publications de 78 nouveaux virus et viroïdes trouvés dans 32 arbres fruitiers depuis 2011. Les informations biologiques utiles à l'évaluation des risques phytosanitaires et publiées parallèlement à la découverte de nouveaux virus ou viroïdes d'arbres fruitiers ont été analysées en tenant compte du cadre de caractérisation biologique proposé pour les nouveaux virus végétaux. En outre, 933 publications citant au moins une publication originale ont été examinées, en mettant l'accent sur l'information biologique pertinente fournie.

Dans la publication initiale, les expériences n'ont été réalisées que de manière sélective, les informations scientifiques fournies comme le développement de tests de détection (94%), le séquençage de génomes entiers, y compris les UTRs (92%), les enquêtes épidémiologiques locales et à grande échelle (68%), les expériences sur l'infectivité et les gammes de plantes d'hôtes en laboratoire (50%), les études d'association avec symptômes (25%), la gamme d'hôte naturelle (23%) et mode

transmission ou l'identification de vecteurs (8%). Au total, 20%des nouvelles espèces virales sont publiées sans aucune information sur leurs caractéristiques biologiques, mais uniquement sur leurs caracteristiques génomiques.

La publication d'un nouveau virus est citée en moyenne 2,8 fois par an. Seulement 18% des citations ont fourni des informations sur la biologie du nouveau virus ou le redécoupage géographique. Ces publications citées n'ont que légèrement amélioré la caractérisation des nouveaux virus, principalement en élargissant la distribution géographique (11%) ou la gamme d'hôte (5%).

La mise en œuvre du framework récemment proposé a été évaluée sur base de la collecte d'informations scientifiques sur la biologie virale dans les publications originales et citées. Des expériences ont été réalisées sur le long terme sur l'épidémiologie mondiale des virus (61%), la transmission et les vecteurs (44%), les symptômes et la gamme d'hôtes (55%), etc. En conclusion, la biologie de la plupart des virus nouvellement découverts et les risques associés pour la santé des plantes restent largement inconnus.

Dans l'ensemble, les virus nouvellement découverts sur les espèces d'arbres fruitiers sont rarement caractérisés biologiquement après leur découverte initiale, ce qui renforce la nécessité d'apporter autant d'informations que possible lors de la publication de nouveaux virus, bien que les expériences de caractérisation biologique puissent prendre du temps et retarder la publication des résultats. Pour toute nouvelle découverte de virus d'arbres fruitiers à l'avenir, il est recommandé de donner la priorité aux expériences complémentaires.

(2) un nouveau pathogène viral : le virus de la mosaïque nécrosante de la pomme identifié à partir de pommiers, peut être lié à la mosaïque de l'aubépine

La mosaïque de la pomme est endémique dans les principales régions productrices de pommes en Chine et est souvent associée à la présence du virus nouvellement découvert de la mosaïque nécrosante de la pomme (apnmv), qui appartient au sous - groupe 3 du genre ilarvirus de la famille des Bromoviridae. Les technologies HTS ont démontrées qu'un arbre d'aubépine présentant des symptômes de mosaïque était infecté par apnmv, ce qui a été confirmé par RT - PCR. Les séquences nucléotidiques complètes de l'arn1 (3378nt), de l'arn2 (2778nt) et de l'arn3 (1917nt) de l'apnmv ont été obtenues à partir de l'aubépine avec des homologies nucléotidiques de 93,8% - 96,8%, 89,7% - 96,1% et 89,8% - 94,6% respectivement avec les isolats infectant les pommiers et les pommiers à baie. Entre l'isolat d'aubépine et les autres isolats, deux régions hypervariables ont été trouvées aux positions 142 - 198 et 780 - 864 de la protéine POL, avec des homologies de séquence de 59,2 - 85,7% et 64,0 - 89,3%, respectivement. Des essais de greffage ont montré que l'apnmv se propage facilement de l'aubépine aux pommiers induisant des chloroses, jaunissement, mosaïques, nécroses et enroulement. De plus, 11 685 aubépines provenant de cinq provinces chinoises ont fait l'objet d'une enquête sur l'incidence de la mosaïque, et seulement six présentaient des symptômes typiques de la mosaïque. Un total de 145 arbres (6 symptomatiques, 68 asymptomatiques et 71 présentant d'autres symptômes) ont été

testés par RT - PCR pour la présence de apnmv. Parmi ceux-ci, 6 arbres symptomatiques, 4 arbres asymptomatiques et 10 autres arbres symptomatiques ont été testés positifs pour l'apnmv. Ensemble, ces résultats démontrent que l'aubépine est un nouvel hôte naturel de l'apnmv dans les principales régions productrices, que sa fréquence d'infection est relativement faible (13,8%, 20 sur 145) et que l'apnmv est probablement l'un des agents pathogènes de la mosaïque de l'aubépine.

Mots-clés: arbre fruitier, HTS, identification de nouveaux virus, désert biologique, risques potentiels, virus de la mosaïque nécrotique de la pomme, nouvel hôte naturel, maladie de la mosaïque d'aubépine.

Abstract

Wanying Hou. (2023). Identification and biological characterization of new viral pathogens affecting fruit trees (PhD thesis). Belgium, Gembloux Agro-Bio Tech, University of Liège.

Abstract — Fruit trees are high value crops which can be infected by viruses/viroids that tend to accumulate due to the vegetative propagation of the plants and the grafting. Some of the viruses cause similar symptoms, and laboratory protocols need to be applied for a specific identification, while other diseases still have an unknown etiology. The identification of the pathogenic viral species and their biological characterization are key information to control potential epidemics of viral diseases. With the advent of high throughput sequencing (HTS) technologies, the pace of discovery of new viral species in symptomatic plants has accelerated. More than 100 virus/viroid species have been reported from fruit trees the last decade. These viruses have been identified from samples presenting symptoms of unknown etiology but also from other symptomatic samples tested negative for other known plant viruses. HTS technologies will allow the reconstruction of partial or complete virus genome without any information on the biology of the virus. Biological characterization of a virus is indeed a complex and long process. These new virus discoveries, sometimes corresponding to the identification of a new viral genome only, have widened the gaps in our understanding of virus biology. The two main objective(s) of this thesis are (i) to critically review the newly discovered fruit trees viruses for answering the question: how biological characterization of a new virus could follow the current pace of virus discovery and to suggest prioritization of complementary experiments to be carried out once a new plant virus is discovered in the near future, and (ii) to report the identification and genome characterization of a newly reported virus, apple necrotic mosaic virus (ApNMV), from a new natural host: hawthorn(*Crataegus* spp) on a tree presenting mosaic symptom.

(1) A retrospective analysis for new fruit tree viruses

The review analyzed 78 publications of new viruses and viroids discovered from 32 fruit tree species since 2011. The biological information useful for a pest risk assessment and published together with the discovery of a new fruit tree virus or viroid has been analyzed taking into account a biological characterization framework proposed for new plant viruses. In addition, the 933 publications citing at least one of these original publications were reviewed, focusing on the biology-related information provided.

In the original publications, biological characterization experiments only had been selectively carried out, the scientific information provided was the development of a detection test (94%), the whole genome sequence including UTRs (92%), local and large-scale epidemiological surveys (68%), infectivity and indicators experiments (50%), association study with symptoms (25%), host range (23%) and transmission mode or vector identification (8%). Twenty percent of the new virus species have been published without any information on their biological characterization but only genome information.

The publication of a new virus is cited 2.8 per year on average. Only 18% of the

citations brought information on the biology or geographical repartition of the new viruses. These citing publications improved only slightly the new virus characterization, and mainly on enlarging the geographical distribution (11%) or host range (5%).

Based the gathering of scientific information on the virus biology in original and citing publications, the fulfilment of recently proposed framework has been evaluated. Long term biological characterization experiments have been carried out in global epidemiology (61%), transmission and vectors (44%), symptoms and host range (55%). In conclusion, the biology of most of these newly discovered viruses and the associated risks for plant health remain largely unknown.

Overall, the biological characterization of a newly discovered virus on fruit tree species is rarely pursued after the first discovery, which reinforce the need to bring as much information as possible when publishing a new virus, even though the biological characterization experiments can be time-consuming and could delay the publication of the results. Minimal recommendations for publishing a new fruit tree virus and prioritization of complementary experiments to be carried out were suggested for any new fruit tree discovery in the future.

(2) The case of a new viral pathogen: apple necrotic mosaic virus identified from apple trees, possibly associated with hawthorn mosaic disease

Apple mosaic disease is widespread in the major apple-producing areas in China, and frequently associated with the presence of the newly identified apple necrotic mosaic virus (ApNMV), belonging to subgroup 3 of *Ilarvirus* genus in the family of *Bromoviridae*. Mosaic symptoms were also observed in hawthorn tree. HTS technologies revealed the hawthorn tree with mosaic symptom was infected by ApNMV, which was confirmed by RT-PCR. The complete nucleotide sequences of RNA1 (3,378 nt), RNA2 (2,778 nt) and RNA3 (1,917 nt) of ApNMV from the hawthorn were obtained, sharing 93.8 - 96.8%, 89.7 - 96.1% and 89.8 - 94.6% nucleotide identities with those from apples and crab apples, respectively. Two hypervariable regions were found which showed 59.2 - 85.7% and 64.0 - 89.3% sequence identities at position 142 - 198 aa and at position 780 - 864 aa in the POL protein, respectively, between the hawthorn isolate and other isolates (apple, crabapple). Grafting test demonstrated ApNMV was easily transmissible from hawthorns to apple trees with severe chlorosis, yellowing, mosaic, curling and necrosis. In addition, a total of 11,685 hawthorn trees were surveyed for the incidence of mosaic disease from five provinces in China, only six were showing typical mosaic symptoms. A total of 145 individual trees (6 symptomatic, 68 asymptomatic and 71 with other symptoms) were tested for the presence/absence of ApNMV by RT-PCR. Among them, 6 symptomatic, 4 asymptomatic and 10 other symptomatic trees tested positive for ApNMV. Taken together, these results demonstrated that the hawthorn tree was identified as a new natural host for ApNMV with a relatively low frequency (13.8%, 20 out of 145) in the main producing areas, and it was likely to be one of the causal pathogens of hawthorn mosaic disease.

Keywords: fruit tree, HTS, new virus identification, biological desert, potential risks, apple necrotic mosaic virus, new natural host, hawthorn mosaic disease.

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I still remembered clearly the day that I have been matriculated as a PhD Student of the joint program between the graduated school of CAAS and liege university six years ago. It had been nearly 10 years after I graduated from professor Li' laboratory. After that since I have been working on manage work in institute of CAAS, due to limit time and enough confidence, I nearly had given up several times to go on my doctor study. Thanks to the encouraging of Prof. Li Shifang, I had kept going and had the chance to meet Prof. Sébastien Massart. My research and thesis writing are carried out under the guidance of them as well as the writing and revision of my published papers. Without their professional guidance, I probably wouldn't have finished my PhD study.

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Tables of Contents

List of Figures

List of Tables

List of Abbreviations

AGCaV	Apple green crinkle associated virus
TFDaV	Temperate fruit decay associated virus
AGV	Apple geminivirus
ApNMV	Apple necrotic mosaic virus
AHVd	Apple hammerhead viroid
ARWV-1	Apple rubbery wood virus -1
ARWV-2	Apple rubbery wood virus -2
ARWaV-1	Apple rubbery wood associated viruses-1
ARWaV-2	Apple rubbery wood associated viruses-2
ALV-1	Apple luteovirus 1
AaLV	Apple-associated luteovirus
PpPV2	Pyrus pyrifolia partitivirus 2
ACFSVd	Apple chlorotic fruit spot viroid
ApRVA	Apple rootstock virus A
MdoVA	Malus domestica virus A
AVCaV	Apricot vein clearing associated virus
CPrV	Caucasus prunus virus
PrVT	Prunus virus T
NSPaV	Nectarine stem pitting-associated virus
CRMaV	Cherry rusty mottle-associated virus
CTLaV	Cherry twisted leaf-associated virus
NeVM	Nectarine virus M
ChALV	Cherry-associated luteovirus
PrVF	Prunus virus F
PLPaV	Peach leaf pitting-associated virus
PaLV	Peach-associated luteovirus
PeVD	Peach virus D
PcVT	Peach virus T
PCLSV	Peach chlorotic leaf spot virus
MuVA	Mume virus A
PrGVA	Prunus geminivirus A
CVF	Cherry virus F
CRV-5	Cherry robigovirus 5

CVTR	Cherry virus Turkey
ChVB	Cherry virus B
PYSaV	Prunus yellow spot-associated virus
PeV1	Peach virus 1
CCDaV	Citrus chlorotic dwarf associated virus
CYVCV	Citrus yellow vein clearing virus
CiLV-C2	Citrus leprosis virus C2
CVEV	Citrus vein enation virus
IrCRSaV	Iranian citrus ringspot-associated virus
CJLV	Citrus jingmen-like virus
CVLV	Citrus virga-like virus
CiLV-N	Citrus leprosis virus N
CCGaV	Citrus concave gum-associated virus
CiCSV	Citrus chlorotic spot virus
CiVA	Citrus virus A
CiYMaV	Citrus yellow mottle-associated virus (CiYMaV)
CTNGmV-1	Citrus Tunisia genomovirus 1
CTNGmV-2	Citrus Tunisia genomovirus 2
BVE	Blackberry virus E
RLBV	Raspberry leaf blotch virus
BVBaV	Blackberry vein banding-associated virus
BVF	Blackberry virus F
BLMaV	Blackberry leaf mottle associated virus
BVA	Blackberry virus A
CuLV	Currant latent virus
CuVA	Currant virus A
BCIV	Blackcurrant idaeovirus
BCLCaV	Blackcurrant leaf chlorosis associated virus
BcLRaV-1	Blackcurrant leafroll-associated virus 1
BCaRV	Black currant associated rhabdovirus
RAVA	Ribes americanum virus A
BCCV-1	Blackcurrant closterovirus 1
BBLV	Blueberry latent virus
BLSV	Blueberry latent spherical virus

BNRBV	Blueberry necrotic ringblotch virus
BVA	Blueberry virus A
BlMaV	Blueberry mosaic associated virus
BFDaV	Blueberry fruit drop associated virus
BGMaV	Blueberry green mosaic-associated virus
MMDaV	Mulberry mosaic dwarf-associated virus
MBV1	Mulberry badnavirus 1
PVd2	Persimmon viroid 2
PeVA	Persimmon virus A
PeLV	Persimmon latent virus
PeCV	Persimmon cryptic virus
PeVB	Persimmon virus B
AcCRaV	Actinidia chlorotic ringspot associated virus
AcV-1	Actinidia virus 1
ASbLV	Actinidia seed-borne latent virus
AcEV-2	Actinidia emaravirus 2

1

General introduction

1 Traditional methods of plant virus identification

Virus a non-cellular microorganism that copies its genome in the living cells of a host. A virus particle consists of DNA or RNA (double or single stranded), and a protein coat. Viroid corresponds to naked circular non-protein-coding genomic RNA. Fruit trees are infected by many viruses and viroids which are mainly transmitted through vegetative propagation and grafting of infected plant material (Maliogka et al., 2018). Some of these pathogens cause severe crop losses and often reduce the productive life of the orchards (Martelli et al., 2011; Di Serio et al., 2018). For example, the plum pox virus (PPV, genus *Potyvirus*) causes "sharka" disease, the most devastating viral disease of stone fruit trees worldwide, which causes severe damages, estimating a total cost at 10 billion Euros worldwide in 30 years (García et al., 2014; Rimbaud et al., 2015). Citrus tristeza virus (CTV; genus *Closterovirus*) is probably the most economically important virus infecting citrus, causing a decline of sour orange rootstock, yellow seedling of lemon and grapefruit, and stem pitting in grapefruit and sweet orange (Moreno et al., 2008; Umer et al., 2019). The disease has led to the death of millions of citrus trees all over the world and has rendered millions of others useless for production (Lee, 2015).

Plant viruses can cause diseases that affect crop production. Unlike fungal and bacterial diseases, virus diseases are very difficult to control due to the lack of effective prevention and control agents. And with the increasing frequency of international exchanges, the globalization of trade, the transportation of plant propagation materials and seeds, etc., will cause the rapid spread of viruses and viroids internationally. Therefore, as much as possible to grasp the occurrence and distribution of viruses, establish rapid and accurate detection technology, and do a good job in the entry inspection and quarantine of plant materials, is particularly important for the prevention and control of plant viral diseases. Viral and viroidal species are identified on the basis of a number of properties including structural, biological, serological, cytopathological, epidemiological, and molecular traits such as genome structure and organization and level of similarity or identity in the nucleotide and amino acid sequences (Martelli et al., 2011). Here, four wildly used methods in identification of viral pathogen were introduced.

1.1 Biological indexing

The biological indexing also known as the indicator plant test, is based on the specific symptom(s) induced by produced by sensitive indicator host infecting with virus (Chi and Mao, 2017). Indicator plants include herbaceous indicator plants and woody indicator plants. Different plant viruses generally have a specific set of indicator plants (Rowhani et al., 2005). In the case of fruit tree viruses, bioassays took advantage of indicator plants can be directly grafted or mechanical inoculation by controlling greenhouse facilities for plant maintenance. Inoculated plants usually require controllable environmental conditions, similar to those in a greenhouse. The need for specific environmental conditions sometimes limits the use of the bioassay

method, especially for large-scale samples (Maliogka et al., 2018).

Biological assay is a very traditional method for plant virus identification. It has been widely used due to needless of expensive instruments and equipment, simple operating and direct observation of the results. Strategies to screen horticultural crops for graft-transmissible agents, particularly viruses, have advanced substantially over the past decade. The bioassay has persisted, and is now the industry standard (Rowhani et al., 2005; Al Rwahnih et al., 2015). However, it is difficult to judge detection results based on symptoms. The growing and development of inoculated plants requires time, as does the expression of symptoms, and can take several weeks, months, or even years for woody plants. Some viruses may induce no apparent symptoms or cause symptomless infection. In addition, different viruses can produce similar symptoms or different strains of a virus cause distinct symptoms. It is still required to combine serological or molecular techniques with biological indexing on indicator plants.

1.2 Electron microscope detection

Electron microscopy (EM) is a universal means and a powerful technique available to virologists today. EM permits providing direct images of viruses for diagnosis and research. The strength of EM lies in its ability to image the whole spectrum of interactions, including resistance and non-reactions in the case of new virus isolates or species (Richert-Pöggeler et al., 2019). EM can determine functional features of viruses and underlying mechanisms of interactions relevant in nature as well as in synthetic virology (Fig1-1). At present, commonly used electron microscopy detection technologies are mainly including: Transmission electron microscope, electron microscopy negative staining and immunoelectron electron microscopy (ISEM).

1.2.1Transmission electron microscope

Transmission electron microscope (TEM) can directly observe the morphological structure of plant viruses, determine the presence or absence of virus particles, and provide the most accurate and direct test results for plant virus detection (Zhang et al., 2013b). TEM provides an immediate overview of actual status, discerning amount and shape of virus(es) present including the unexpected(Gentile and Gelderblom, 2014). Since its “open view” nature, using TEM serves as a decisive tool should subsequently be used to further identify virus genus and species (Gentile and Gelderblom, 2014).

1.2.2 Electron microscopy negative staining

Negative-staining (NS), a rapid, simple and conventional technique of electron microscopy (EM), has been commonly used to initially study the morphology and structure of proteins for half a century (Zhang et al., 2013a). Negative staining, the embedding of a specimen in a layer of dried heavy metal solution, was introduced early on as a quick and easy specimen preparation technique that significantly increases the specimen contrast (Ohi et al., 2004).Initially, biological specimens were prepared for EM by NS, the embedding of a specimen in a layer of dried heavy metal

solution, was introduced early on as a quick and easy specimen preparation technique (Stahlberg and Walz, 2008). Images of negatively stained molecules are amenable to image averaging techniques that allow finer details of the molecule to be visualized (Ohi et al., 2004).

1.2.3 Immuno-electron microscopy

Immuno-electron microscopy (IEM) is based on serological principles as ELISA, and can be used for further virus identification during TEM diagnosis. IEM has the advantage that it works directly with raw serum with no further purification of immunoglobulins or conjugation steps are required. Taking advantage of the adsorptive properties of antigen and antibody, by concentrating the virus and observing it with the electron microscope, the sensitivity of detection can be further increased (Richert-Pöggeler et al., 2019).

The EM is an important tool in the diagnosis of plant virus's infection, although it is only practical when a few samples are to be examined. It can provide direct detection results by directly observing the morphology and structure of plant viruses, even though we have entered the molecular stage in the field of viruses. Since it requires expensive equipment and experienced technician, and usually just can identify a virus to family or genus. The high equipment costs and the need to maintain an experienced staff are hampering its timely renewal (Kitajima, 2004).

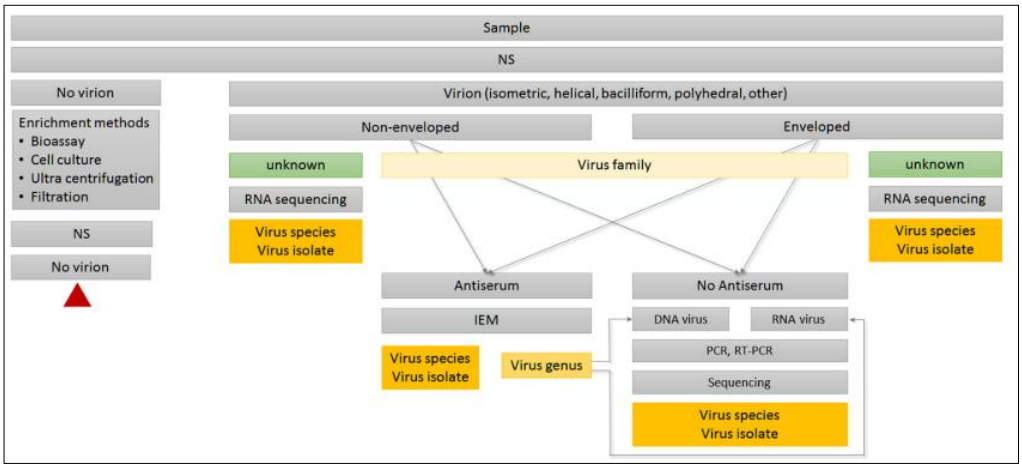


Figure 1-1 Decision tree for routine diagnosis using TEM. Red triangle indicates terminal node. IEM, Immuno-electron microscopy; NS, negative staining (Richert-Pöggeler et al., 2019).

1.3 Serological method

Serological detection methods are widely used for plant virus detection which have been developed from the last 70's. They are based on the specific reaction between a viral antigen and an antibody. The most common antibodies used in virus diagnostics are polyclonal antibodies (PAbs) produced in rabbits, sheep or goats. PAbs are

produced when the animal is exposed to an antigen injected as purified (or partially purified) virus. In contrast, monoclonal antibodies (MAbs) are produced by a single, immortalized B lymphocyte clone. The MAbs are almost always produced from B lymphocytes that are extracted from the antigen-exposed mouse and fused with myeloma cells (Fig 1-2). Some of the main characteristics of antibodies are their specificity, affinity and avidity. The specificity is defined by how well an antibody can recognize a specific epitope and not another (Meng et al., 2017). Enzyme-linked immunosorbent assay (ELISA), lateral flow devices (LFD) and tissue blot immunosorbent assay (TBIA) are powerful tools for the detection and diagnosis of plant viruses (Chang et al., 2011).

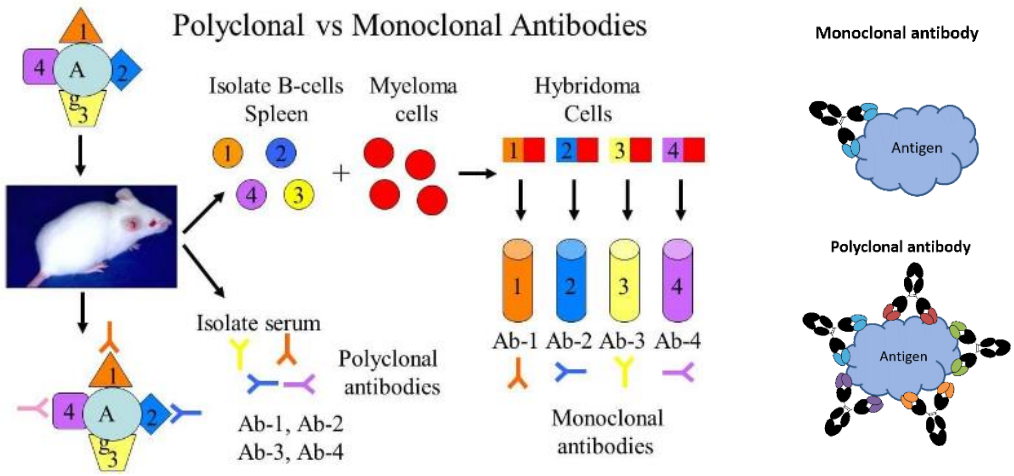


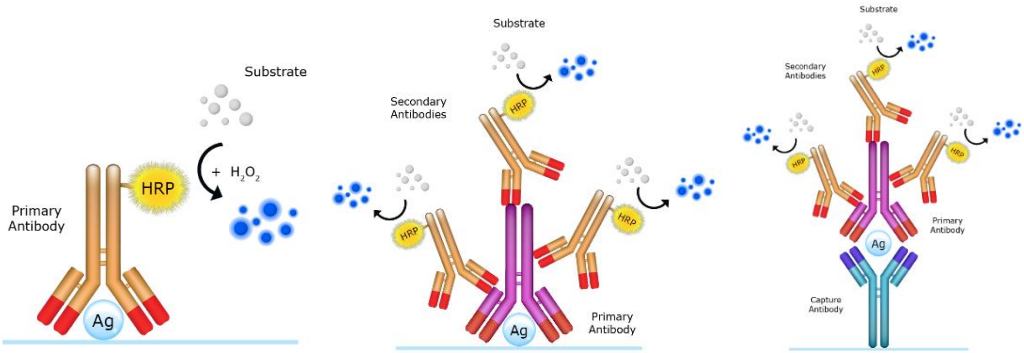
Figure 1-2 Monoclonal and polyclonal antibodies producing scheme

1.3.1Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) had been first reported to detect a plant virus in 1977.It based on the antibody binding specificity to detect substances such as peptides and proteins(Clark et al., 1977). Its use is now very common in plant virus detection, with antibodies specific to the coat protein of the target virus. Types of ELISA include direct method, indirect method, double antibody sandwich method (Fig1- 3) (Meng et al., 2017).

Direct ELISA means the antigen is coated on a solid carrier, the enzymelabeled anti body is added after blocking, the unbound enzymelabeled antibody is washed away, and the substrate is added to develop color. The color depth is proportional to the am ount of coated antigen and the amount of enzyme-labeled antibody. Indirect ELISA was an enzyme labled anti Ig as a second antibody to detect the antigen antibody complex on the solid face. This avoids the necessity of making specifice enzyme conjugates for each antigen to be teseted and eliminates the extreme specificty, thus allowing for quanitiative evaluation of strain relationships (Abd El-Aziz, 2019). DAS-ELISA using the analogy of a sandwich, two different antibodies form the bread,

while the virus coat protein or the virion (antigen) is the filling. DAS-ELISA is a protocol used when the second antibody is not conjugated with an enzyme or has been conjugated with a tag such as biotin. After the introduction of the enzyme-labeled secondary antibody, the detection signal will be amplified dozens of times.



Direct ELISA Indirect ELISA DAS-ELISA

Figure 1-3 ELISA Procedures of different types

ELISA is specificity, rapid and sensitive detection method, also due to the advantage of detecting large number of samples simultaneously, it has been widely used in detection of plant viruses, but it requires laboratory equipment by exchanging a 96-well polyethlene plat to a nitrocellulose filter or chromatography paper, these thechiques require many steps and complicated operations (Tsuda, 1992). The sensitivity of ELISA varies depending on the organism, sample freshness, and titre (Martinelli et al., 2015). It also has several disadvantages, such as it cannot be applied on viruses lacking coat proteins and a false negative result for some samples in low concentrations. In addition, it needs high quality antibody and some different viruses have similar serological reaction(Schwendicke et al., 2019).

1.3.2 Lateral-flow devices

Lateral flow devices (LFDs) are qualitative immunochromatographic tests for the rapid and specific detection of target analytes.The basis of lateral-flow devices (LFD) is specific antigen is ‘sandwiched’ between the immobilized antibody and the antibody sensitized particles and immune complexes. As the amount of captured antigen increases, the concentration of accumulated particles also increases. The reaction site can be seen by eye once a high enough density of particles is reached (Danks and Barker, 2000). The filter paper strips coated with antibody can be stored at room temper. It enable detection have been fabricated for on-site detection (Fang and Ramasamy, 2015). The speed and simplicity of lateral-flow devices have been demonstrated for plant-pathogen diagnosis (Danks and Barker, 2000).The main disad vantages of LFD is their relatively low sen-sitivities and poor limit of detection, which primarily restricts their use to detection of samples with highly abundant analytes (Katis et al., 2018).

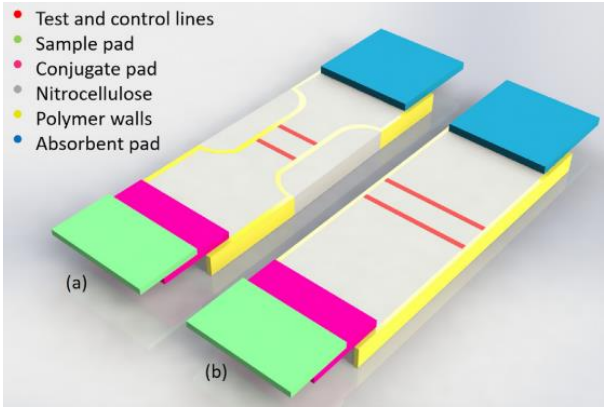


Figure 1-4 Schematic of the constricted flow device and a standard lateral flow device.(a) the constricted flow device (b) a standard lateral flow device (Katis et al., 2018).

1.3.3 Tissue blot immunosorbent assay (TBIA)

Tissue blot is a process of transfer of protein antigens from a freshly cut tissue surface to nitrocellulose membrane. It is achieved simply by bringing a freshly cut tissue surface in direct contact with a dry nitrocellulose membrane (Burns, 2009). The advantages of the tissue blotting techniuqes for detection of plant pathogens is that the tissues blot nitrocelleloes membranes can be prepared in any laboratory, greenhoues, or even fileds at any locations by persons with just a few instructions. Furthermor, the tissue blot membranes can be stored and / or transported and processed 2-3 weeks after the samples are applied. The techniques also offers the advantage that a large number of samples can be processed in a short time (Lin, N.S.; Hsu, Y.H ; Hsu, 1990). A drawback of this technique is that concentration of antigen in the new tissue fluid limits the detection sensitivity for a large volume of relatively concentrated antiserum is required (Martelli and Uyemoto, 2008).The selection of the correct tissue was critical for DTBIA detection, this would limit the use of DTBIA when suitable tissue is not available, particularly in winter (Martín et al., 2002).

Immunofluorescence technology also has certain drawbacks in practical applications. This technique requires a well-equipped immunofluorescence microscope, which is very time consuming to operate, and the autofluorescence from plants also interferes with the application of this technology (Madeley and Peiris, 2002).

1.4 Molecular methods

Molecular biological detection is based on hybridization or amplification of viral DNA or RNA to identify the presence of virus. It can be more sensitive, since the virus are detected at the nucleic acid level (López et al., 2009). It can detect all types of viruses and viroid and is suitable for carry out large-scale sample analysis. Most used

molecular method for plant virus identification can be grouped as follows: hybridization-based and nucleic acid amplification methods (conventional PCR and RT-PCR).

1.4.1 Nucleic acid hybridization (NAH)

The nucleic acid hybridization technique is based on the process of hybridization. It needs a nucleic acid probe with the sequences of which are the same or highly similar to those of virus. The probe is labeled with markers that are easy to detect. A virus can be detected through the probe marker when it specifically interacts with its probe through base complementation. This method was first developed for the detection of potato spindle tuber viroid (Owens and Diener, 1981) and adapted to virus detection (Garger et al., 1983). The most common molecular hybridization format for the detection of viruses is dot-blot hybridization using digoxigenin-labelled probes. Furthermore, multiple RNA riboprobes or poly probes have been used to detect different viruses (Ivars et al., 2004). It can also be applied to the specific detection of amplicons generated after amplification techniques based on PCR, thereby increasing their sensitivity and specificity levels (Bertolini (López et al., 2009).The commonly used hybridization technique in virus identification including southern hybridization, northern hybridization, and fluorescence in situ hybridization(FISH) (Yamagishi et al., 2006; Pahalawatta et al., 2008; Zhao et al., 2009; Zhang et al., 2015).

1.4.2 PCR and RT-PCR

Polymerase chain reaction (PCR) is an in vitro method to amplify targeted nuclei acid sequences, DNA fragments of interest can be enzymatically amplified in vitro by the PCR complementary oligonucleotide primers to the target sequence and the synthesis of multiple copies of complementary DNA of the sequence between the primers using heat-stable DNA polymerase (Mullis et al., 1986). As PCR is based on DNA, it is not directly applicable to most plant viruses that have RNA genomes. However, a cDNA can be made to the desired region of the RNA genome using a primer and reverse transcriptase, and this used as the initial template. This procedure, now widely used, is termed reverse transcription PCR (RT-PCR). PCR (and RT-PCR) has proved to be a very power- ful tool for virus detection and diagnosis. It can be used to directly produce a DNA product of predicted size that can be confirmed by gel electrophoresis and sequencing (Hull, 2014). With the rapid improvement of technology, some new virus detection methods have appeared based on the PCR technique. Such such as real- time PCR and multiplex PCR (Wittwer et al., 1997; James et al., 2006).

The PCR method enables the rapid and accurate identification of a particular virus. Therefore, PCR-based molecular detection of a virus is currently the method of choice for its correct identification. However, a false positive results from contamination can result in high sensitivity of the reaction (Webster et al., 2004). Also, a prior knowledge of at least some fragments of the viral nucleotide sequence is required for primers design.

2 High throughput sequencing technologies for virus

detection

Since the first discovery of plant virus, technologies used to identify them have evolved considerably but can be grouped in two categories. Targeted virus detection methods include molecular biological detection and serological methods which depend on a prior knowledge of the biological characteristics, genomic properties, physicochemical properties or serological characteristics of the virus. Untargeted protocols include electron microscopy and high throughput sequencing (HTS, also called next-generation sequencing-NGS or deep sequencing). Since HTS has steadily evolved in recent years, it now provides a powerful alternative for virus detection, including unknown or unexpected viral species (Barba et al., 2013).

2.1 Establishment and application of HTS platform

The traditional Sanger sequencing technology which was developed by Frederick Sanger in 1977 encountered a revolutionary impact, while more high throughput and faster HTS technology have been developed since 2005 (Margulies et al., 2005), The launch of the Roche 454 sequencing platform of Roche Corporation of the United States created a precedent for high-throughput sequencing. Since then, Illumina and ABI of the United States have successively launched Solexa (Porreca et al. 2007) and SOLiD (Ondov et al., 2008; Shendure and Ji, 2008) sequencing technologies. The birth of HTS technology is a landmark event in the field of genomics (Mardis, 2008, 2011, 2013; Boonham et al., 2014).

Compared with traditional Sanger sequencing, the second-generation sequencing technology has greatly improved the sequencing speed and throughput, and the sequencing cost has also been greatly reduced. Take the human genome sequencing project as an example. In 2001, a draft of the human genome was published, announcing the preliminary completion of the human genome project. However, this study took 15 years and cost nearly three billion US dollars (Collins et al., 2003). Today with the latest HiSeq X sequencing platform, 45 human genomes can be sequenced in a single day, and each genome can be sequenced for only $ 1,000. Compared with traditional sequencing methods, there is a quantitative improvement and a qualitative leap. The decryption of multi-species genetic information enters the era of big data of genomic information.

At present, the application of HTS technology in the field of molecular biology includes: Whole-Genome Sequencing (Sundquist et al., 2007; Butler et al., 2008; Gresham et al., 2008), Exome Sequencing, Targeted regions sequencing, TRS, De Novo Sequencing, RNA-Seq, Small RNA and non-coding RNA sequencing (Wang et al., 2009), DNA methylation sequencing in the field of epigenomics (Brunner et al., 2009)，Ribosomal map and ChiP sequencing (Park, 2009), It provides important data information for the disclosure of genetic information and gene expression regulation and other basic biological research (Nakazato et al., 2013).

In 2009, HTS began to be used in the field of plant virology (Adams et al., 2009; Al Rwahnih et al., 2009; Kreuze et al., 2009), including the discovery of new viruses and viroids, the detection of known viruses, the analysis of genomic diversity and

evolution. HTS technology has accelerated the discovery and identification of unknown viruses to an unpreceded speed. The application of HTS in the detection of plant viruses have overcome the shortcomings of traditional detection methods for plant viruses and brought about a revolution in detection technology.

2.2 Comparison of high-throughput sequencing platforms

In the past 10 years, the platform of HTS has undergone continuous improvement. The amount of data for a single sequencing reaction has also increased from 1G in 2005 to 1T, an increase of 1,000 times. The major developments focused on reducing the time-to-result and enlarging the output to lower the sequencing price. Several technologies have been developed throughout time based on different sequencing methods(Maliogka et al., 2018) (Table 1-1).

Table 1-1 Comparison of HTS platforms

Method	Principle	Read length (bp)	Output/run	Time
Roche 454	Pyrosequencing	400-500	700 bp	23 h
Illumina HiSeq 2500	SBS	125	1000 Gb	1-6 d
Illumina MiSeq	SBS	100-150	15 Gb	5-55 h
Ion Torrent	SBS	100-200	2 Gb	3.5-5.5 h
ABI SOLiD	SBH	35-75	95 Gb	2-4 h
Pacific Biosciences	SMRT	>1000	75 Mb	0.5-5 h

SBS: Sequencing by synthesis; SBH: Sequencing by hybridization; SMRT: Single molecular real-time sequencing.

2.3 HTS protocols for plant viruses

The main processes used of HTS to detect plant viruses include: 1) sample preparation; 2) library construction; 3) sequencing; 4) data analysis. Among them, the high-quality extraction of nucleic acids is the key step in the sample preparation process, which will directly affect the construction of the library and the depth of sequencing. However, compared with the host plant genome, the content of virus molecules can be extremely low. How to efficiently extract virus-derived nucleic acids in order to enrich the sample in sequences of viral origin? It is a key question that has been explored by many researchers. It is particularly critical to enrich sequences from virus sources as much as possible, and to select a suitable sequencing platform to improve the depth of sequencing and the sensitivity of detection. At present, according to different principles, HTS commonly used sequencing nucleic acids include the following:1) Total RNA, 2) Double stranded RNA, 3) Virion-associated nucleic acids, 4) sRNA small interfering RNA. For viruses with circular genomes, a rolling circle amplification (RCA) can also be carried out.

2.3.1 Total RNA or DNA

The most direct way to study the types of viruses in susceptible plants is to sequence the total RNA extracted and to perform high-throughput sequencing after ribodepletion. In theory, all RNA or DNA viruses can be detected, and the virus population can be studied. The first publication for plant viruses successfully detected new viruses from grapes and ornamental plants by extracting total RNA for high-

throughput sequencing (Adams et al., 2009; Al Rwahnih et al., 2009). However, the biggest disadvantage of this method is that the huge majority of the sequences come from the host genome rather than the viral genome sequence. In this complex sequence background, low levels of plant viruses might be more difficult to detect.

2.3.2 Double stranded RNA

Most RNA viruses produce double-stranded RNA (dsRNA) intermediates in the replication or genome assembly process, which is a unique feature of these viruses. Therefore, using dsRNA as a sequencing template can enrich the sample in virus-specific sequences (Roossinck et al., 2010; Roossinck, 2012). For the identification of grapevine syrah virus-1, total RNA and dsRNA were both applied to prepare the sample for sqequencing. As a result, dsRNA as a sequencing template generated more virus-derived reads and presented a higher sensitivity. In 2010, Roossinck established a sequencing process using dsRNA as a sequencing template to study the viral population through HTS (Roossinck et al., 2010). However, the disadvantage of this sequencing method is that it cannot effectively detect some DNA viruses, and the preparation of double-stranded RNA currently uses the method of CF-11 specifically binding dsRNA to extract, which takes a long time and requires much more laboratory steps (Rna and Morris, 1984).

2.3.3 Virion-associated nucleic acids

The idea of concentrating the sample in virus particles from which nucleic acids are extracted and sequenced was first applied from animal and human tissues (Allander et al., 2001; Jones et al., 2005), then used in plant virus in 2006 (Zhang et al., 2006). The general protocol includes the following steps: isolation of the virus particles by ultracentrifugation or filtration, followed by a RNase and DNase treatment to degrade the unencapsulated nucleic acid, and finally extract the nucleic acid molecules from the virus particles (Roossinck et al., 2015). The obtained extract can further be prepared for sequencing and sequenced. The advantage of detecting viruses by purifying VANA is that it can remove the host genome sequence, eliminate irrelevant sequence interference, and achieve the purpose of maximizing the enrichment of virus sequences. The disadvantage is that it is very difficult to detect viroid or non-encapsidated viruses. In addition, the sample preparation process is long and complex.

2.3.4 Small RNA

When the virus invades the host cell, a common defense mechanism from the plant to control the virus replication relies on RNA silencing. During replication and transcription, the virus produces double-stranded RNA, which can be cleaved into small interfering RNA molecules (Small interfering RNAs, siRNAs) by the host's endonuclease (Dicer-like Protein). In case of infection, a huge proportion of small RNA sequences in the cell can be of viral origin thanks to this defense mechanisms. This property was exploited first on plant viruses in 2009 (REFERENCE) by isolating the small RNA fragments form diseased plants and sequencing them. In a few years, more than 30 new viruses have been discovered using this method (Roossinck et al., 2015), of which there are 11 geminiviruses, respectively from sweet potato, citrus,

grape, apple, mulberry and other crops (Loconsole et al., 2012b; Bernardo et al., 2013; Poojari et al., 2013; Krenz et al., 2014; Mbanzibwa et al., 2014; Liang et al., 2015). This protocol is therefore also very effective to detect single stranded DNA geminiviruses. The disadvantage of this approach is the small size of the viral reads that may complicate the genome assembling process, especially for novel viruses(Maliogka et al., 2018).

3. Comparison of the performance criteria of the methods detecting plant viruses

All virus detecting methods have advantages and disadvantages related to sensitivity, specificity, feasibility, rapidness, and cost. The advantages and disadvantages of the virus detection methods are summarized in table, according to descriptions provided by Lopez et al. (2009) and EPPO standard PM7/125(López et al., 2009; Europe, 2015).

For the bioassay based on the specific symptom(s) should be easy to infect and express specific symptoms in a short time which depend on different cultivar and virus spices. The need for specific environmental conditions limits the use of the bioassay method especially for large-scale samples. It is important to note that a bioassay is needed to verify the infectivity and biological properties of a new virus.

For serological method is specificity, rapid and sensitive detection method, also due to the advantage of detecting large number of samples simultaneously, it has been widely used in detection of plant viruses. Lateral flow devices (LFD) allow disease diagnosis in the field within minutes solved the problem of the user may have no appropriate expertise, may be unfamiliar with the handling of diagnostic equipment and have no facilities or equipment to hand. The main disadvantages of LFD are relatively low sensitivities outside the plants' vegetative period because the titre of some viral pathogens is usually very low.

For molecular method: The nucleic acid hybridization technique will be 8 to10 times greater than that achieved by the use of ELISA (Astuti et al., 2015). PCR/RT-PCR is more sensitive for it can detect trace amounts of the template in small samples because the template is exponentially amplified more than a million-fold. PCR and RT-PCR is performed in a small reaction volume and only requires a few hours. As such, it is simpler and faster than nucleic acid hybridization methods. PCR is more sensitive than ELISA. The development of specific molecular tests is strongly dependent on the knowledge of the pathogen genome and its molecular diversity. Compared with nucleotide acid amplification methods, HTS has no advantages related to cost, time, detection throughput, or ease of operation, due to its complex library construction, the expensive sequencing platform, and the need for professional bioinformatics analysis. However, due to its sequence independence and high sensitivity, HTS detection is a powerful tool for discovering virus.

The proper method must meet detection requirements related to accuracy, reliability, and time. In some situations, the most important requirement is accuracy; in others, it could be time. For instance, if we are testing a propagating material, such as a mother

fruit plant used to produce seedlings, the mother plant should be virus-free. In this case, accuracy is the most important consideration. Thus, method(s) with high sensitivity, such as RT-PCR and NGS, is optimal.

Table 2-2 Comparison of different virus detection methods

Method	Biological Assay	ELISA	LFD	NASH	PCR/RT-PCR	HTS
Sensitivity	Depending on cultivar	Middle	Middle	High	Very high	Highest but limited by contamination
Specificity - inclusivity	Low	Depending on antibodies	Depending on antibodies	Depending on probes	Depending on primers/probes, annealing temperature and reagent composition	Very high if the nucleic acids of a virus present in the sample
Specificity - exclusivity	Low	Middle	Middle	Depending on probes	Depending on primers, annealing temperature and reagent composition	Very high (highest if complete genome sequenced)
Time to results	Weeks to years	Hours to days	Minutes	Hours to days	Hours to days	Days to weeks
Technical difficulty	Easy	Middle	Easy	Complex	Complex	Very complex
Equipment required	Greenhouse space	Low-tech lab	No lab	High tech	High tech	High tech
Ability to analyse many samples in parallel	Weak	Good	Good	Good	Good	Good

4 Viruses affecting fruit

Fruit tree are high value crops which have been widely grown around the world. Nevertheless, they can be infected by numerous plant viruses, sometimes at very high prevalence due to vegetative propagation and grafting. Some of these pathogens cause severe crop losses (Martelli et al., 2011). However, the identification, detection, and characterization of the causal agents responsible of symptoms was a difficult task and progressed slowly before the advent of high throughput sequencing (HTS) technologies during the last decade.

4.1. Viruses infecting pome

Pome fruit include apple (*Malus*), quince (*Quince*), and pear (*Pyrus*) are temperate species and belong to the family *Rosaceae*. Apple trees, arguably, the most important fruit trees of the temperate world have a global production of 86.44 million metric tons according to the Food Agricultural Organization (FAO). Most pome fruit trees do not root from cuttings; therefore, vegetative propagation is difficult without grafting which is commonly applied. The combination of vegetative propagation and grafting between a rootstock. It is favorable to the accumulation of viruses as they can be easily transmitted from a mother plant. As a consequence they can be infected by

viruses in high propotion, potentially resulting in significant economic losses to all sectors of the production chain (Shank, 2012).Fifteen viruses and viroids have been first identified from pome fruit trees before 2011(Martelli et al., 2011). Some of these pathogens cause severe crop losses and often reduce the productive life of the orchards (Martelli et al., 2011). For example, Apple chlorotic leaf spot virus (ACLSV, genus *Trichovirus*) infects most fruit trees of *Rosaceae* family, which include apple, pear, quince, it reduces tree vigor (up to 50% on pear), yield (up to 40% on pear), and quality of fruits(Umer et al., 2019).

4.2. Viruses infecting stone fruit

Stone fruit trees (genus *Prunus*, family *Rosaceae*) , include apricot (*Prunus armeniaca*), peach (*Prunus persica*), almond *(Prunus dulcis)* , plum (*Prunus domestica*) and sweet cherry (*Prunus avium*) are temperate species. They probably originated in Central Asia, with secondary centers in Eastern Asia, Europe, and North America. It includes about 430 species of deciduous or evergreen trees and shrubs naturally widespread throughout temperate regions (Park, 1990). Plums are cultivated over a wide range of climatic conditions. World production of plums was estimated at 8. 83 million tonnes. Nearly 40 different virus/viroid were infected stone fruit trees (Martelli et al., 2011). Plum pox virus (PPV, genus *Potyvirus*) causes “sharka” disease, particularly the most devastating viral disease of stone fruit trees worldwide, which causes severe damages, estimating a total cost at 10 billion Euros worldwide in 30 years (García et al., 2014; Rimbaud et al., 2015).

4.3. Viruses infecting citrus

Citrus fruit trees (genus *Citrus*, family *Rutaceae*), include sweet orange (*Citrus sinensis*), lemon (*Citrus limon*), grapefruits (*Citrus x paradisi*), tangerines(*Citrus reticulata*), and limes (*Citrus aurantiifolia*) are native to Southeast Asia, New Caledonia, and Australia. They are now grown throughout the tropics and subtropics worldwide. Current annual worldwide citrus production is estimated at over 140 million tons, with more than half of this corresponding to orange production (FAOSTAT, http://faostat.fao.org). The yield and quality of these products are affected by many pathogens, including a large number of viruses.

Nearly 20 different virus/viroid had been identified infecting citrus fruit trees before 2011(Serra et al., 2008), Some of these pathogens cause severe crop losses. Citrus tristeza virus (CTV; genus *Closterovirus*, family *Closteroviridae*) is probably the most economically important virus infecting citrus, causing a decline of sour orange rootstock, yellow seedling of lemon, and stem pitting in sweet orange (Moreno et al., 2008; Umer et al., 2019). The disease has led to the death of millions of citrus trees all over the world and has rendered millions of others useless for production (Lee, 2015). Citrus leprosis virus C, C2 and N (CiLV-C and -C2, genus *Cilevirus*; CiLV-N, genus *Dichorhavirus*, family *Rhabdoviridae*), also have significant economic impacts on citrus crops including oranges, grapefruits, and tangerines, resulting in stunted growth and one-third or more losses of fruit yields (Rodrigues et al., 2003).

4.3. Viruses infecting rubus

Rubus comprises hundreds of species which is divided into 15 subgenera with blackberries and raspberries in the *Idaeobatus* subgenera. Rubus species are propagated vegetatively and are subject to infection by viruses during development, propagation, and fruit production stages. More than 30 viruses detected affecting *Rubus* spp (Martin et al., 2013). Virus complexes have been identified as the major cause of diseases in blackberries and raspberries. For example, blackberry yellow vein-associated virus (BYVaV; genus *Trialeurodes*, family *Closteroviridae*) in association with several black berry viruses contribute to blackberry yellow vein disease which may lead to plant death (Tzanetakis et al., 2006). Rubus yellow net virus (RYNV; genus *Badnavirus*, family *Caulimoviridae*) likely contributes to raspberry mosaic disease (Martin et al., 2013).

4.4 Viruses infecting ribes

The genus *Ribes* L., known as currants and gooseberries, contains more than 150 species. Four main crops, black currant (*Ribes nigrum*), red currant (*Ribes rubrum*), white currant (*Ribes spicatum*) and gooseberry (*Ribes uvacrispa*) were domesticated from European species. Currants are widely cultivated in more than 30 countries across temperate zones of Europe, Asia, South America, Australia, and New Zealand (Hummer and Dale, 2010). About 99% of the world's production comes from Europe. In 2018, the total world acreage of black and red currant exceeded 120,000 ha, yielding more than 650,000 tons and producer prices ranged from 0.13 to 5.75 EUR/ kg (FAOSTAT 2020) (Spak et al., 2021).

Nearly 20 virus species identified from *Ribes* spp. plants before 2011. Blackcurrant reversion virus (BRV; genus *Nepovirus*, family *Secoviridae*) associated with blackcurrant reversion disease has been reported to cause major losses in some cultivars of currants from all countries where blackcurrant is grown commercially. In New Zealand, reversion has had a devastating effect on blackcurrant, forcing many growers to stop growing the crop altogether. Gooseberry vein-banding associated virus (GVBaV; genus *Badnavirus*, family *Caulimoviridae*). occurs commonly in Ribes species worldwide and, under experimental conditions, is known to decrease growth and yield in gooseberry and red currant by 15% (Bragard et al., 2019b; SŠpak et al., 2020; Spak et al., 2021).

4.5 Viruses infecting minor fruit trees

Blueberries belong to the genus *Vaccinium* in family *Ericaceae*. North America is the world's leading blueberry producer in the early 1990s (Isogai et al., 2012) and more than 248,000 Tons of highbush blueberry were produced in 2011.Approximately 655,000 metric tons of highbush blueberries were produced worldwide in 2016 (Martin and Tzanetakis, 2018). Cultivation is expanding within North America and other parts of the world raising concern regarding distribution of existing viruses as well as the appearance of new viruses (Martin et al., 2012; Thekke-Veetil and Ho, 2019). Blueberry plants can be infected by more than 15 virus species (Martin and Tzanetakis, 2015). Blueberry necrotic ring blotch virus (BNRBV; genus *Blunervirus*,

family *Bromoviridae*) associated with necrotic ring blotch in south-eastern states of the USA showing severe foliage damage and potentially lowering yields (Quito-Avila et al., 2013).

The genus *Actinidia* (commonly known as kiwifruit), whose members occur naturally in China, is composed of over 50 species (Veerakone et al., 2018). China is the largest producer of kiwifruit with 56% of global kiwifruit production. Like some other fruit trees, kiwifruit is propagated by grafting of selected buds or scions on seedlings or self-rooted rootstocks. The utilization of infected materials for plant propagation often causes the accumulation of viruses in kiwifruit trees and dissemination of viruses, At least 17 viruses have been reported to infect kiwifruit trees, causing many types of diseases (Zheng et al., 2017b; Veerakone et al., 2018; Wang et al., 2020; Hammond et al., 2021).

Japanese persimmon (*Diospyros kaki* Thunb) originated in China and has been grown for centuries in East Asian countries and has become popular recently in many other countries. This plant has been cultivated commercially mainly in China, Korea and Japan, and recently also in Brazil, Italy, Israel and other countries. More than 10 virus/viroid species had been identified from persimmons (Ito et al., 2013a; Morelli et al., 2015; Ito and Sato, 2020).

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2

Objectives and thesis structure

Fruit trees are high value crops which are infected by many viruses and viroids that tend to accumulate over the generations due to their vegetative propagation and the practice of grafting. Some of these viruses cause severe crop losses and often reduce the productive life of the orchards. The identification, detection, and characterization of the causal agent(s) of viral disease symptoms could be challenging, due to the low titer of several viruses, their heterogeneous distribution between tissues or over time within the tree, the frequent mixed infections, the absence of universal primers for the detection of all viruses, the occurrence of symptomless infection and the impact of cultivar on the symptom development. The advent of high-throughput sequencing (HTS) technologies during the last years has dramatically changed research on viral and virus-like pathogens. These technologies allow the detection and identification of known or unknown agents without any prior information.

This document started with an introduction on the general context of available methods for identification new viral pathogens, the traditional methods and the new technologies based on HTS are summarized for virus identification, then the general information of virus/viroid agents affecting fruit trees (Chapter 1).

The pace of virus discovery since the advent of HTS technologies also raises an important question: How the virologists address the biological characterization of a new virus with the genome sequence information as a starting point? Biological characterization of a new virus is a complex and lengthy process requiring comprehensive knowledge on epidemic potential, possible alternative hosts in ecosystems, symptomatology on various cultivars and host species, vectors and modes of transmission, geographical distribution, interactions with other viruses. Therefore, the first objective of this thesis is to critically review and analyze in depth the “deluge” of new viruses discovered after HTS application with a focus on (i) the scientific information on the virus biology that was published together with the genome sequence when a fruit tree virus was discovered and (ii) the contend of the scientific publications citing the original publication reporting the first publication of a fruit tree viruses (Chapter 3).

The second objective of this thesis is to identification of new host plants for known viral species using high-throughput sequencing technologies. For the case of newly reported apple necrotic mosaic virus (ApNMV) identified from a new natural host: hawthorn, and possibly associated with hawthorn mosaic disease. The case of ApNMV: high throughput sequencing protocol was performed and a new *Ilarvirus* (apple necrotic mosaic virus) was identified, during the field survey of fruit trees viral diseases, hawthorn trees affecting mosaic disease viral symptoms of unknown etiology. After recovering the whole genome sequence, the virus was further tested easily transmissible from hawthorns to apple trees with severe chlorosis, yellowing, mosaic, curling and necrosis and with high associated with hawthorn mosaic symptoms plants in main producing areas, indicating it was likely to be the causal pathogen of hawthorn mosaic disease (Chapter 4).

Finally, a conclusion and future prospects for the results above are proposed (Chapter 5).

3

Is there a “biological desert” with the discovery of new plant viruses? A retrospective analysis for new fruit tree viruses

In this chapter, critically review and analyze in depth (1) the scientific information on the virus biology that was published together with the genome sequence when a fruit tree virus was discovered and (2) the contend of the scientific publications citing the original publication reporting the first publication of a fruit tree viruses.

From Hou, WY., Li, Sf., Massart, S. (2020). Is there a "biological desert" with the discovery of new plant viruses? A retrospective analysis for new fruit tree viruses. *Journal of Frontiers in Microbiology,*11,1-15.

Abstract: High throughput sequencing technologies accelerated the pace of discovery and identification of new viral species. Nevertheless, biological characterization of a new virus is a complex and long process, which can hardly follow the current pace of virus discovery. This review has analyzed 78 publications of new viruses and viroids discovered from 32 fruit tree species since 2011. The scientific biological information useful for a pest risk assessment and published together with the discovery of a new fruit tree virus or viroid has been analyzed. In addition, the 933 publications citing at least one of these original publications were reviewed, focusing on the biology-related information provided. In the original publications, the scientific information provided was the development of a detection test (94%), whole-genome sequence including UTRs (92%), local and large-scale epidemiological surveys (68%), infectivity and indicators experiments (50%), association with symptoms (25%), host range infection (23%), and natural vector identification (8%). The publication of a new virus is cited 2.8 times per year on average. Only 18% of the citations reported information on the biology or geographical repartition of the new viruses. These citing publications improved the new virus characterization by identifying the virus in a new country or continent, determining a new host, developing a new diagnostic test, studying genome or gene diversity, or by studying the transmission. Based on the gathered scientific information on the virus biology, the fulfillment of a recently proposed framework has been evaluated. A baseline prioritization approach for publishing a new plant virus is proposed for proper assessment of the potential risks caused by a newly identified fruit tree virus.

Key words: fruit trees; new virus; biological characterization; virus diseases; potential risks

1 Introduction

The fruit trees are high-value crops grown worldwide. Stone, pome, citrus, rubus, ribes, blueberry mulberry, kiwifruit, and persimmon fruit trees represent the major cultivated species. Nevertheless, numerous plant viruses can infect them, sometimes at a very high prevalence. The virus infection can originate from vegetative propagation and grafting of infected cultivars, and might be exacerbated during the perennial life cycle by horizontal transmission accelerating the mixing and infection of viruses of individual plants (Czotter et al., 2018). Some of these pathogens cause severe crop losses and often reduce the productive life of the orchards. For example, the plum pox virus (PPV, genus *Potyvirus*) causes "sharka" disease, the most devastating viral disease of stone fruit trees worldwide, which causes severe damages, estimating a total cost at 10 billion Euros worldwide in 30 years (García et al., 2014; Rimbaud et al., 2015). Citrus tristeza virus (CTV; genus *Closterovirus*) is probably the most economically important virus infecting citrus, causing a decline of sour orange rootstock, yellow seedling of lemon and grapefruit, and stem pitting in grapefruit and sweet orange (Moreno et al., 2008; Umer et al., 2019). The disease has led to the death of millions of citrus trees all over the world and has rendered millions of others useless for production (Lee, 2015).

The identification, detection, and characterization of the causal agent(s) of viral disease symptoms could be challenging, due to the low titer of several viruses, their heterogeneous distribution between tissues or over time within the tree, the frequent mixed infections, the absence of universal primers for the detection of all viruses, the occurrence of symptomless infection, and the impact of the cultivar on the symptom development (Czotter et al., 2018; Maliogka et al., 2018). Another challenge for a complete viral indexing of a diseased tree was the intrinsic genome variability of plant viruses, which complicates the design of inclusive primers able to detect any isolate of a species or a specific genus or family through classical molecular test (Massart et al., 2014). Therefore, it was required to combine several tests like electron microscopy, serological or molecular techniques, and biological assays on indicator plants to achieve a complete indexing and the identification of viral species infecting the diseased trees with symptoms of unknown etiology.

The advent of high throughput sequencing (HTS) technologies during the last decade has dramatically changed research on viral and virus-like agents. HTS technologies are a potential universal screening method for plant virus detection, allowing for the theoretical detection and identification of any known or unknown agents. Until recently, there were many fruit tree diseases with unknown etiology, although a viral origin was suspected. Nevertheless, the development of HTS technologies has drastically changed the situation (Zheng et al., 2017a). In fact, they have accelerated the pace of discovery and identification of new viral species and the characterization of their genome (Massart et al., 2014). As an example, until 2011, the genomes of 50 different viruses from pome and stone fruit species were sequenced (Martelli et al., 2011), representing an average of 1.2 viruses per year since the development of sequencing technologies. From 2012 to 2016, three and seven new viruses were found in pome and stone fruits (Rubio et al., 2017), respectively (average: two per year).

From 2017, the number of newly identified viruses reached 28, representing an average of 9.3 novel species per year. Up to now, HTS technologies allowed the identification and genome characterization of nearly 40 novel viruses from pome and stone fruits in a few years.

The pace of virus discovery since the advent of HTS technologies also raises an important question: How do virologists address the biological characterization of new viruses with the genome sequence information as a starting point? Indeed, these new viruses identified by HTS technologies are often lacking information on their biology and the risk they can pose on fruit production. This is an important concern for carrying out a proper risk assessment. For example, a panel of experts highlighted in a recent report that for several viruses, especially those recently discovered, the pest categorization based on pest risk analysis is associated with high uncertainties due to the absence of data on biology and distribution (Bragard et al., 2019a). Biological characterization of a new virus is a complex and lengthy process requiring comprehensive knowledge on epidemic potential, possible alternative hosts in ecosystems, symptomatology on various cultivars and host species, vectors and modes of transmission, geographical distribution, and interactions with other viruses (Massart et al., 2017). Definitively linking a novel pathogen candidate with observed disease symptoms, according to Koch's postulates, is not easy or sometimes turns out to be impossible for some viruses. Koch's postulates are based on the one pathogen–one disease paradigm of infection biology and are inadequate in cases of diseases with polymicrobial causes. Therefore, the suitability of alternate strategies based on epidemiological observations and appropriate statistics for determining causal relationships of disease have been proposed when other experimental demonstrations of causation cannot be readily achieved (Fox, 2020).

A decade after the first discovery of a phytovirus by HTS technologies (Kreuze et al., 2009), it is now scientifically relevant and timely to address important questions related to the identification of new fruit tree viruses: When a new virus is discovered by HTS technologies, what information is published? To which extent do the scientists explore the biology when publishing a newly discovered virus: genome variability, prevalence, transmission, and host range?

After the original publication reporting the discovery of a new fruit tree virus, the high pace of virus discoveries also raises another question: Once a new virus is discovered and published, does it trigger additional experiments or surveys by the virologists to complete its biological characterization? Indeed, resources are limited for plant virologists, and the current abundance of newly identified viruses might limit their downstream biological characterization. This publication also evaluated how a recently proposed framework for the evaluation of biosecurity, commercial, regulatory, and scientific impacts of plant viruses and viroids identified by HTS technologies is fulfilled (Massart et al., 2017).

The objectives of this publication were, therefore, to critically review and perform and in deep analysis of (1) the scientific information on the virus biology that was published together with the genome sequence when a fruit tree virus was discovered, and (2) the content of the scientific publications citing the original publication

reporting the first publication of a fruit tree virus. We focus our analysis on the newly discovered viruses since 2011 and from the major fruit tree species worldwide. The gathered information was categorized, and general conclusions are provided. Overall, this analysis contributes to the establishment of a baseline and prioritization of complementary experiments to be done once a new fruit tree virus is discovered in the near future.

2 Screening of the scientific literature and categorization of experiments

2.1 Analysis of the publications reporting a new fruit tree virus

Thirty-two fruit tree species which listed in identified host species in Table S3-1 were included in our analysis. The NCBI nucleotide database was surveyed with the keywords of common name of the fruit tree species to identify virus or viroid species submitted for the first time since January 1, 2011. A first list of virus names was elaborated, and the corresponding publications retrieved. In addition, a literature survey was also carried out using Scopus with the keywords of the virus names retrieved from NCBI to complete the publication list.

All the publications reporting the discovery of a new virus or viroid species from January 1, 2011 to April 1, 2020 were screened. The information provided for the characterization of the new viral species has been classified into 13 categories:

- Complete genome: whole viral genome has been sequenced, including the UTR region at the 3'- and 5'-terminus.
- Primer design: virus-specific oligonucleotide primers and (RT)-PCR protocol were designed for RT-PCR detection and are described in the publication or the supplementary material.
- Genome diversity: several complete or near-complete genome sequences from different isolates were published and compared.
- Gene diversity: several partial or complete gene sequences (like the polymerase or the coat protein) were sequenced and aligned.
- Local survey: after the discovery, samples were collected in the same location (either a commercial orchard, research station, or germplasm collection) to evaluate the prevalence of the virus.
- Large scale survey: after the discovery, samples were collected from different locations, producing regions in one country or different countries.
- Association with symptoms: whatever the scale of the survey, the sampling was carried out on symptomatic and asymptomatic trees to evaluate the association between the virus presence and symptoms.
- Co-infection with other viruses: samples infected by a new virus are also infected by other known viruses which have been checked by RT-PCR.
- Infectivity bioassays: inoculation of plants using an infectious clone or graft inoculation to a host from the same cultivar or different cultivars of fruit tree species.

- Indicators: transmission to several herbaceous indicators were attempted, even if not successful.

- Symptomatology: symptoms have been observed on the grafted plants in a greenhouse experiment or grafted plants, including host plants and indicators, which have been mechanically inoculated with sap from symptomatic samples or transfers by a natural vector.

- Transmission: at least one natural vector (mite, aphid) has been identified. It might have been used for transmission assays.

- Host range: the virus has been detected on at least another plant species during a survey or successfully inoculated to another plant species (not an indicator).

2.2 Analysis of the peer review articles citing the publication of a new fruit tree virus

The peer-review publications citing one of the publications describing a newly identified fruit tree virus were also reviewed and analyzed in depth. They were retrieved based on the Scopus citations. First, the citation of each publication reporting the discovery of a new fruit tree virus was analyzed as follows: the total number of citations and number of citations per year from the initial publication. The information provided in the citing publication was classified into 18 categories in TableS3-2. Nine categories brought additional information on the biological characterization (transmission, survey, genome diversity) or new host and new country reported of the new virus, while other nine categories are not related to the biological or geographical characterization of the new virus.

3. Publications of new fruit tree viruses from 2011

A total of 78 scientific peer-review publications describing the discovery of a new virus or viroid species in the studied fruit tree species were identified between January 1, 2011 to April 1, 2020. These publications reported 81 new virus and three new viroid species among which 13 viruses and 2 viroids for pome fruit species, 22 viruses for stone fruit species, 14 viruses for *Citrus* sp., 8 viruses for *Ribes* sp., 6 viruses for *Rubus* sp., 17 viruses and one viroid for other minor fruit trees. The full name and abbreviations of all these new virus species is listed in Table S1-S3.

For the most identified fruit tree species, fifteen viruses from apple trees and thirteen viruses from sweet orange, at the opposite only 2 from pear, nectarine and lemon trees while a single virus from pear, Japanese pear, David's peach, Japanese apricot, plum, raspberry and American blackcurrant. Thirteen viruses (TFDaV, CPrV, PrVT, CTLaV, ChALV, PrVF, PrGVA, CVF, CVTR, CCGaV, CiVA, BcLRaV-1, BCCV-1) identified from more than one host species in the first publications.

4. Analysis of the scientific information added when a new viral species is discovered

The scientific information provided in the original publication describing a new

virus is highly variable. The minimal information corresponded to a recent publication of HTS sequences gathered into contigs without any biology confirmation of the results (Wright et al., 2020). On the other hand, a comprehensive biological characterization of the newly identified viruses was provided for temperate fruit decay associated virus (TFDaV). In a publication regarding TFDaV, forty-five complete viral genomes of TFDaV were sequenced and analyzed from different host species: apple, pear, and grapevine. Samples displaying virus-like symptoms collected at different regions of the country were evaluated by PCR and rolling-circle amplification (RCA). The ability of TFDaV to infect apple and pear tree seedlings and to cause growth reduction was confirmed by infectivity tests using the cloned viral genome (Basso et al., 2015). Another example is HTS applied to the citrus yellow mottle-associated virus (CiYMaV), which had been discovered from field samples that mainly showed virus-like symptoms. After characterizing the genome of CiYMaV, several aspects of its biology had been evaluated, including host range, symptomatology, association with symptoms, and epidemiology. Bioassays were performed by graft- and mechanical inoculation on eight citrus species and seven herbaceous species, symptoms of oak-leaf pattern and vein yellowing was observed, with CiYMaV detected in all symptomatic plants. The full CP gene of CiYMaV was amplified using a specific primer pair to study sequence diversity. A detection method was designed specifically for CiYMaV and revealed high prevalence (62%) in 120 citrus trees from the Punjab Province in Pakistan, where the novel virus was found mainly in mixed infections with CYVCV (45%) or CTV (9.2%). However, a preliminary survey on samples from 200 citrus trees from the Yunnan province in China failed to detect CiYMaV in this region (Wu et al., 2020).

Our analysis showed that almost all of the discovered genomes (76 viruses, 92%) (Fig 3-1) were amplified, cloned, and Sanger sequenced, including the UTR regions. The complete genome sequence retrieved was not confirmed by Sanger sequencing for only seven viral species, including PcVT and CVF, which had almost the whole genome sequenced but lacked UTR regions (Jo et al., 2018; Koloniuk et al., 2018a). ARWaV-1, ARWaV-2, IrCRSaV, CJLV, and CVLV had only sequenced partial fragments published (Sadeghi et al., 2016; Matsumura et al., 2017; Wright et al., 2018a). Until recently, there was no reporting of unconfirmed HTS sequences without any characterization (Wright et al., 2020).

Detection primers based on the assembled sequences were designed for 78 viruses (94%). The primers were only used to confirm the virus presence in the original sample or experimentally inoculated plants for 32 new viruses. For example, the detection primers designed for CJLV and CVLV had only been used to confirm the presence of the viruses identified in the RNA-seq and sRNA libraries (Matsumura et al., 2017). The PCR-based protocols were applied in the frame of local or large-scale surveys for 57 new viruses. Several primers or protocols were designed and tested for 14 new viruses. Nineteen publications gave little importance to these primers as their sequences were described in the supplementary material.

More than a single genome was sequenced for nine viruses (11%). In this case, the genome diversity was always analyzed. A maximum of 45 complete viral genomes of

TFDaV were sequenced, including 17 from apple, 26 from pear and two from grapevine (Basso et al., 2015). On the other hand, a complete genome of two isolates of peach-associated luteovirus (PaLV) were obtained and compared (Wu et al., 2017). In total, three publications reported genomes of isolates from very distant geographical locations (Martin et al., 2011; Marais et al., 2015a; Koloniuk et al., 2018b). For three viruses, the genome of isolates was obtained and compared in different host species. One is TFDaV, and the other two are PrVT and PrGVA (Al Rwahnih et al., 2018).

The diversity has been analyzed at genes or partial gene levels for 15 (18%) new viruses. The comparison was based on partial genome sequences obtained by classical Sanger sequencing. The sequenced fragment ranged from a partial gene to several genes. For apricot vein clearing-associated virus (AVCaV), PCR amplicons of 330 bp from the replicase gene were obtained from two samples (Elbeaino et al., 2014). In contrast, the nucleotide sequence of the entire CP from 58 different citrus yellow mottle-associated virus (CiYMaV) isolates was determined and aligned (Wu et al., 2020). The population diversity of blackberry vein banding-associated virus (BVBaV) was studied on 25 isolates for three genes (Thekke-Veetil et al., 2013).

A local survey of prevalence was carried out for 25 (30%) new species. The number of tested samples were also variable, ranging from four to more than 200. For example, four sweet cherry trees maintained at the Yamagata horticultural experiment station were evaluated to the study ChVB (Yaegashi et al., 2020). On the other hand, the local survey incidence of PrGVA included 215 samples collected from the National Clonal Germplasm Repository, selected trees represented a diverse array of *Prunus* sp. (Al Rwahnih et al., 2018). Tested samples usually ranged from 10 to 100 in other species. Less than 10 samples were tested for five viruses: NSPaV, ChVB, PeVB, PVd2, and BCI. Two (PrGVA, BCCV-1) had been tested in different host species.

The discovery was completed by a large-scale survey, including samples collected from different locations for 41 (49%) new species. The extent of the survey was highly variable, ranging from tens to hundreds of samples. For example, in a publication with cherry virus F (CVF), a small survey on nine cherry trees form four locations in different countries was carried out (Koloniuk et al., 2018a). Moreover, 524 samples collected from several areas in the U.S.A were surveyed for the presence of blueberry mosaic associated virus (BLMaV) in both wild and cultivated trees (Hassan et al., 2017). In the large scale survey on the prevalence of Mume virus A (MuVA), a total of 285 samples from 11 *Prunus* sp. trees from China, Japan, Czech Republic, Azerbaijan, Kazakhstan, Italy, and France were screened (Marais et al., 2018). Eighty peach samples collected from two germplasm nurseries located in different provinces in China were tested for the incidence of PLPaV (He et al., 2017). In total, more than one hundred samples were surveyed for 18 species, and less than 30 samples tested in five species. Eleven species (ARWV-1, ARWV-2, TFDaV, CPrV, PrVT, PrVF, MuVA, CVTR, CCGaV, CiVA, and BCCV-1) were tested for different host species, while 12 (AGCaV, ApNMV, CPrV, PrVT, MuVA, CVF, CRV-5, CiYMaV, RLBV, BlMaV, BFDaV, and MBV1) were tested for samples collected from different countries.

The sampling was carried out on symptomatic and asymptomatic trees to evaluate the association between the virus presence and symptoms in 56 (67%) species, including 15 at a local scale, 10 both at a local and large scale, and 31 at a large scale. Depending on the survey results, a high association with symptoms was found for 21 (25%) virus species: AGCaV, ApNMV, ARWV-1, ARWV-2, ARWaV-1, ARWaV-2, CCGaV, CYVCV, CiLV-C2, IrCRSaV, CiLV-N, CiYMaV, RLBV, BVBaV, BLMaV, BNRBV, BlMaV, BFDaV, MMDaV, AcCRaV, and AcEV-2. A possible latent infection had been concluded for eight (10%) species: AGV, AaLV, PrGVA, BBLV, BLSV, BVA, PeCV, and ASbLV. An association between symptoms and virus infection were confused in 27 (32%) species.

The association with symptoms in 25% species based on much higher incidence in symptomatic plants than in asymptomatic plants, regardless of the scale of the survey. For example, the incidence of ApNMV was 83% in symptomatic samples and 37% in asymptomatic samples from 359 samples in a survey of mosaic-diseased apple trees from major apple-producing provinces in China (Noda et al., 2017). CYVCV was considered to be highly associated with symptoms, according to the results from seven samples: four symptomatic plants and three asymptomatic plants from a local survey (Loconsole et al., 2012a). AcEV-2 was identified from a kiwifruit tree showing leaf mottle and chlorosis symptoms. Meanwhile, most AcEV-2-infected kiwifruit trees showed viral disease-like symptoms (Wang et al., 2020).

Latent infection was found in 10% species, and this was based on most of the positive samples that were symptomless. For example, PeCV had a high incidence in symptomless plants, while the original symptoms (vein necrosis) could not be linked to its presence (Morelli et al., 2015). BLSV was also considered to be latent as it did not cause any obvious symptoms in the highbush blueberry in a single infection (Isogai et al., 2012).

The association between symptoms and virus infection remained unclear in 32% species after a large-scale survey due to several reasons. The low incidence (<1%) or absence of virus (such as CPrV, MuVA, PeV1, AVCaV, CRV-5, PLPaV, and PVd2) in the survey impeded any association based on sufficient number of samples. Another limitation of the association is the presence of other viruses in the infected trees. This phenomenon was reported for 12 species (TFDaV, MdoVA, ChALV, PaLV, PcVT, ChVB, BVF, BCIV, BCaRV, BCCV-1, PrVT, and PeVB) and underlined the need to complete the survey on the newly identified virus with other known viruses. Symptom variability can also hamper the establishment of an association in eight species (ALV-1, NSPaV, NeVM, CVF, PrVF, CiVA, CVTR, and MBV1).

The presence of co-infecting viruses was reported and confirmed by RT-PCR for 28 (34%) viral species. Among them, a prevalence survey of four (AGCaV, ALV-1, CYVCV, AcEV-2) was carried out surveying for other co-infecting viruses. Another 24 species mentioned co-infection in the original sequenced plant but did not survey for another co-infecting virus or report the names of the co-infecting virus species.

Infectivity bioassays in the correspondent hosts were carried out for 30 (36%) viral species. All of the bioassays resulted in positive RT-PCR detection, and 12 reported observed symptoms. Infectious clones were constructed for TFDaV, consisting of

circular single-stranded DNA of family *Geminiviridae* and AHVd belonging to *Pelamoviroid*. Growth reduction was observed in apple and pear plants following biolistic inoculation with the cloned TFDaV (Basso et al., 2015a). But, no symptoms were observed in AHVd clone inoculated plants (Serra et al., 2018a). CiLV-C2, CiLV-N, and RLBV were successfully transmitted by mites, while 25 others were only successfully inoculated by grafting. For BLSV, six cultivars were successfully infected through graft transmissions; meanwhile, mechanical inoculation with purified BLSV particle into forty seedlings of highbush blueberry failed (Isogai et al., 2012). Three publications (CCDaV, CiYMaV, and PLPaV) confirmed the presence of co-infecting viruses on grafted plants (Loconsole et al., 2012b; He et al., 2017; Wu et al., 2020). Eight publications did not mention weather another virus along with the studied one (NSPaV, CCDaV, CYVCV, CiLV-C2, CVEV, IrCRSaV, CiLV-N, and RLBV).

Transmission to herbaceous indicators have been attempted for 21 viruses (25%). More than one herbaceous plant species was inoculated for 13 species. Ten transmissions allowed the observation of symptoms. However, nine failed to inoculate (TFDaV, PrVF, MuVA, PrGVA, ChVB, BLMaV, BVA, BFDaV, and PeCV). Two species (AGV and BVF) had successful infections, although none of the infected plants displayed visible symptoms. Among the 10 species with observed symptoms, CiLV-N and CiCSV were transmitted by mites while eight (PLPaV, CYVCV, IrCRSaV, CiYMaV, RLBV, BCLCaV, BLSV, and AcCRaV) were mechanically transmitted. Among the nine failed experiments, only TFDaV was biolistically-inoculated with the SpeI-linearized and recircularized genome onto *Nicotiana benthamiana* (Basso et al., 2015b). The other eight failed experiments were mechanical inoculation.

Symptoms have been observed for 16 (19%) viral species. These including symptoms observed on both grafted host plants and herbaceous indicators in six (PLPaV, CYVCV, IrCRSaV, CiLV-N, CiYMaV, and RLBV), only observed on experimental host plants in six (TFDaV, NSPaV, CCDaV, CiLV-C2, CVEV, and CCGaV), and only on herbaceous indicators plants in four (AcCRaV, BLSV, BCLCaV, and CiCSV).

More than one host species within the trees studied was identified for 19 (23%) viruses. Thirteen (TFDaV, CPrV, PrVT, CTLaV, ChALV, PrVF, PrGVA, CVF, CVTR, CCGaV, CiVA, BcLRaV-1, BCCV-1) were naturally infected in different host species identified, and six (MuVA, CCDaV, CYVCV, CVEV, IrCRSaV, and CiYMaV) were successfully inoculated to another plant species in infectivity bioassays. Three species (CCGaV, CiVA, and PrGVA) that were identified in different host species naturally were also infectable while inoculated on another plant species (Navarro et al., 2018b). Only TFDaV was found to naturally infect different host species which belong to different genera, while another 18 infected different host species belonging to the same genus.

The possible natural transmission mode and vectors were investigated for 7 (8%) viral species. Among them, potential natural vectors have been identified for five species (CiLV-C2, CiLV-N, CiCSV, RLBV, and BVA). Four of them successfully transmitted from one host plant to another through a natural vector (mite or aphid)

while one (BVA) failed with cotton aphids (Isogai et al., 2013). Only BBLV tested seed transmission and BCLCaV mentioned pollen transmission (Martin et al., 2011; James and Phelan, 2017).

Seventeen viruses (20%), including PpPV2, ApRVA, PeVD, PcVT, PCLSV, PYSaV, CJLV, CVLV, CTNGmV-1, CTNGmV-2, BVE, CuLV, CuVA, RAVA, BGMaV, PeLV, and AcV-1 lacked information regarding the biological characterization of epidemiological survey, infectivity or indicator assays, symptomatology, transmission, and host range. However, they did have genetic annotations or information regarding detection confirmation when first published.

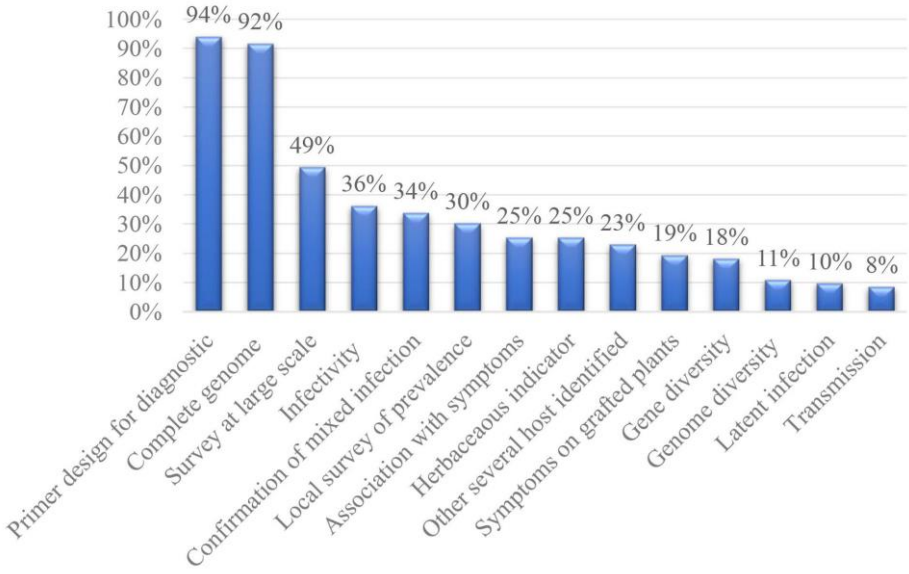

Figure 3-1 Scientific information added when a new fruit tree viral species is discovered

5. Biology progress after the discovery of new fruit tree virus

The 78 original publications of a new fruit tree virus have been cited 933 times (Table S3-2), representing an average of 12 citations per publication. If these numbers are reported per year from the initial publication, the average citation is 2.8 per year. The most frequently cited publication was on CCDaV, which had been cited by 70 other peer-review publications since 2012, with an average of 8.8 citations per year (Loconsole et al., 2012b).

The percentages (Fig 3-2) for each category calculated from Table S2. Only 18 % of citation covered information on the biology or geographical repartition of the newly

discovered viruses. This is an average of two citations per new virus (and a yearly number of 0.5). Among them, 3.6% and 1.2% reported the presence in a new country or host, respectively. The development of a diagnostic method was mentioned in 2.8% of the citations, while 0.9% studied genome variability by sequencing new isolates. Only 1.1% of the citations corresponded to epidemiological surveys, and another 1.1% focused on transmission. Finally, 1.6% focused on the interaction factor between virus and host, and 5.1% were related to risk assessment. The most cited for biology is a publication on citrus yellow vein clearing virus (CYVCV) which was first identified as the putative viral causal agent of yellow vein clearing disease (YVCD) in lemon trees in Pakistan (Loconsole et al., 2012a). In total, 22 publications (43%) were cited by publications on the biology of CYVCV. Five publications reported CYVCV in new countries and new host. Six publications developed diagnostic methods for CYVCV and four identified transmission vectors. Four publications focused on epidemiology and variability, and monitoring of CYVCV in China indicated a low level of sequence heterogeneity among CYVCV isolates of different geographic origins and hosts (Zhou et al., 2017). The interaction factor of and host response to CYVCV was also further investigated in three publications.

The other nine categories, which were not related to the biological or geographical characterization of a new virus, corresponded to 82% of all citations. Nearly half of the citations corresponded to reviews (22.9%) or the discovery of another virus (24.3%). In addition, 23% were related to the study of a known virus. The other categories represented less than 5%. Most of the citing publications were, therefore, not related to virus biology and repartition. Moreover, they did not provide useful information to better evaluate the phytosanitary risks posed by the new viruses.

The detection of the new virus in another country on the same continent was reported for six species: ApNMV, CYVCV, CCDaV, CRMaV, RLBV, and PeCV. ApNMV was identified in Japan and reported from China and Korea (Cho et al., 2017; Xing et al., 2018b). CYVCV was identified from Turkey, and reported in China, Pakistan, and India (Chen et al., 2014; Cao et al., 2016; Yu et al., 2017; Meena et al., 2019). CCDaV, identified from Turkey, was reported in China (Guo et al., 2015; Karanfil and Korkmaz, 2019a). CRMaV was identified from Canada but also reported in South Carolina (Poudel and Scott, 2017). RLBV, identified from Scotland, was reported in Montenegro, Bulgaria, Finland, and Serbia (Mavrič Pleško et al., 2014; Zindović et al., 2015; Dong et al., 2016; Jevremović et al., 2019). PeCV, identified from Italy, was also reported in North Macedonia, Spain, and Turkey (Morelli et al., 2015; Morelli and Arli-Sokmen, 2016; Ruiz-García et al., 2017; Jevremović and Paunović, 2019).

A new virus was detected in another continent for 14 species: AGCaV, AHVd, ARWV-1, ALV-1, NSPaV, CVF, ChALV, PrVF, PaLV, CVEV, BlMaV, PeVA, MBV1, and AcV-1. AGCaV, identified from Australia and Canada, was reported in Korea (Cho et al., 2016). AHVd had been reported in the United States, Japan, Italy, Spain, and New Zealand since it had been identified as a new viroid species (Szostek et al., 2018; Chiumenti et al., 2019; Sanderson and James, 2019). ALV-1 and ARWV-1, identified from the United States, were confirmed to be co-infected with more than two other viruses in apple rootstocks in Korea (Lim et al., 2019). NSPaV, discovered

from nectarine cultivars imported from France to California, was reported in Italy, China, Korea, and Japan (Candresse et al., 2017; Davaajargal etal., 2017; Lu et al., 2017; Sorrentino et al., 2018). CVF, identified from The Czech Republic and Greece, was reported in Canada (James et al., 2019). ChALV, identified from The Czech Republic, was reported in South Korea (Igori et al., 2017a). PrVF had been reported in Canada, Belgium, and The Czech Republic since it was first identified (Šafářová et al., 2017; James et al., 2018a; Tahzima et al., 2019). PaLV, identified from the USDA Appalachian Fruit Research Station (ARS) in West Virginia, was reported in Italy and China (Sorrentino et al., 2018; Zhou et al., 2018b). CVEV, identified from Spain, was reported in Japan and China (Huang et al., 2015; Nakazono-Nagaoka et al., 2017). BlMaV was confirmed from three states in the US and British Columbia, and Canada, and reported in Japan and Serbia (Thekke-Veetil et al., 2014; Jevremović et al., 2015; Isogai et al., 2016). PeVA was identified from Japan and also reported in Italy (Ito et al., 2013a). MBV-1 was identified in mulberry from Lebanon, Turkey, and Italy, but also reported in Iran (Alishiri et al., 2016). AcV-1, identified from Italy, was reported in China (Blouin et al., 2018; Wen et al., 2019).

A new host was identified for nine species: AGCaV, ApNMV, AVCaV, NSPaV, ChALV, PrVF, CYVCV, CCGaV, CiVA, CiCSV and CiLV-C2. A new strain of AGCaV, which was identified from apple trees, was first reported in quince (*Cydonia oblonga*) (Morelli et al., 2017). ApNMV, which was isolated from apple, was reported in crabapple (*Malus.*spp) (HU et al., 2019). AVCaV only infected one out of 39 varieties of apricot while identified, but was reported in four additional species (*P. salicina, P. mume, P. domestica, P. persica*) (Abou Kubaa et al., 2014; Kinoti et al., 2017). NSPaV was identified in nectarine trees and reported to infect peach and Japanese apricot trees (Candresse et al., 2017; Davaajargal etal., 2017; Lu et al., 2017; Sorrentino et al., 2018). A highly divergent South Korean (SK) isolate of ChALV, which was identified from sweet and sour cherry trees, was also reported in peach trees (Igori et al., 2017a). PrVF, identified from three prunus species (*P. avium, P. domestica,* and *P. cerasifera*), was reported in a new natural host, sour cherry (*P. cerasus*) (Koloniuk et al., 2018a). CYVCV, identified in lemon trees, proved to naturally infect different citrus (Kinnow mandarin, sweet oranges) and weed species (Önelge et al., 2016; Meena et al., 2019). CCGaV was identified from citrus trees and reported in apple trees (Wright et al., 2018a). CiVA was identified in citrus and then reported in pear (Svanella-Dumas1 et al., 2019). Besides affecting sweet orange CiCSV also infects *Taliparitiliaceum* and *Agave desmettiana* (Chabi-Jesus et al., 2018; Chabi-Jesus et al., 2019). CiLV-C2 has been reported naturally infecting *Hibiscus rosa-sinensis*, *Dieffenbachia* sp. and *Swinglea glutinosa* (Roy et al., 2015, 2018).

A high association with symptoms, based on the survey results on the incidence in symptomatic and asymptomatic plants in the first publication, was confirmed for six species (ApNMV, AGCaV, NSPaV, CYVCV, RLBV, BlMaV, and CCGaV) when reported in a new country or host. ApNMV, which was first correlated with the apple mosaic symptoms in Japan, was also reported at a high incidence in apple trees with mosaic symptoms in China and Korea (Cho et al., 2017; Xing et al., 2018b). AGCaV, which was associated with apple green crinkle disease (AGCD) in Australia was reported in apples showing severe symptoms of AGCD in Canada, and reported to co-

infect with other malus viruses in apples, showing small leaves or growth retardation in Korea, and in a new host quince with severe disease (Cho et al., 2016; Morelli et al., 2017). CYVCV was first described as the putative viral causal agent of yellow vein clearing disease (YVCD) in lemon trees from Turkey, and was also associated with YVCD in other countries and other citrus species (Chen et al., 2014; Cao et al., 2016; Yu et al., 2017; Meena et al., 2019). RLBV was associated with symptoms of leaf blotch disorder in raspberry plants and reported in samples that showed virus-like symptoms (including chlorotic mottling and yellow blotches) in four counties. BlMaV, which was confirmed in mosaic samples collected from three states in the US and British Columbia, Canada, was later reportedly associated with blueberry mosaic disease (BMD) in Japan and Serbia. CCGaV, identified to have an association with citrus concave gum disease (CG) affecting citrus trees, was found at a high incidence in apple-decline affected trees (Wright et al., 2018a).

Six publications focused on transmission related to four species: CYVCV, CiLV-C2, CiLV-N, and BNRBV. Four citing publications identified the transmission vector of CYVCV (Zhou et al., 2015; Zhang et al., 2018, 2019a, 2019b). One publication related to CiLV-C2 studied transmission (León et al., 2017). One publication was conducted to determine how BNRBV spreads in the field (Robinson et al., 2016).

Eight publications focused on genome diversity related to six species: AHVd, CYVCV, BCLCaV, BlMaV, MBV1, and AcV-1. Two publications analyzed the genetic diversity of genome sequences from variants of AHVd (Chiumenti et al., 2019; Sanderson and James, 2019b). Genetic stability among CYVCV isolates from different geographic origins were analyzed (Zhen et al., 2015; Chen et al., 2017). Analysis of HTS derived paired-end reads revealed the existence of bridge reads encompassing the 3'- and 5'-terminus of RNA-2 or RNA-3 of BCLCaV (James et al., 2018b). The genome diversity of BlMaV was examined using 61 isolates collected from North America and Slovenia (Thekke-Veetil et al., 2015). One publication characterized MBV1-derived small RNAs (Chiumenti et al., 2016). One publication compared CP sequences of different AcV-1 isolates with an isolate that was reported in New Zealand, and showed sequences identities of CP nucleotides and amino acids among these isolates, which were 84.8%-97.1% and 89.7%-99.6%, respectively (Peng et al., 2020).

Sixteen publications focused on the diagnostic method for 14 species: AGCaV, NSPaV, PaLV, PeVD, CCDaV, CYVCV, CiLV-C2, CVEV, BE, BVBaV, BBLV, BLSV, BNRBV, and BlMaV. Real-time RT-PCR assays were developed for two viruses (ASGV and AGCaV) infecting pome fruit (Beaver-Kanuya et al., 2019). Multiplex RT-PCR was developed to simultaneously detect three new viruses (NSPaV, PaLV, and PeVD) that infect peach (Xu et al., 2019). A loop-mediated isothermal amplification assay was established for CCDaV (Liu et al., 2017). Six publications developed diagnostic methods for CYVCV (Chen et al., 2016; Liu et al., 2017; Zhao et al., 2017; Bin et al., 2018; Liu et al., 2019; Meena et al., 2020), and four developed diagnostic methods for CiLV-C2 (Choudhary et al., 2013, 2014, 2015, 2017). A quantitative RT-PCR approach for the quantification of CVEV was also developed (Wang et al., 2016). One publication describes methods for the extraction of nucleic

acids for molecular testing from a range of different berry fruit crops, and lists oligonucleotide primers that were developed to amplify a large number of berry fruit viruses related to BVE, BBLV, BNRBV, and BlMaV (Macfarlane et al., 2015). One publication focused on reliable detection assays for BlMaV (Thekke-Veetil and Tzanetakis, 2017).

Six publications focused on epidemiological surveys related to nine species: ApNMV, CCDaV, CYVCV, BVE, BLMaV, BBLV, BlMaV, BFDaV, and AcCRaV. A large scope epidemiological survey in China demonstrated that ApNMV was highly associated with mosaic disease in apple trees (Xing et al., 2018b). One publication investigated the potential spread of CCDaV in commercial orchards, and showed that Turkish and Chinese samples clustered into different groups (Karanfil and Korkmaz, 2019b). Monitoring the presence of CYVCV in China indicated that there is a low level of sequence heterogeneity among CYVCV isolates from different geographic origins and hosts (Zhou et al., 2017). The incidence of BVE and BLMaV in two large-scale blackberry plantings in South Carolina demonstrated the transmission of mites (Poudel et al., 2018). A survey for blueberry viruses was carried out in the U.S and included BBLV, BlMaV, and BFDaV (Martin and Tzanetakis, 2018). The incidence of six viruses in kiwifruit was studied, including one new species AcCRaV (Zhao et al., 2019).

Five publications focused on co-infection synergisms between different virus or viroid species. The citing number was larger than the number of publications, while simultaneously citing several new species. For example, one publication included five new viruses in their analysis of co-infection patterns in peach (NSPaVF, NeVM, PLPaV, PaLV, and PeVD) (Jo et al., 2018).

The citing number was also much larger than the number of review publications. Five systematic literature publications of the European Food Safety Authority performed a listing of non-EU and pest categorization, which were classified into the category of risk assessment, and cited 32 new species, including seven pome, 12 stone, eight ribes, and five rubus viruses. Seven of them (ApNMV, TFDaV, CRMaV, CTLaV, BCaRV, BCLCaV, and RAVA) satisfied the criteria to be considered as Union quarantine pests. With the exception of the impact in the EU territory, on which the Panel was unable to conclude, 11 species (CPrV, MuVA, NSPaV, NeVM, PLPaV, PeVD, PrVF, PrVT, BVBaV, BVE, and BVF) satisfied the other criteria to be considered as potential Union quarantine pests. AGCaV satisfied the criteria to be considered as Union quarantine pests with the possible exception of being absent from the EU territory or having a restricted presence and being under official control. PrGVA met the criterion of having a negative impact in the EU. For those recently discovered, the categorization is associated with high uncertainties, mainly because of the absence of data on their biology, distribution, and impact (Bragard et al., 2019b, 2019c, 2019d, 2019a, 2020).

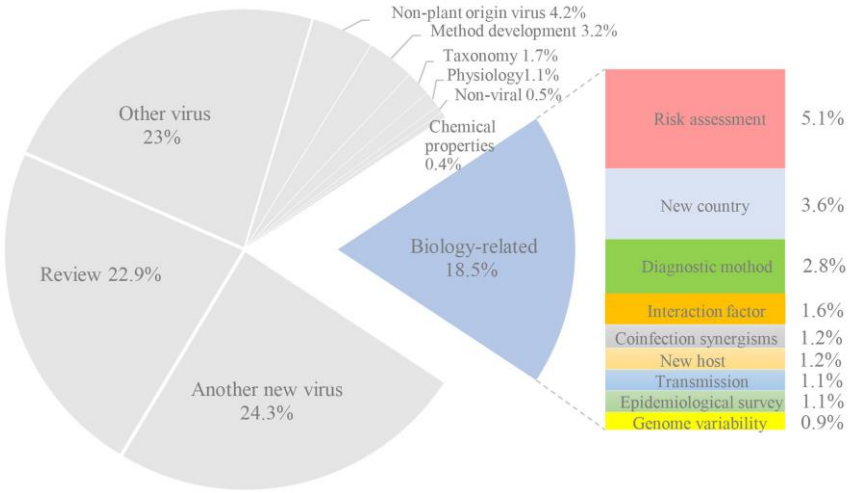

Figure 3-2 Publications citing a new fruit tree viral species

6. Evaluation of a previous framework for biological characteristics

The information provided in the first publication of a fruit tree virus and the citing publications was compared to a recently proposed framework for the efficient characterization of new phytoviruses (Massart et al., 2017). This framework proposed a three-step biological characterization of a new plant virus with regular exchanges with the phytosanitary authorities. Figure 3-3 shows how this framework has been completed for the viruses analyzed in this review in the original publications and those citing them.

6.1 Framework completion when publishing a new fruit tree virus

As shown in Figure 3-3, the rate of framework completion is very high for all the information needed during the first step. Only one publication failed to confirm the detection, while all the other publications described the sample context. A bibliographical search was always carried out but with differences between publications. Some publications explored the biology of related viruses while others did not. The genome integration was never studied because the identified viruses did not present any risk of integrated sequences in the host genome, which is particularly important for the *Caulimoviridae* family. Even in the absence of possible integration, the presence of virions was observed by electron microscopy for 11 species, representing 13% of the publications.

Regarding the second step, nearly all the original publications already sequenced the full genome (92%) and designed a diagnostic test (94%) for the new viral species.

Meanwhile, only 30% performed a local epidemiological survey. This low percentage is nevertheless compensated by the fact that half of the publications completed a large-scale survey, among which 37% were only at large scale. This means that an epidemiological survey was carried out in 67% of the first publications. So, the rate of completion of this second step is also very high with the first publication.

For the third step, half of the publications (42) analyzed the symptoms or host range. An analysis of symptoms was carried out for 30 species. This includes the evaluation of symptom association during the epidemiological survey for 21 species (25%); virus incidence in symptomatic and asymptomatic plants and the observation of symptoms on inoculated plants for 16 species (19%). For eight species, both analyses were carried out. The host range was evaluated for 19 species (23%). The host range was completed by symptom analysis for seven species. The transmission mode was studied for 35 viruses (42%). It was carried out either successfully with indicator plants (12 viruses, 14%), host plant candidates, or other cultivars of the same species (30 viruses, 36%) by mechanical transmission, grafting, or possible vectors (for six species). For eight species, both indicator plants and crop species were inoculated. The seed transmission was only studied for one species (BBLV). As expected, the completion rate of the third step of the framework is, therefore, significantly lower than the two first steps.

6.2 Additional characterization performed by the citing publications

Figure 3-3 shows that the improvement in the framework fulfillment by the citing publication was variable depending on the steps, but overall progressed percentage remained low. The information category that was most often covered by the citing publication involved improving the knowledge of the diagnostic method in 14 species. Among them only one species BVE (1%) did not have a published diagnostic protocol or primers in the first publication.

In addition, new progress was made regarding global epidemiology with the report of a new country or continent for nine viruses (11%): AcV-1, PeCV, PeVA, CVEV, CYVCV, CCDaV, PaLV, CRMaV, and NSPaV; which only tested samples collected from the discovery location in the first publications.

A new host was reported for eight species, and only four of them (5%), AGCaV, ApNMV, AVCaV, and NSPaV, did not have their host range tested in the first publications. The other four had different host species identified when first published. New progress of transmission and vectors had been obtained for four species. Two of them (2%), CYVCV and BNRBV, were not included in any transmission experiments when first published. The other two, CiLV-C2 and CiLV-N, had identified possible natural vectors in the first publications. No new species progressed with symptomatology in the citing publications when compared to the first publications.

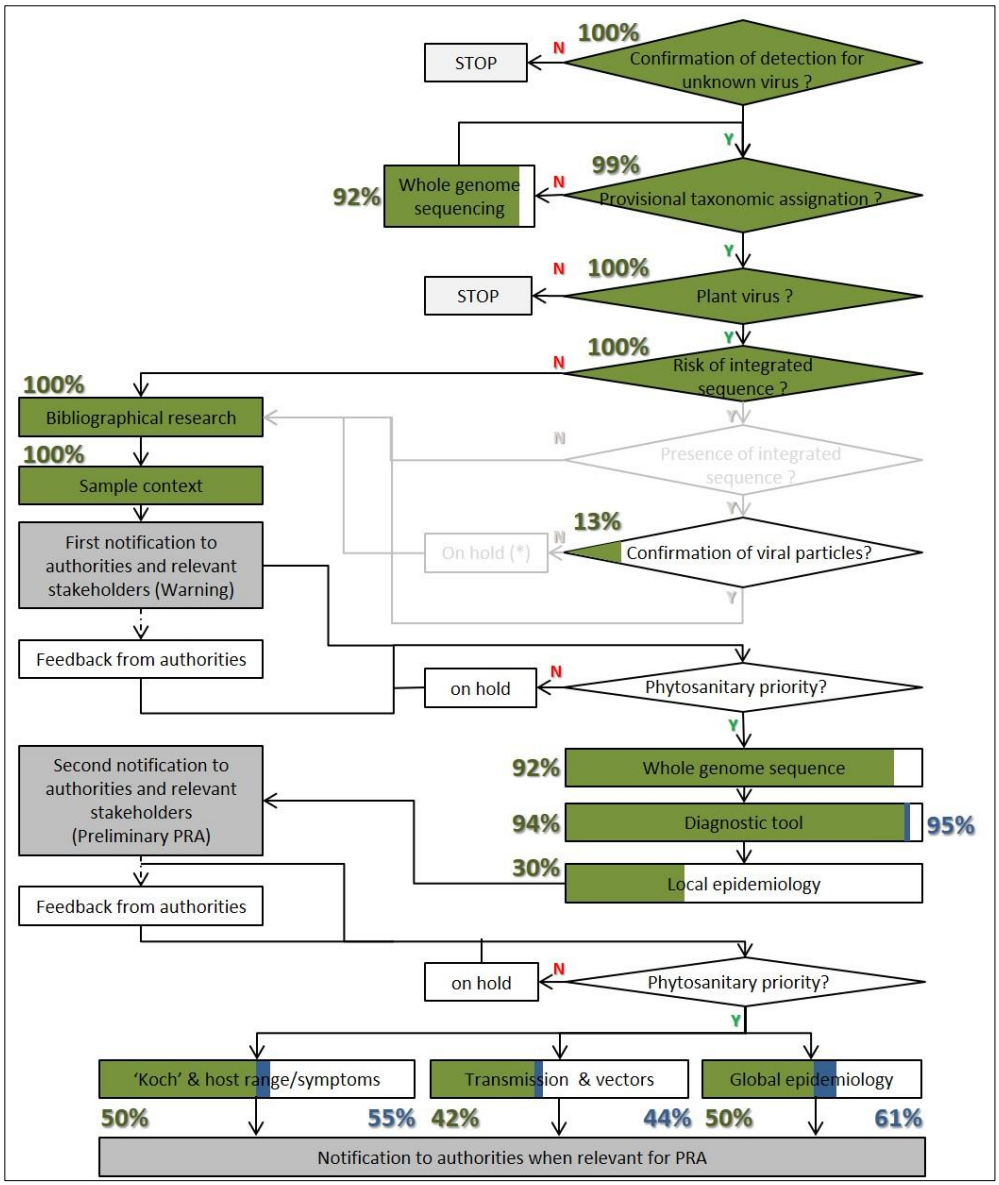

Figure 3-3 Fulfillment evaluation of a previous published framework for efficient characterization of new phytoviruses (Massart et al., 2017) for the newly discovered fruit tree viruses.Green box and percentage numbers represent the rate of framework completion in the first publication of a new fruit tree virus. Blue box and percentage numbers represent the rate of framework completion for the citing publications and the original one.

7. Conclusions

This large-scale, retrospective analysis of the biological information provided by the publications reporting the discovery of new fruit tree viruses and their citing publications highlights important trends in the characterization of these new viruses with the publication of the genome sequence.

First, various categories of information are always or nearly always provided: full or nearly full genome, confirmation of detection by an independent technique, and the design of diagnostics primers. Electron microscopy has been carried out in 13% of the publications. Even if this information was scientifically sound and demonstrated the presence of a viral particle, it provided limited information on the biology of the virus and the associated risks for plant health. An epidemiological survey is carried out in more than two-thirds of the publications. However, its extent was highly variable in geographical range, from only the same orchard to several continents and the number of analyzed trees, from a few to hundreds.

Further biological characterization experiments (transmission, host range and symptom) were only selectively carried out in the publications. For 20% of the discovered species, there was no information at all on the biological characterization, and the publications focused on genetic information. Biological characterization requires more resources and a longer time than genetic characterization. The association of the new species with symptoms might be limited by the low prevalence of symptomatic plants in a survey or the difficulty in reproducing symptoms experimentally. Symptom variability due to cultivar or environmental effects is also a bottleneck to associating a new virus with a disease. In addition, it can be further complicated by the presence of co-infections with other viruses in the orchards.

Therefore, there is a trade-off between quickly publishing partial information and building a robust characterization of a newly identified virus before publishing. Journals have very different policies on this subject. For example, a recent publication presenting only the contigs generated without any biological confirmation has been recently accepted (Wright et al., 2020), while other journals require biological characterization experiments for accepting the publication of a new virus. Whatever the policy of the journal, our retrospective analysis suggests that in more than 90% of the cases, the confirmation of the detection of the new virus by an independent technique and the characterization of the full or nearly full genome have been presented for a peer-review publication of a new fruit tree virus. This information can be recommended as the very minimal information needed for publication. Another important element that has been neglected is the availability of the HTS raw data supporting the virus discovery in public databases to allow the further use of these data by the scientific community.

Beyond the minimal recommendation for scientific publication, an epidemiological survey will provide very useful information with minimal resources and time compared to biological experiments and should be highly recommended. The surveys would indicate the prevalence of the virus in the orchards and several regions/countries. For example, a very recent publication showed that a new foveavirus on *Rubus* spp. was restricted to a single province in Turkey (Gazel et al.,

2020). In addition, the epidemiological survey can identify new hosts (Basso et al., 2015a) and study the association of the virus with symptoms (Fox, 2020). So, the survey should include healthy trees as well as symptomatic trees, presenting either the same symptoms as the original tree or not. In addition, testing other viruses during the survey is recommended, provided there is a risk of co-infection that could puzzle the analysis of the results.

The lack of resources can be partially solved by improving pre-publication data sharing between research groups. Indeed, a much richer biological characterization could be achieved when a virus is detected by several groups from different host plant presenting diverse symptoms in several countries using multiple tests and sequencing strategies. This can give quickly valuable information on the host range, field symptomatology and geographical spread of the virus. In addition, such collaborations significantly reduce the burden of publications and minimizes the collective effort to publish new data as the confirmatory experiment and the biological characterization experiments (host range, transmission, symptomatology) can be shared between partners. For example, HTS data sharing allowed for a better understanding of the geographical spread of a new *closterovirus* (Koloniuk et al., 2018b). The pre-publication data sharing could also include biological experiments, as was recently shown for the European wheat striate mosaic virus (Sõmera et al., 2020). Overall, pre-publication data sharing enables better risk evaluation and can limit the risks of unnecessary regulatory action.

Our analysis also showed that, in general, the citing publications provided very little additional information on the biological characterization of a newly discovered virus, except for enlarging the geographical spread (11%) or host range (5%). The biological characterization of a newly discovered virus is therefore rarely pursued, which reinforces the need to provide as much information as possible when publishing a new virus, even though the biological characterization experiments can be time-consuming and could delay the publication of the results.

Our analysis also allowed a preliminary evaluation of a published framework for the biological characterization of new plant viruses (Massart et al., 2017) with the reality of publications. According to the current situation with fruit tree viruses, the first step, including discovery, confirmation, taxonomic assignation, and sample documentation, and the second step, corresponding to whole-genome sequencing, diagnostic test development, and local epidemiology, could be gathered together in a single step before publication. The necessity to share both the genome sequence and the raw data, either publicly after publication or within an informal consortium before the publication, should be emphasized. In addition, the host range evaluation should be considered independently from the symptomatology as symptom expression could be very variable and difficult to analyze compared to the ability of the virus to infect a plant. Finally, the analysis of symptoms should include both biological experiments in controlled conditions and association studies from field surveys as proposed in the framework.

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Supplementary Materials

Table S3-1 Publications reporting a new fruit tree virus

Virus name (abbr.) (ref.)	Identified host	Design primers	Complete genome	Global survey	Infectivity	Mix-infect	Local survey	Association with symptoms	Indicators	Host range	Symptomatology	Gene diversity	Genome diversity	Transmission
Apple green crinkle associated virus (AGCaV) (James et al., 2013)	apple [*Malus* sp.]	■	■	■		■	■	■						
Temperate fruit decay associated virus (TFDaV) (Basso et al., 2015c)	apple [*Malus* sp.], pear [*Pyrus* sp.], Grapevine	■	■	■	■				■	■	■		■	
Apple geminivirus (AGV) (Liang et al., 2015)	apple [*Malus* sp.]	■	■	■		■			■					
Apple necrotic mosaic virus (ApNMV) (Noda et al., 2017)	apple [*Malus* sp.]	■	■	■			■	■						
Apple hammerhead viroid (AHVd) (Serra et al., 2018b)	apple [*Malus* sp.]	■	■		■								■	
Apple rubbery wood virus -1(ARWV-1) (Rott et al., 2018)	apple [*Malus* sp.]	■	■	■			■	■						
Apple rubbery wood virus -2 (ARWV-2) (Rott et al., 2018)	apple [*Malus* sp.]	■	■	■			■	■						
Apple rubbery wood associated virus-1 (ARWaV-1) (Wright et al., 2018b)	apple [*Malus* sp.]			■			■	■				■		
Apple rubbery wood associated virus-2 (ARWaV-2) (Wright et al., 2018b)	apple [*Malus* sp.]			■			■	■				■		
Apple luteovirus 1 (ALV-1) (Liu et al., 2018)	apple [*Malus* sp.]	■	■	■	■	■	■							

Virus name (abbr.) (ref.)	Identified host	Design primers	Complete genome	Global survey	Infectivity	Mix-infect	Local survey	Association with symptoms	Indicators	Host range	Symptomatology	Gene diversity	Genome diversity	Transmission
Apple-associated luteovirus (AaLV) (Shen et al., 2018)	apple [*Malus* sp.]	■	■				■							
Pyrus pyrifolia partitivirus 2 (PpPV2) (Osaki and Sasaki, 2018)	Japanese pear [*Pyrus pyrifolia*]	■	■											
Apple chlorotic fruit spot viroid (ACFSVd) (Leichtfried et al., 2019)	apple [*Malus* sp.]	■	■		■									
Apple rootstock virus A (ApRVA) (Baek et al., 2019)	apple [*Malus* sp.]	■	■											
Malus domestica virus A (MdoVA) (Koloniuk et al., 2020)	apple [*Malus* sp.]	■	■	■		■								
Apricot vein clearing associated virus (AVCaV) (Elbeaino et al., 2014)	apricot [*Prunus armeniaca*]	■	■	■								■		
Caucasus prunus virus (CPrV) (Marais et al., 2015b)	apricot [*Prunus armeniaca*]	■	■	■						■				
Prunus virus T (PrVT) (Marais et al., 2015a)	apricot [*Prunus armeniaca*]	■	■	■		■				■		■	■	
Nectarine stem pitting-associated virus (NSPaV) (Bag et al., 2015)	nectarine [*Prunus persica*]	■	■		■		■				■			
Cherry rusty mottle-associated virus (CRMaV) (Villamor et al., 2015)	sweet cherry [*Prunus avium*]	■	■			■								
Cherry twisted Leaf-associated virus (CTLaV) (Villamor et al., 2015)	sweet cherry [*Prunus avium*], apricot [*Prunus armeniaca*]	■	■			■				■				

Virus name (abbr.) (ref.)	Identified host	Design primers	Complete genome	Global survey	Infectivity	Mix-infect	Local survey	Association with symptoms	Indicators	Host range	Symptomatology	Gene diversity	Genome diversity	Transmission
Nectarine virus M (NeVM) (Villamor et al., 2016)	nectarine [*Prunus persica*]	■	■		■	■	■							
Cherry-associated luteovirus (ChALV) (Lenz et al., 2017)	sweet cherry [*Prunus avium*], sour cherry [*Prunus cerasus*]	■	■	■		■				■				
Prunus virus F (PrVF) (Villamor et al., 2017)	sweet cherry [*Prunus avium*], plum [*Prunus domestica*], cherry plum [*Prunus cerasifera*]	■	■	■	■				■	■				
Peach leaf pitting-associated virus (PLPaV) (He et al., 2017)	peach [*Prunus persica*]	■	■	■	■	■	■		■		■	■		
Peach-associated luteovirus (PaLV) (Wu et al., 2017)	peach [*Prunus persica*]	■	■		■	■	■						■	
Peach virus D (PeVD) (Igori et al., 2017b)	peach [*Prunus persica*]	■	■											
Peach virus T (PcVT) (Jo et al., 2018)	peach [*Prunus persica*]	■				■								
Peach chlorotic leaf spot virus (PCLSV) (Zhou et al., 2018a)	peach [*Prunus persica*]	■	■											
Mume virus A (MuVA) (Marais et al., 2018)	Japanese apricot [*Prunus mume*]	■	■	■	■				■	■				
Prunus geminivirus A (PrGVA) (Al Rwahnih et al., 2018)	sweet cherry [*Prunus avium*], apricot [*Prunus armeniaca*]	■	■		■		■		■	■			■	

Virus name (abbr.) (ref.)	Identified host	Design primers	Complete genome	Global survey	Infectivity	Mix-infect	Local survey	Association with symptoms	Indicators	Host range	Symptomatology	Gene diversity	Genome diversity	Transmission
Cherry virus F (CVF) (Koloniuk et al., 2018a)	sweet cherry [*Prunus avium*], sour cherry [*Prunus cerasus*]	■		■		■				■		■		
Cherry robigovirus 5 (CRV-5) (Wu et al., 2019)	sweet cherry [*Prunus avium*]	■	■	■	■	■	■							
Cherry virus Turkey (CVTR) (Çağlayan et al., 2019)	sweet cherry [*Prunus avium*], sour cherry [*Prunus cerasus*]	■	■	■		■				■		■		
Cherry virus B (ChVB) (Yaegashi et al., 2020)	sweet cherry [*Prunus avium*]	■	■			■	■		■					
Prunus yellow spot-associated virus (PYSaV) (Hou et al., 2019)	David's peach [*Prunus davidiana*]	■	■											
Peach virus 1 (PeV1) (Zhou et al., 2020)	peach [*Prunus persica*]	■	■	■	■	■								
Citrus chlorotic dwarf associated virus (CCDaV) (Loconsole et al., 2012b)	lemon [*Citrus limon*]	■	■		■	■				■	■			
Citrus yellow vein clearing virus (CYVCV) (Loconsole et al., 2012a)	lemon [*Citrus limon*]	■	■		■	■	■	■	■	■	■			
Citrus leprosis virus C2 (CiLV-C2) (Roy et al., 2013)	sweet orange [*Citrus sinensis*]	■	■	■	■			■			■			■
Citrus vein enation virus (CVEV) (Vives et al., 2013)	sweet orange [*Citrus sinensis*]	■	■		■					■	■			
Iranian citrus ringspot-associated virus (IrCRSaV) (Sadeghi et al., 2016)	sweet orange [*Citrus sinensis*]	■			■		■	■	■	■	■			

Virus name (abbr.) (ref.)	Identified host	Design primers	Complete genome	Global survey	Infectivity	Mix-infect	Local survey	Association with symptoms	Indicators	Host range	Symptomatology	Gene diversity	Genome diversity	Transmission
Citrus jingmen-like virus (CJLV)(Matsumura et al., 2017)	sweet orange [*Citrus sinensis*]	■												
Citrus virga-like virus (CVLV) (Matsumura et al., 2017)	sweet orange [*Citrus sinensis*]	■												
Citrus leprosis virus N (CiLV-N)(Ramos-González et al., 2017)	sweet orange [*Citrus sinensis*]	■	■	■	■			■	■		■			■
Citrus concave gum-associated virus (CCGaV) (Navarro et al., 2018a)	sweet orange [*Citrus sinensis*], clementine [*Citrus clementina*], tangerine [*Citrus reticulata*]	■	■	■	■			■		■	■			
Citrus chlorotic spot virus (CiCSV) (Chabi-Jesus et al., 2018)	sweet orange [*Citrus sinensis*]	■	■						■		■			■
Citrus virus A (CiVA) (Navarro et al., 2018b)	sweet orange [*Citrus sinensis*], clementine [*Citrus clementina*] mandarin [*Citrus reticulata*]	■	■	■	■					■				
Citrus yellow mottle-associated virus (CiYMaV) (Wu et al., 2020)	sweet orange [*Citrus sinensis*]	■	■	■	■	■		■	■	■	■	■		
Citrus Tunisia genomovirus 1 (CTNGmV-1) (Chabi-Jesus et al., 2020)	sweet orange [*Citrus sinensis*]	■	■										■	
Citrus Tunisia genomovirus 2 (CTNGmV-2) (Chabi-Jesus et al., 2020)	sweet orange [*Citrus sinensis*]	■	■											

Virus name (abbr.) (ref.)	Identified host	Design primers	Complete genome	Global survey	Infectivity	Mix-infect	Local survey	Association with symptoms	Indicators	Host range	Symptomatology	Gene diversity	Genome diversity	Transmission
Blackberry virus E (BVE) (Sabanadzovic et al., 2011)	blackberry [*Rubus armeniacus*]		■											
Raspberry leaf blotch virus (RLBV) (McGavin et al., 2012)	raspberry [*Rubus parvifolius*]	■	■	■	■			■	■		■			■
Blackberry vein banding-associated virus (BVBaV) (Thekke-Veetil et al., 2013)	blackberry [*Rubus armeniacus*]	■	■	■				■				■		
Blackberry virus F (BVF) (Shahid et al., 2017)	blackberry [*Rubus armeniacus*]	■	■	■		■			■			■		
Blackberry leaf mottle associated virus (BLMaV) (Hassan et al., 2017)	Blackberry [*Rubus armeniacus*]	■	■	■	■			■	■			■		
Blackberry virus A (BVA) (Hassan et al., 2018)	blackberry [*Rubus armeniacus*]	■	■	■		■						■		
Currant latent virus (CuLV) (Petrzik et al., 2016)	red currant [*Ribes rubrum*]	■	■											
Currant virus A (CuVA) (Petrzik et al., 2016)	red currant [*Ribes rubrum*]	■	■											
Blackcurrant idaeovirus (BCIV) (Thekke-Veetil et al., 2017)	blackcurrant [*Ribes nigrum*]	■	■			■	■							
Blackcurrant leaf chlorosis associated virus (BCLCaV) (James and Phelan, 2017)	blackcurrant [*Ribes nigrum*]	■	■		■				■		■			■
Blackcurrant leafroll-associated virus 1 (BcLRaV-1) (Koloniuk et al., 2018b)	blackcurran [*Ribes nigrum*], red currant [*Ribes rubrum*]		■							■			■	

Virus name (abbr.) (ref.)	Identified host	Design primers	Complete genome	Global survey	Infectivity	Mix-infect	Local survey	Association with symptoms	Indicators	Host range	Symptomatology	Gene diversity	Genome diversity	Transmission
Black currant associated rhabdovirus (BCaRV) (Wu et al., 2018)	blackcurrant [*Ribes nigrum*]	■	■				■					■		
Ribes americanum virus A (RAVA) (Thekke-Veetil et al., 2018)	American blackcurrant [*Ribes americanum*]	■	■											
Blackcurrant closterovirus 1 (BCCV-1) (Zheng et al., 2018)	blackcurrant [*Ribes nigrum*], white currant [*Ribes spicatum*], gooseberry [*Ribes uva-crispa*],	■	■	■			■			■				
Blueberry latent virus (BBLV) (Martin et al., 2011)	blueberry [*Vaccinium corymbosum*]	■	■	■									■	■
Blueberry latent spherical virus (BLSV) (Isogai et al., 2012)	blueberry [*Vaccinium corymbosum*]	■	■		■		■		■		■			
Blueberry necrotic ringblotch virus (BNRBV) (Quito-Avila et al., 2013)	blueberry [*Vaccinium corymbosum*]	■	■	■				■						
Blueberry virus A (BVA) (Isogai et al., 2013)	blueberry [*Vaccinium corymbosum*]	■	■	■	■				■					■
Blueberry mosaic associated virus (BlMaV) (Thekke-Veetil et al., 2014)	blueberry [*Vaccinium corymbosum*]	■	■	■				■						
Blueberry fruit drop associated virus (BFDaV) (Diaz-Lara and Martin, 2016)	blueberry [*Vaccinium corymbosum*]	■	■	■		■		■	■			■		
Blueberry green mosaic-associated virus (BGMaV) (Thekke-Veetil and Ho, 2019)	blueberry [*Vaccinium corymbosum*]		■											

Virus name (abbr.) (ref.)	Identified host	Design primers	Complete genome	Global survey	Infectivity	Mix-infect	Local survey	Association with symptoms	Indicators	Host range	Symptom atology	Gene diversity	Genome diversity	Transmi ssion
Mulberry mosaic dwarf-associated virus (MMDaV) (Ma et al., 2015)	mulberry [*Morus nigra*]	■	■	■				■						
Mulberry badnavirus 1 (MBV1) (Elbeaino et al., 2013)	white mulberry [*Morus alba*]	■	■	■	■							■		
Persimmon viroid 2 (PVd2) (Ito et al., 2013b)	American persimmon [*Diospyros virginiana*]	■	■		■		■							
Persimmon virus A (PeVA) (Ito et al., 2013a)	Japanese persimmon [*Diospyros kaki*]	■	■		■									
Persimmon latent virus (PeLV) (Ito et al., 2013a)	Japanese persimmon [*Diospyros kaki*]	■	■											
Persimmon cryptic virus (PeCV) (Morelli et al., 2015)	Japanese persimmon [*Diospyros kaki*]	■	■				■		■					
Persimmon virus B (PeVB) (Ito et al., 2015)	American persimmon [*Diospyros virginiana*]	■	■			■	■						■	
Actinidia chlorotic ringspot associated virus (AcCRaV) (Zheng et al., 2017c)	kiwifruit [*Actinidia deliciosa*]	■	■	■	■	■		■	■		■			
Actinidia virus 1 (AcV-1) (Blouin et al., 2018)	kiwifruit [*Actinidia deliciosa*]	■	■											
Actinidia seed-borne latent virus (ASbLV) (Veerakone et al., 2018)	kiwifruit [*Actinidia deliciosa*]	■	■			■	■							
Actinidia emaravirus 2 (AcEV-2) (Wang et al., 2020)	kiwifruit [*Actinidia deliciosa*]	■	■	■		■		■				■		

Note: blue color cell represent identified biological characteristics.

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Table S3-2 Citing publications of identification a new fruit tree virus

Host	Virus	Cited number	New country	New host	Detection method	Variability	Transmission	Epidemiology	Interaction	Coinfection	Risk assessment	Another New virus	Review	Other virus	Non-plant origin virus	Method develop	Taxonomy	Plant physiology	Non-viral	Chemical properties
Pome	Apple green crinkle associated virus (AGCaV)	16	1	1	1						2	3		7						1
	Temperate fruit decay associated virus (TFDaV)	19									2	4	7	1	3	2				
	Apple geminivirus (AGV)	25							1		2	12	4	3		2	1			
	Apple necrotic mosaic virus (ApNMV)	8	1	1				1			2		1			2				
	Apple hammerhead viroid (AHVd)	9	1			2					1		2	2						1
	Apple rubbery wood virus -1(ARWV-1)	11	1									5	2	1	1		1			
	Apple rubbery wood virus -2 (ARWV-2)																			
	Apple rubbery wood associated viruses-1 (ARWaV-1)	4										3		1						
	Apple rubbery wood associated viruses-2 (ARWaV-2)																			
	Apple luteovirus-1 (ALV-1)	4	1									1		1				1		
	Apple-associated luteovirus (AaLV)	9									2	5	2							
	Pyrus pyrifolia partitivirus 2 (PpPV2)	5									2	2		1						
	Apple chlorotic fruit spot viroid (ACFSVd)	1												1						
	Apple rootstock virus A (ApRVA)	0																		
Pome	Malus domestica virus A (MdoVA)	0																		

3. Biological desert of new fruit tree viruses

Host	Virus	Cited number	New country	New host	Detection method	Variability	Transmission	Epidemiology	Interaction	Coinfection	Risk assessment	Another New virus	Review	Other virus	Non-plant origin virus	Method develop	Taxonomy	Plant physiology	Non-viral	Chemical properties
Stone	Apricot vein clearing associated virus (AVCaV)	18		2							2	6	3	4		1				
	Caucasus prunus virus (CPrV)	21									2	6	4	8	1					
	Prunus virus T (PrVT)	18									2	4	4	5	2	1				
	Nectarine stem pitting-associated virus (NeVM)	26	2	3	1					1	2	5	8	2		1				1
	Cherry rusty mottle-associated virus (ChALV)	14	1									7	5	1						
	Cherry twisted leaf-associated virus (CTLaV)																			
	Nectarine virus M (NeVM)	29									2	7	8	8	3					1
	Cherry-associated luteovirus (ChALV)	13		1							1	4	3	4						
	Prunus virus F (PrVF)	17	4	1							1	2	5	4						

Host	Virus	Cited number	New country	New host	Detection method	Variability	Transmission	Epidemiology	Interaction	Coinfection	Risk assessment	Another New virus	Review	Other virus	Non-plant origin virus	Method develop	Taxonomy	Plant physiology	Non-viral	Chemical properties
	Peach leaf pitting-associated virus (PLPaV)	12								1	1	2	3	5						
	Peach-associated luteovirus (PaLV)	10	2		1					2	2	3								
	Peach virus D (PeVD)	8			1					1	1	2	2	1						
	Peach virus T (PcVT)	10										1	3	6						
	Peach chlorotic leaf spot virus (PCLSV)	1										1								
	Mume virus A (MuVA)	2									1		1							
	Prunus geminivirus A (PrGVA)	4									1	1		2						
Stone	Cherry virus F (CVF)	5			1								1	3						
	Cherry robigovirus-5 (CRV-5)	0																		

3. Biological desert of new fruit tree viruses

Host	Virus	Cited number	New country	New host	Detection method	Variability	Transmission	Epidemiology	Interaction	Coinfection	Risk assessment	Another New virus	Review	Other virus	Non-plant origin virus	Method develop	Taxonomy	Plant physiology	Non-viral	Chemical properties
	Cherry virus Turkey (CVTR)	0																		
	Cherry virus B (ChVB)	0																		
	Prunus yellow spot-associated virus (PYSaV)	1											1							
	Peach virus 1 (PeV1)	0																		
Citrus	Citrus chlorotic dwarf associated virus (CCDaV)	70	2		1			1				22	14	20	4	4	2			
	Citrus yellow vein clearing virus (CYVCV)	51	4	1	6	2	4	2	3			4	16	4	1	4				
	Citrus leprosis virus C2 (CiLV-C2)	48		1	4		1			1	1	7	13	13	2	4		1		
	Citrus vein enation virus (CVEV)	37	2		1				2			9	16	6	1					
	Iranian citrus ringspot associated virus (IrCRSaV)	0																		

Host	Virus	Cited number	New country	New host	Detection method	Variability	Transmission	Epidemiology	Interaction	Coinfection	Risk assessment	Another New virus	Review	Other virus	Non-plant origin virus	Method develop	Taxonomy	Plant physiology	Non-viral	Chemical properties
	Citrus jingmen-like virus (CJLV)	18							1			2	4	6	2			1	2	
	Citrus virga-like virus (CVLV)																			
	Citrus leprosis virus N (CiLV-N)	15							2	1		1	3	5			1	1	1	
	Citrus concave gum-associated virus (CCGaV)	17		1								6	3	2	3		2			
	Citrus chlorotic spot virus (CiCSV)	8											2	4			1		1	
	Citrus virus A (CiVA)	6										3	1	1	1					
Citrus	Citrus yellow mottle-associated virus (CiYMaV)	0																		
	Citrus Tunisia genomovirus 1 (CTNGmV-1)	0																		
	Citrus Tunisia genomovirus 2 (CTNGmV-2)	0																		

3. Biological desert of new fruit tree viruses

Host	Virus	Cited number	New country	New host	Detection method	Variability	Transmission	Epidemiology	Interaction	Coinfection	Risk assessment	Another New virus	Review	Other virus	Non-plant origin virus	Method develop	Taxonomy	Plant physiology	Non-viral	Chemical properties
Rubus	Blackberry virus E (BVE)	14			1			1			1	4	2	4	1					
	Raspberry leaf blotch emaravirus (RLBV)	42	4						2		1	7	8	17		1		2		
	Blackberry vein banding-associated virus (BVBaV)	16			1						1	4	5	5						
	Blackberry virus F (BVF)	5									1	1	2	1						
	Blackberry leaf mottle associated virus (BLMaV)	15						1			1	5	5	2		1				
	Blackberry virus A (BVA)	2										1					1			
Ribes	Currant latent virus (CuLV)	5									1	1	1	2						
	Currant virus A (CuVA)	9									1	7	1							
	Blackcurrant idaeovirus (BCIV)	4									2			2						
	Blackcurrant leaf chlorosis	6				1					2	1	1	1						

Host	Virus	Cited number	New country	New host	Detection method	Variability	Transmission	Epidemiology	Interaction	Coinfection	Risk assessment	Another New virus	Review	Other virus	Non-plant origin virus	Method develop	Taxonomy	Plant physiology	Non-viral	Chemical properties
	associated virus (BCLCaV)																			
	Blackcurrant leafroll-associated virus 1 (BcLRaV-1)	3									1		1	1						
	Black currant associated rhabdovirus (BCaRV)	8									2	5	1							
	Ribes americanum virus A (RAVA)	3									1		1	1						
Ribes	Blackcurrant closterovirus 1 (BCCV-1)	2									1			1						
Blueberry	Blueberry latent virus (BBLV)	45			2			1				10	11	13	7		1			
	Blueberry latent spherical virus (BLSV)	12			1							3	5	3						
	Blueberry necrotic ringblotch virus (BNRBV)	16			1		1					3	2	2	4	2			1	

3. Biological desert of new fruit tree viruses

Host	Virus	Cited number	New country	New host	Detection method	Variability	Transmission	Epidemiology	Interaction	Coinfection	Risk assessment	Another New virus	Review	Other virus	Non-plant origin virus	Method develop	Taxonomy	Plant physiology	Non-viral	Chemical properties
	Blueberry virus A (BVA)	3										1				1		1		
	Blueberry mosaic associated virus (BlMaV)	14	2		2	1		1	2			2	2	1			1			
	Blueberry fruit drop associated virus (BFDaV)	3						1	1			1								
	Blueberry green mosaic-associated virus (BGMaV)	0																		
Mulberry	Mulberry mosaic dwarf-associated virus (MMDaV)	25							1			8	4	8	1		1	2		
	Mulberry badnavirus 1(MBV1)	3			1	1							1							
Persimmon	Persimmon viroid 2 (PVd2)	26								1		5	13	3		3	1			
	Persimmon virus A (PeVA)	19	1							1		7	2	5	1	1		1		
	Persimmon latent virus (PeLV)																			

Host	Virus	Cited number	New country	New host	Detection method	Variability	Transmission	Epidemiology	Interaction	Coinfection	Risk assessment	Another New virus	Review	Other virus	Non-plant origin virus	Method develop	Taxonomy	Plant physiology	Non-viral	Chemical properties
	Persimmon cryptic virus (PeCV)	6	3							1			1	1						
	Persimmon virus B (PeVB)	2								1		1								
Actinidia	Actinidia chlorotic ringspot-associated virus (AcCRaV)	22						1				8	3	7			3			
	Actinidia virus 1(AcV-1)	7	2			1						1		2	1					
Actinidia	Actinidia seed-borne latent virus (ASbLV)	6										1	1	4						
	Actinidia emaravirus 2 (AcEV-2)	0																		
	Total citations number	933	34	11	26	8	10	10	15	11	48	227	214	215	39	30	16	10	5	4

Note: Categories brought information on the biological characterization of the new virus:

- New country: reported in different counties for the first publication, including the first report papers.

- New host: a new natural host was found to be infected by the new virus, with an enlarged known host range.

- Detection method: a new molecular diagnostic method was developed for detecting the new virus, including methods used to detect other viruses simultaneously.

- Variability (Genome variability): a new strain isolated from different cultivars or different geographical locations and genetic diversity of genome sequences or variants, and stability of gene have been analyzed.

- Transmission: investigation of factors affecting virus transmission, such as natural vectors or modes of transmission.

- Epidemiology (Epidemiological survey): samples collected from different producing regions in one country or different countries have been detected for the virus incidence, including the incidence of other viruses infected in the same samples simultaneously.

- Interaction (Interaction factor): analysis of a response factor in the host after infection or identification of potential virulence factors in the virus.

- Co-infection: analysis of co-infection while viral communities.

- Risk assessment (Potential risk): assessment of potential epidemic risk.

Categories did not bring biological or geographical characterization of the new virus

- Review: review publication or book chapter, which has reviewed the research progress or trends.

- Another new virus: the citing publications identified another novel virus whatever the host.

- Other virus: research focused on some other known viruses. An example: the first literature of apple geminivirus (AGV) was cited by the publication, "Identification of the potential virulence factors and RNA silencing suppressors of mulberry mosaic dwarf associated geminivirus" (Yang et al., 2018), but this citing did not focus on AGV and was classified into this category of other virus.

- Non-plant origin virus: viromes studies on fungi, bacteria, or other non-plant samples. An example: "Virome characterization of a collection of Sclerotinia sclerotiorum from Australia" (Mu et al., 2018) cited nectarine virus M (NeVM).

- Method develop: method improvement which is not directly related to the new virus, or some universal method. An example: "Identification of viruses and viroids by next-generation sequencing and homology-dependent and homology-independent algorithms" (Wu et al., 2015) focused on a universal method and cited citrus yellow vein clearing virus (CYVCV).

- Taxonomy: taxonomy topics. An example, "ICTV virus taxonomy profile: Fimoviridae" (Elbeaino et al., 2018) cited Actinidia chlorotic ringspot-associated virus (AcCRaV).

- Plant physiology: focus on physiology or nutrition factors. An example: "Potential role of weather, soil and plant microbial communities in rapid decline of apple trees" (Singh et al., 2019) cited apple luteovirus 1 (ALV-1).

- Non-viral: fungi, bacteria, or other non-viral pests. An example: "Aphid species composition in populations from citrus orchards in a region of the island of Crete" (Kalaitzaki et al., 2019) focused on aphid cited citrus jingmen-like virus (CJLV).

- Chemical properties (Chemistry): chemistry properties topics. An example: “Direct visualization of the native structure of viroid RNAs at single-molecule resolution by atomic force microscopy” (Moreno et al., 2019) cited apple hammerhead viroid (AHVd).

Table S3-3 Summary of the new viruses infecting fruit trees

	Virus/viroid name (abbr.)	Family	Genus	Group	Size (nt/bp)	Host
1	Apple green crinkle associated virus (AGCaV)	*Betaflexiviridae*	*Foveavirus*	ssRNA(+)	9,266	apple; quince*
2	Temperate fruit decay associated virus (TFDaV)	*Geminiviridae*	*Unassigned*	ssDNA	3,442 (circular)	apple; pyrus; grapevine;
3	Apple geminivirus (AGV)	*Geminiviridae*	*Unassigned*	ssDNA	2,932 (circular)	apple; crabapple*
4	Apple necrotic mosaic virus (ApNMV)	*Bromoviridae*	*Ilarvirus*	ssRNA(+)	3,378; 2,767; 1,956	apple;
5	Apple hammerhead viroid (AHVd)	*Avsunviroidae*	*Pelamoviroid*	ssRNA(+)	433 (circular)	apple;
6	Apple rubbery wood virus -1 (ARWV-1)	*Phenuiviridae*	*Rubodvirus*	ssRNA(-)	7,200; 1,600; 1,300	apple;
7	Apple rubbery wood virus -2 (ARWV-2)	*Phenuiviridae*	*Rubodvirus*	ssRNA(-)	7300-7400; 1600; 1100-1500	apple;
8	Apple rubbery wood associated viruses-1 (ARWaV-1)	*Phenuiviridae*	*Rubodvirus*	ssRNA(-)	/	apple;
9	Apple rubbery wood associated viruses-2 (ARWaV-2)	*Phenuiviridae*	*Rubodvirus*	ssRNA(-)	/	apple;
10	Apple luteovirus 1 (ALV-1)	*Luteoviridae*	*Luteovirus*	ssRNA(+)	6,001	apple;
11	Apple-associated luteovirus (AaLV)	*Luteoviridae*	*Unassigned*	ssRNA(+)	5,890	apple;
12	Pyrus pyrifolia partitivirus 2 (PpPV2)	*Partitiviridae*	*Alphapartitivirus*	dsRNA	1,945; 1,877	Japanese pear;
13	Apple chlorotic fruit spot viroid (ACFSVd)	*Pospiviroidae*	*Apscaviroid*	ssRNA(+)	354 (circular)	apple;

	Virus/viroid name (abbr.)	Family	Genus	Group	Size (nt/bp)	Host
14	Apple rootstock virus A (ApRVA)	*Rhabdoviridae*	*Nucleorhabdovirus*	ssRNA(-)	14,043	apple;
15	Malus domestica virus A (MdoVA)	*Closteroviridae*	*Velarivirus*	ssRNA(+)	17,003	apple;
16	Apricot vein clearing associated virus (AVCaV)	*Betaflexivirida*	*Unassigned*	ssRNA(+)	7,315	apricot; Japanese plums*; Japanese apricot*; plum*; nectarine*
17	Caucasus prunus virus (CPrV)	*Betaflexiviridae*	*Prunevirus*	ssRNA(+)	8,255	Japanese plums; almond
18	Prunus virus T (PrVT)	*Betaflexiviridae*	*Tepovirus*	ssRNA(+)	6,835	plum; sweet cherry; cherry plum
19	Nectarine stem pitting-associated virus (NSPaV)	*Luteoviridae*	*Luteovirus*	ssRNA(+)	4,491	nectarine; peach*; Japanese apricot*
20	Cherry rusty mottle-associated virus (CRMaV)	*Betaflexiviridae*	*Robigovirus*	ssRNA(+)	8,397	sweet cherry
21	Cherry twisted leaf-associated virus (CTLaV)	*Betaflexiviridae*	*Robigovirus*	ssRNA(+)	8,426-8,431	sweet cherry; apricot
22	Nectarine virus M (NeVM)	*Tymoviridae*	*Marafivirus*	ssRNA(+)	6,421-6,701	nectarine
23	Cherry-associated luteovirus (ChALV)	*Luteoviridae*	*Luteovirus*	ssRNA(+)	5,857	sweet cherry; sour cherry
24	Prunus virus F (PrVF)	*Secoviridae*	*Fabavirus*	ssRNA(+)	6,165; 3,622	sweet cherry; plum; cherry plum; sour cherry*
25	Peach leaf pitting-associated virus (PLPaV)	*Secoviridae*	*Fabavirus*	ssRNA(+)	6,357; 3,861	peach
26	Peach-associated luteovirus (PaLV)	*Luteoviridae*	*Luteovirus*	ssRNA(+)	5,819	peach
27	Peach virus D (PeVD)	*Tymoviridae*	*Marafivirus*	ssRNA(+)	6,612	peach
28	Peach virus T (PcVT)	*Tymoviridae*	*Marafivirus*	ssRNA(+)	6,618 (partial)	peach
29	Peach chlorotic leaf spot virus (PCLSV)	*Betaflexiviridae*	*Trichovirus*	ssRNA(+)	7,465-7,466	peach
30	Mume virus A (MuVA)	*Betaflexivirida*	*Capillovirus*	ssRNA(+)	7,644	Japanese apricot; peach[#]
31	Prunus geminivirus A (PrGVA)	*Geminiviridae*	*Grablovirus*	ssDNA	3,174 (circular)	cherry; apricot; plum; peach[#]
32	Cherry virus F (CVF)	*Secoviridae*	*Fabavirus*	ssRNA(+)	5,664 (partial); 2,608	sweet cherry; sour cherry
33	Cherry robigovirus 5 (CRV-5)	*Betaflexiviridae*	*Robigovirus*	ssRNA(+)	8,384	sweet cherry
34	Cherry virus Turkey (CVTR)	*Betaflexivirida*	*Robigovirus*	ssRNA(+)	8,464	sweet cherry; sour cherry

	Virus/viroid name (abbr.)	Family	Genus	Group	Size (nt/bp)	Host
35	Cherry virus B (ChVB)	*Betaflexiviridae*	*Foveavirus*	ssRNA(+)	8,806	sweet cherry
36	Prunus yellow spot-associated virus (PYSaV)	*Unassigned*	*Gratylivirus*	ssRNA(+)	6,072	David's peach
37	Peach virus 1 (PeV1)	*Rhabdoviridae*	*Alphanucleorhabdovirus*	ssRNA(+)	13,949	peach
38	Citrus chlorotic dwarf associated virus (CCDaV)	*Geminiviridae*	*Unassigned*	ssDNA	3,640 (circular)	lemon; sour orange[#]; Mexican lime[#]; Eureka lemon[#]; Duncan grapefruit[#]
39	Citrus yellow vein clearing virus (CYVCV)	*Alphaflexiviridae*	*Mandarivirus*	ssRNA(+)	7,531	lemon; sour orange[#]; Mexican lime[#]; Eureka lemon[#]; Duncan grapefruit[#]; Kinnow mandarin*; sweet oranges*; Weed species*
40	Citrus leprosis virus C2 (CiLV-C2)	*Kitaviridae*	*Cilevirus*	ssRNA(+)	8,717; 4,989	sweet orange; *Hibiscus rosa-sinensis**; *Dieffenbachia* sp. *; *Swinglea glutinosa**
41	Citrus vein enation virus (CVEV)	*Luteoviridae*	*Enamovirus*	ssRNA(+)	5,983	sweet orange; Mexican lime[#]
42	Iranian citrus ringspot-associated virus (IrCRSaV)	*Rhabdoviridae*	*Cytorhabdovirus*	ssRNA(+)	/	sweet orange; Trifoliate orange[#]; Mexican lime[#]; Grapefruit[#]; sour orange[#]
43	Citrus jingmen-like virus (CJLV)	*Flaviviridae*	*Flavivirus*	ssRNA(+)	/	sweet orange
44	Citrus virga-like virus (CVLV)	*Virgaviridae*	*Virgavirus*	ssRNA(+)	/	sweet orange
45	Citrus leprosis virus N (CiLV-N)	*Rhabdoviridae*	*Dichorhavirus*	ssRNA(-)	6,268; 5,847	sweet orange
46	Citrus concave gum-associated virus (CCGaV)	*Phenuiviridae*	*Phlebovirus*	ssRNA(-)	6,681; 2,703	sweet orange; Clementine; Tangerine; Sweet orange[#]; Grapefruit[#]; Dweet tangor[#]; Rough lemon[#]
47	Citrus chlorotic spot virus (CiCSV)	*Rhabdoviridae*	*Dichorhavirus*	ssRNA(-)	6,518; 5,987	sweet orange; *Taliparitiliaceum*; *Agave desmettiana**
48	Citrus virus A (CiVA)	*Unassigned*	*Coguvirus*	ssRNA(-)	6,691; 2,740	sweet orange; Clementine; Mandarin; Grapefruit[#]; Rough lemon[#]; Dweet tangor [#]; pear*
49	Citrus yellow mottle-associated virus (CiYMaV)	*Alphaflexiviridae*	*Mandarivirus*	ssRNA(+)	7,529	sweet orange; Chandler pummello[#]; Daidai sour orange[#]; Dweet tangor[#]; Eureka lemon[#]; Mexican lime[#];

	Virus/viroid name (abbr.)	Family	Genus	Group	Size (nt/bp)	Host
						Morocco sour orange[#]; Rough lemon[#]; Sweet orange[#]
50	Citrus Tunisia genomovirus 1 (CTNGmV-1)	*Genomoviridae*	*Gemykolovirus*	ssDNA	2191 (circular)	sweet orange
51	Citrus Tunisia genomovirus 2 (CTNGmV-2)	*Genomoviridae*	*Gemycircularvirus*	ssDNA	2156 (circular)	sweet orange
52	Blackberry virus E (BVE)	*Alphaflexivirida*	*Allexivirus*	ssRNA(+)	7,718	blackberry
53	Raspberry leaf blotch virus (RLBV)	*Bunyaviridae*	*Emaravirus*	ssRNA(+)	7,062; 2,135; 1,365; 1,675; 1,718	raspberry
54	Blackberry vein banding-associated virus (BVBaV)	*Closteroviridae*	*Ampelovirus*	ssRNA(+)	18,643	blackberry
55	Blackberry virus F (BVF)	*Caulimoviridae*	*Badnavirus*	dsDNA	7,663	blackberry
56	Blackberry leaf mottle associated virus (BLMaV)	*Bunyaviridae*	*Emaravirus*	ssRNA(-)	7,050; 2,271; 1,510; 1,504; 1,224	blackberry
57	Blackberry virus A (BVA)	*Betaflexiviridae*	*Vitivirus*	ssRNA(+)	7,285	blackberry
58	Currant latent virus (CuLV)	*Secoviridae*	*Cheravirus*	ssRNA(+)	6,603; 3,292	red currant
59	Currant virus A (CuVA)	*Betaflexivirida*	*Capillovirus*	ssRNA(+)	7,925	red currant
60	Blackcurrant idaeovirus (BCIV)	*Bromoviridae*	*Idaeovirus*	ssRNA(+)	5,349; 2,278	blackcurrant
61	Blackcurrant leaf chlorosis associated virus (BCLCaV)	*Bromoviridae*	*Idaeovirus*	ssRNA(+)	5,349; 2,280	blackcurrant
62	Blackcurrant leafroll-associated virus 1 (BcLRaV-1)	*Closteroviridae*	*Closterovirus*	ssRNA(+)	16,996-17,313	blackcurrant; red currant
63	Black currant associated rhabdovirus (BCaRV)	*Rhabdoviridae*	*Nucleorhabdovirus*	ssRNA(+)	14,432	blackcurrant
64	Ribes americanum virus A (RAVA)	*Betaflexiviridae*	*Unassigned*	ssRNA(+)	7,106	American blackcurrant
65	Blackcurrant closterovirus 1 (BCCV-1)	*Closteroviridae*	*Closterovirus*	ssRNA(+)	17,320	blackcurrant; red currant; white currant; gooseberry
66	Blueberry latent virus (BBLV)	*Amalgamaviridae*	*Amalgavirus*	ssRNA(+)	3,431	blueberry
67	Blueberry latent spherical virus (BLSV)	*Secoviridae*	*Nepovirus*	ssRNA(+)	7,960; 6,344	blueberry

	Virus/viroid name (abbr.)	Family	Genus	Group	Size (nt/bp)	Host
68	Blueberry necrotic ringblotch virus (BNRBV)	*Bromoviridae*	*Blunervirus*	ssRNA(+)	5,906; 3,935; 2,588; 1,724	blueberry
69	Blueberry virus A (BVA)	*Closteroviridae*	*Unassigned*	dsRNA	17,798	blueberry
70	Blueberry mosaic associated virus (BlMaV)	*Ophioviridae*	*Ophiovirus*	ssRNA(-)	7,963; 1,934; 1,570	blueberry
71	Blueberry fruit drop associated virus (BFDaV)	*Caulimoviridae*	*Unassigned*	dsDNA	9,850 (circular)	blueberry
72	Blueberry green mosaic-associated virus (BGMaV)	*Betaflexiviridae*	*Vitivirus*	ssRNA(+)	7,477	blueberry
73	Mulberry mosaic dwarf-associated virus (MMDaV)	*Geminiviridae*	*Unassigned*	ssDNA	2,952 (circular)	mulberry
74	Mulberry badnavirus 1 (MBV1)	*Caulimoviridae*	*Badnavirus*	dsDNA	6,945 (circular)	white mulberry
75	Persimmon viroid 2 (PVd2)	*Pospiviroidae*	*Apscaviroid*	ssRNA	358 (circular)	American persimmon
76	Persimmon virus A (PeVA)	*Rhabdoviridae*	*Cytorhabdovirus*	ssRNA(+)	13,467	Japanese persimmon
77	Persimmon latent virus (PeLV)	*Unassigned*	*Unassigned*	dsRNA	7,475	Japanese persimmon
78	Persimmon cryptic virus (PeCV)	*Partitiviridae*	*Deltapartitivirus*	dsRNA	1,577; 1,491	Japanese persimmon
79	Persimmon virus B (PeVB)	*Closteroviridae*	*Unassigned*	ssRNA(+)	18,569; 18,030	American persimmon
80	Actinidia chlorotic ringspot associated virus (AcCRaV)	*Fimoviridae*	*Emaravirus*	ssRNA(+)	7,061; 2,267; 1,678; 1,664; 1,476	kiwifruit
81	Actinidia virus 1 (AcV-1)	*Closteroviridae*	*Unassigned*	ssRNA(+)	18,848	kiwifruit
82	Actinidia seed-borne latent virus (ASbLV)	*Betaflexiviridae*	*Prunevirus*	ssRNA(+)	8,192	kiwifruit
83	Actinidia emaravirus 2 (AcEV-2)	*Fimoviridae*	*Emaravirus*	ssRNA(+)	7,079; 2,252; 1,387; 1,514; 1,744; 1,233	kiwifruit

Note: "/" refers to undetermined; " #" refers to host identified by grafting in first publication; "*" refers to host identified in citing publications.

4

RNA-seq reveals hawthorn tree as a new natural host for apple necrotic mosaic virus, possibly associated with hawthorn mosaic disease

In this chapter, the pathogen of a diseased hawthorn sample with leaf mosaic symptom was identified by small RNA sequencing with the presence of the newly identified apple necrotic mosaic virus (ApNMV), Grafting test demonstrated ApNMV was easily transmissible from hawthorns to apple trees reproducing severe chlorosis, yellowing, mosaic symptoms, Incidence survey in the main producing areas, indicating it was likely to be the causal pathogen of hawthorn mosaic disease.

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Abstract: Apple mosaic disease is widespread in the major apple-producing areas in China, and frequently associated with the presence of the newly identified apple necrotic mosaic virus (ApNMV), belonging to subgroup 3 of *Ilarvirus* genus in the family of *Bromoviridae*. Mosaic symptoms were also observed in hawthorn tree. Deep sequencing revealed the hawthorn tree with mosaic symptom was infected by ApNMV, which was confirmed by RT-PCR. The complete nucleotide sequences of RNA1 (3,378 nt), RNA2 (2,778 nt) and RNA3 (1,917 nt) of ApNMV from the hawthorn were obtained, sharing 93.8 - 96.8%, 89.7 - 96.1% and 89.8 - 94.6% nucleotide identities with those from apples and crab apples, respectively. Two hypervariable regions were found which showed 59.2 - 85.7% and 64.0 - 89.3% sequence identities at position 142 - 198 aa and at position 780 - 864 aa in the POL protein, respectively, between the hawthorn isolate and other isolates (apple, crabapple). Grafting test demonstrated ApNMV was easily transmissible from hawthorns to apple trees with severe chlorosis, yellowing, mosaic, curling and necrosis. In addition, a total of 11,685 hawthorn trees were surveyed for the incidence of mosaic disease from five provinces in China, only six were found showing typical mosaic symptoms. Total 145 individual trees (6 symptomatics, 68 asymptomatics and 71 with other symptoms) were tested for the presence/absence of ApNMV by RT-PCR. Among them, 6 symptomatic, 4 asymptomatic and 10 other symptomatic trees tested positive for ApNMV. Taken together, these results demonstrated that the hawthorn tree was identified as a new natural host for ApNMV with a relatively low frequency (13.8%, 20 out of 145) in the main producing areas, and it was likely to be the causal pathogen of hawthorn mosaic disease.

Key words: RNA-seq, hawthorn tree, apple necrotic mosaic virus, new natural host, hawthorn mosaic disease.

1 Introduction

Ilarviruses, belonging to the Bromoviridae family, are worldwide viral pathogens which cause significant economic losses of many fruit trees such as apple, peach, plum, cherry and apricot through direct or indirect reduction of yield and fruit quality (Pallas et al., 2012, 2013). Apple mosaic virus (ApMV), American plum line pattern virus (APLPV), prune dwarf virus (PDV), and prunus necrotic ringspot virus (PNRSV) are the main pathogens infecting fruit trees (Pallas et al., 2012). These viruses may cause similar symptoms to some degree which could be misleading in preliminary field diagnosis (Desvignes, 1999; Çağlayan et al. 2011; Hammond, 2011; Myrta et al. 2011; Paunovic et al. 2011; Petrzik and Lenz, 2011; Pallás et al. 2012). Among these four ilarviruses, both ApMV and PNRSV can cause mosaic disease in apple trees (Petrzik and Lenz, 2011; Hu et al. 2016). In recent years, apple necrotic mosaic virus (ApNMV), a newly identified ilarvirus, also shows a high relationship with apple mosaic disease in apple trees in China (Xing et al. 2018).

ApNMV was firstly isolated from apple leaves infected with mosaic disease in Japan, but occurs much less frequently in Japan (Noda et al. 2017). On the contrary, ApNMV is widespread in apple-producing areas in China (Xing et al. 2018). Usually, apple leaves infected by ApNMV are found to exhibit obvious symptoms including chlorosis, pale yellow mosaic to bright cream-colored irregular spots, rings or brownish necrotic spots. Bands and/or line patterns along the main veins were observed with uneven distribution on apple leaves (Noda et al. 2017; Xing et al. 2018).

ApNMV genome consists of three single-stranded positive-sense RNA segments, namely RNA1, RNA2 and RNA3 (Noda et al. 2017). RNA1 encodes a replicase protein containing a conserved methyltransferase/helicase (MET/HEL) domain (3171 nt; 1056 amino acids [aa]). RNA2 is monocistronic and encodes a non-structural protein, RNA polymerase (POL) of 855 - 867 aa, which contains the highly conserved motif sequence of glycine-aspartic acid-aspartic acid (GDD) and plays an essential role in virus replication while RNA3 contains two open reading frames (ORF) encoding a movement protein (MP, 280 - 281 aa) at 5′ terminal and a coat protein (CP, 219 aa) at 3′ terminal, respectively (Noda et al. 2017; Xing et al. 2018). The dendrogram analysis indicated a close genetic relationship of ApNMV with ApMV and PNRSV. To date, ApNMV was also reported in apple trees in South Korea (Cho et al. 2017) and India (only partial sequences from NCBI. Nabi and Baranwal, 2019), and was detected in crabapple trees (Malus spp., family Rosaceae), an ornamental apple germplasm resource (Hu et al. 2019).

Hawthorn (*Crataegus* spp., family *Rosaceae*) has an economically value for nutrition and medicine, and is widely planted in China. However, until now, only apple chlorotic leaf spot virus (ACLSV), a latent virus in most commercial cultivars, was reported to be the main virus in hawthorn trees (Sweet, 1980; Wang and Dai, 2014; Guo et al. 2016). In this study, the high-throughput sequencing (HTS) of RNA, an unbiased technique allowing us to detect a broad range of viruses in plants (Nagano et al. 2015), was used to identify the viruses in hawthorn leaves showing mosaic symptoms. ApNMV was tested positive in hawthorn trees, and its genome was sequenced and characterized. To the best of our knowledge, this is the first report of

ApNMV in hawthorn trees, revealing hawthorn trees as a new natural host for ApNMV.

2 Materials and methods

2.1Plant materials and virus isolates

The hawthorn leaves were collected from the main hawthorn-producing provinces and regions in China, including Hebei, Jilin, Liaoning, Shandong and Xinjiang. The collected samples were stored at low temperature using frozen blue ice and immediately frozen using liquid nitrogen at -196℃ upon arrival in the laboratory. They were further stored at -80℃ until total RNA was extracted.

2.2Library preparation and RNA sequencing

A mixed library that contained two leaf samples from Xinjiang province showing mosaic symptoms was constructed. Total RNA was extracted from the symptomatic leaves using TRIzol reagent (Tiangen, Beijing). RNA degradation was monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Ribosomal RNA was removed by Epicentre Ribo-zeroTM rRNA Removal Kit (Epicentre, USA), and mRNA from the tested plants was enriched using the poly-T attached to magnetic beads. The sequencing library was generated using the rRNA-depleted mRNA of total RNA by NEBNext® UltraTM Directional RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations. The library was sequenced using an Illumina Hiseq 4000 platform and generated 150 bp paired-end reads.

2.3Analysis and assembly of RNA-seq data

Raw reads from the Illumina platform were trimmed with the minimum quality of 25, then paired and duplicate reads were removed, the processed reads were assembled de novo into larger contigs by the SPAdes assembler Method with a k-mer of 32 using commercial software Genious (www.geneious.com). The final contigs were screened against the Refseq genomes of viruses and viroids database from NCBI (https://www.ncbi.nlm.nih.gov/genome/ viruses/) using tblastx programs in Genious.

2.4Total RNA extraction and reverse transcription of hawthorn leaf samples

Total RNA was extracted using an RNAprep Pure Plant Kit (TianGen, Beijing, China) following the manufacturer's instructions, and then treated with DNase I for 15 min at room temperature to remove DNA. The first-strand cDNAs were synthesized by reverse-transcription (RT) reactions using M-MLV reverse transcriptase (Promega, Beijing, China) with random hexamer primers according to the manufacturer's protocol.

2.5Detection of ApNMV and ASPV in hawthorn trees by RT-PCR

The polymerase chain reaction (PCR) was performed using the specific primer sets ApNMV-F/-R and ApN-F2/-R2 for the detection of ApNMV, as described before (Xing et al. 2018), and specific primer sets szASPV-F/R and szASPV-F2/R2 for apple stem pitting virus (ASPV). For the detection of ASPV, the mastermix of samples was denatured at 94℃ for 4 min and followed by 35 cycles of PCR (15 cycles of nested PCR) amplification with Taq polymerase (Bingda, Beijing, China) at 94℃ for 30 sec, 55 ℃ (54℃ for the nested PCR) for 30 sec and 72℃ for 40 sec (30 sec for the nested PCR) and a final step of 10 min at 72℃. PCR products were observed by 1% agarose gel electrophoresis using 1×TAE buffer (0.04 M Tris–acetate, 1 mM EDTA), purified by gel DNA purification kit (Axygen, Hangzhou, China) and sequenced by Sangon Biotech (Shanghai) Co., Ltd.

2.6Cloning of the complete nucleotide sequences of ApNMV isolates from hawthorn trees

To obtain the complete nucleotide sequence of an ApNMV isolate from a hawthorn tree, E. coli polymerase (NEB, Beijing, China) was used to add poly A tails to 3' ends of viral RNAs 1-3 molecules. M-MLV reverse transcriptase was used for the synthesis of first-strand cDNAs with a random hexamer primer. Firstly, three primer sets ApnS1/fullRNA1-R1, ApnS1/fullRNA1-R1 and RNA3-mcF/fullRNA1-R1 were designed to amplify the partial genomes of ApNMV RNAs 1-3, respectively. The amplification of cDNA ends of ApNMV isolates were carried out using the SMARTer RACE 5'/3' Kit (Clontech, USA) following the manufacturer's instructions using the primers of 5R1GSP1/ 5R1GSP2, 5R2GSP1/ 5R2GSP2, and 5R3GSP1/ 5R3GSP2 for 5'-end sequences, and 3R1GSP1, 3RACE-R2-Ft and 3ApN-RACE2 for 3'-end sequences, respectively. Finally, the sequenced cDNA fragments were assembled and complete nucleotide sequences of the three RNA segments of ApNMV from a hawthorn tree were obtained. All amplification reactions were performed using Q5 high-fidelity DNA polymerase (NEB, Beijing, China). All primers used in this study are listed in the Supplementary Table 1.

2.7Phylogenetic analysis

Nucleotide sequences of ApNMV isolated from hawthorn trees based on the MET/HEL, POL, MP and CP were aligned with the homologous sequences of other ApNMV isolates from GenBank using ClustalW, and the phylogenetic relationships of the four aligned ORFs sequences were assessed by MEGA version 6 (Tamura et al. 2013) using the neighbor-joining method (Saitou and Nei, 1987) with 1,000 bootstrap replications.

2.8Transmission test of ApNMV by grafting

The hawthorn shoots which showed mosaic symptom form Xinjiang Province were collected as virus sources. Transmission experiments of ApNMV were conducted by bud grafting from hawthorn trees to four one-year-old and one three-year-old apple

plantlets, which were free of ApNMV. The plants were maintained at about 25℃ in the greenhouse with 16 h/8 h (day/night). The symptoms on the tested plants were under continuous observation, and RT-PCR together with Sanger dideoxy sequencing was used to confirm the infection of ApNMV on these grafting transmitted plants.

3 Results

3.1 Symptoms and occurrence of mosaic disease on hawthorn trees in China

A survey on symptoms and occurrence of mosaic disease on hawthorn trees was conducted in the main hawthorn-producing provinces and regions (such as Hebei, Jilin, Liaoning, Shandong and Xinjiang) of China. A total of 11,685 hawthorn trees were surveyed, only six trees (2/10,000 in Chengde, Hebei; 1/135 in Gongzhuling, Jilin; 1/1500 in Shenyang, Liaoning; 0/20 in Linyi, Shandong; 2/30 in Luntai, Xinjiang) were showing typical mosaic symptoms. The mosaic symptoms in hawthorn trees varied from mild, moderate to severe chlorosis, yellowing, pale yellow mosaic to bright cream-colored irregular spots, rings. Bands and/or line patterns were observed with uneven distribution on hawthorn leaves (Fig. 4-1 A-D). The symptoms are often restricted to parts of the whole hawthorn trees. The symptoms of mosaic disease on hawthorn trees were similar to those on apple leaves caused by ApNMV that we speculated to have a crucial role of ApNMV in causing mosaic disease on hawthorn trees. In addition, other symptoms were also found, including chlorosis, yellowing or necrosis on the leaf margins but obviously different from the typical symptoms of mosaic disease (Fig. 4-1 E-G).

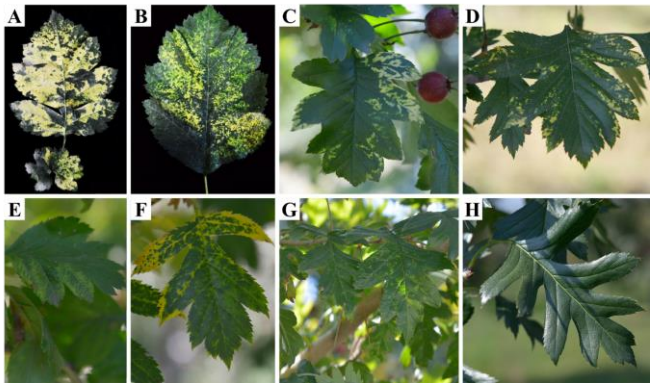

Figure 4-1 The investigated symptoms on hawthorn trees in the fields.Typical symptoms caused by mosaic diseases with yellowing, mosaic, line pattern, pale yellow mosaic to bright cream-colored irregular spots, rings (A-D); E-G showed other symptoms, including chlorosis, yellowing or spots on the leaves but obviously different from the typical symptoms of mosaic disease; and asymptomatic hawthorn leaf (H).

3.2 RNA-seq analysis of hawthorn leaves with mosaic symptoms

In total, 43,252,990 clean reads that had passed the quality control were produced after having been trimmed, pared and merged. The assembly generated 171,338 contigs ranging from 55 to 45,814 nt. Three contigs of 3,357, 2,748 and 1,934 nucleotides, respectively, were found to share high nucleotide and amino acid similarity with ApNMV, and the additional two contigs of 9,310 and 1,474 nucleotides had highly identities with apple stem pitting virus (ASPV). Using blast against the viral sequences in the GenBank Virus Reference Database (http://www.ncbi.nlm.nih.gov), the identities of the three contigs were observed with ApNMV ranging from 96.9% (RNA1), 95.6% (RNA2), 94.5% (RNA3), covered the genome sequence of ApNMV available from NCBI on 100% (RNA1), 100% (RNA2), 99% (RNA3), respectively. Meanwhile, one contig of 9,310 nt shared 81.6% identity with ASPV on 99% coverage, and the other of 1,934 nt showed 90.8% nucleotide identity with ASPV on 59% coverage.

3.3 Complete nucleotide sequence and genome characteristics of ApNMV isolated from detected hawthorn tree

To confirm the HTS result and to understand the differences of ApNMV isolates from hawthorn and apple trees, we cloned and sequenced the complete nucleotide sequences of ApNMV isolated from the same hawthorn tree with mosaic symptoms from Xinjiang province. Its genome consisted of three RNA molecules, ranging in size from 3,378 nt (RNA1, accession no. MN656198), 2,778 nt (RNA2, accession no. MN656199) and 1,917 nt (RNA3, accession no. MN656200), respectively. The nucleotide sequence identities of RNAs 1 - 3 obtained from hawthorn trees, apple trees and crabapple trees were 93.8 - 96.8%, 89.7 - 96.1% and 89.8 - 94.6%, respectively, as shown in Table 4-1. The identities of MET/HEL, POL, MP and CP of ApNMV isolated from the hawthorn tree with other isolates were 93.7 - 96.9%, 89.5 - 96.1%, 88.4 - 94.5% and 91.8 - 96.8%, respectively, based on the nucleotide sequences, and were 97.8 - 98.7%, 92.1 - 96.8%, 90.4 - 94.6% and 94.5 - 97.3% based on the amino acid sequences, respectively. The MET/HEL was highly conserved while the POL and MP showed great variation among the four proteins. Two hypervariable regions were found at position 142 - 198 aa and at position 780 - 864 aa in the POL encoded by RNA2, which shared 59.2 - 85.7% and 64.0 - 89.3% sequence identities between the hawthorn isolate and other isolates (apple, crabapple), respectively (Fig. 4-2).

After the complete viral genome was obtained, clean reads were mapped to the ApNMV genome, indicating a nearly whole coverage (Supplementary Fig.4-1). The nt identity percentage values were 99.7% (RNA1), 99.0%(RNA2) and 97.9% (RNA3) between the sequence obtained with HTS and the individual clones obtained by 5'-3' RACE and RT-PCR, respectively, which showed the ApNMV variability in the infected tissue.

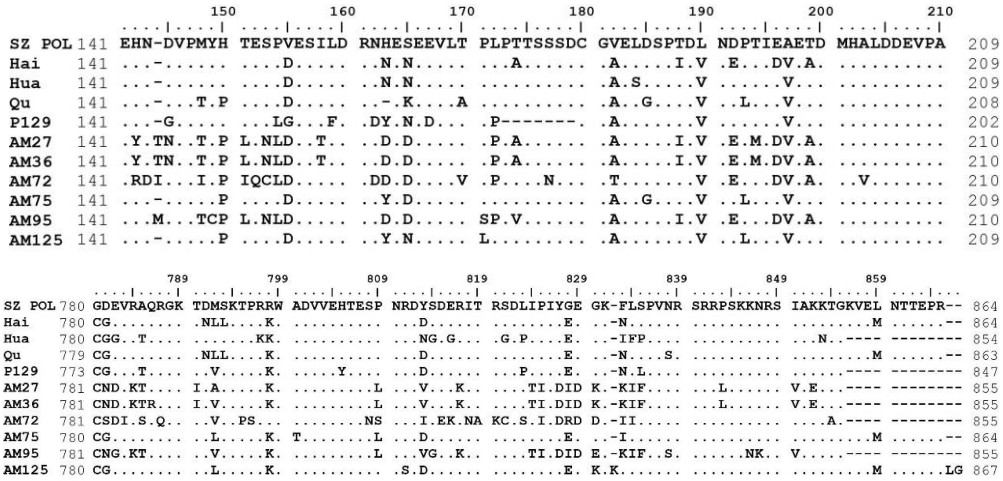

Figure 4-2 Two hypervariable regions in the POL protein among ApNMV isolates.SZ POL was obtained in this study. It showed 59.2 - 85.7% and 64.0 - 89.3% sequence identities at position 142 - 198 aa and at position 780 - 864 aa, respectively, between the hawthorn isolate and other isolates (apple, crabapple)

Table 4-1 The sequence identities of ApNMV isolated from hawthorn tree with others at nucleotide and amino acid level

Isolate[1]	Host	RNA1 (%)			RNA2 (%)			RNA3 (%)				
		Full length	MET/HEL		Full length	POL		Full length	MP		CP	
			nt[2]	aa[3]		nt[2]	aa[3]		nt[2]	aa[3]	nt[2]	aa[3]
Hai	crabapple	96.3	96.4	98.3	93.6	93.4	96.5	91.5	89.0	**90.4**	95.9	95.0
Hua	**apple**	**93.8**	**93.7**	98.3	95.0	94.9	95.9	90.4	89.2	91.8	94.2	95.0
Qu	**apple**	96.7	96.7	98.6	**96.1**	**96.1**	96.2	**94.6**	94.1	93.9	**96.8**	95.4
P129	**apple**	96.0	96.1	97.9	95.1	95.0	95.7	93.5	92.6	92.9	95.5	**97.3**
AM27	**apple**	95.2	95.2	97.9	90.8	90.8	93.0	**89.8**	**88.4**	91.1	94.4	95.4
AM36	**apple**	95.1	95.2	98.0	90.7	90.7	93.0	90.2	88.5	91.4	94.5	95.4
AM72	**apple**	96.6	96.6	98.2	**89.7**	**89.5**	**92.1**	**89.8**	89.7	91.8	**91.8**	95.4
AM75	**apple**	**96.8**	**96.9**	**98.7**	96.0	95.9	**96.8**	94.5	94.2	**94.6**	96.5	96.3
AM95	**apple**	95.5	95.5	97.9	90.6	90.4	93.1	90.0	88.6	91.8	93.8	**94.5**
AM125	**apple**	**96.8**	96.8	**97.8**	**96.1**	96.0	96.6	94.2	**94.5**	93.6	95.8	95.9

Note: [1]the accession numbers of RNA1, RNA2 and RNA3 of ApNMV isolates used in this study are Hai (MG924894, MG924897 and MG924900), Hua (MG924895, MG924898 and MG924901), Qu (MG924896, MG924899 and MG924902), P129 (LC108993 - LC108995), AM27 (KY808376 -KY808378), AM36 (KY808379 - KY808381), AM72 (KY808382 - KY808384), AM75 (KY808385 - KY808387), AM95 (KY808388 - KY808390) and AM125 (KY808391 - KY808393),

respectively; [2]nt represents the sequence alignment at nucleotide level; [3]aa represents the sequence alignment at amino acid level; The highest and the least were indicated in bold type.

3.4 Phylogenetic analysis of ApNMV from hawthorn trees with other isolates

Phylogenetic analysis of the ApNMV hawthorn isolate with others isolated from crabapple and apple trees was performed using the neighbor-joining method on the selected amino acid sequences of the MEL/HEL, POL, MP and CP genes. Results showed the ApNMV hawthorn isolate had a closer genetic relationship with P129 isolate based on the POL and CP while it seemed to be more closely related to AM75 and Qu isolates based on MET/HEL and MP (Fig. 4-3), consistent with phylogenetic relationship constructed based on the nucleotide sequences or using maximum likelihood method (data not shown). But no host- or geography-related conclusion could be drawn from the phylogenetic analysis.

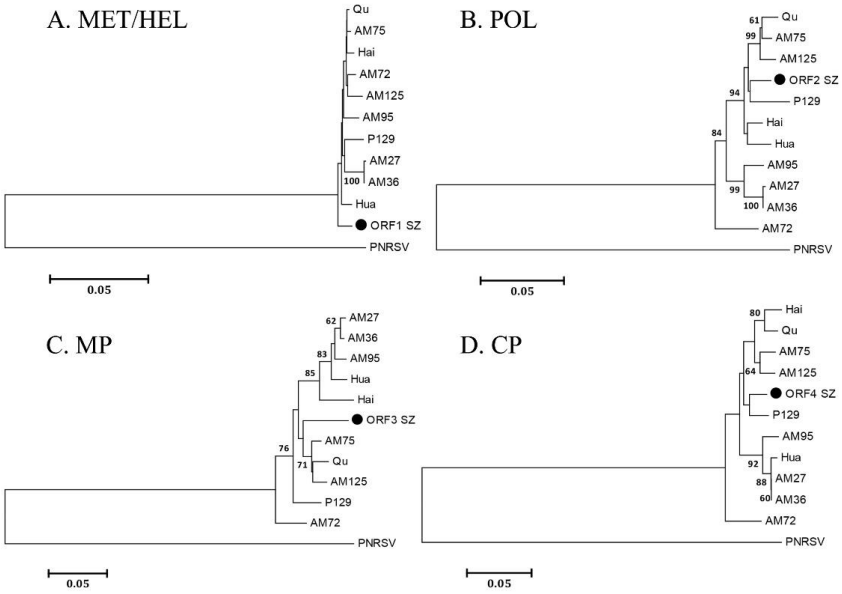

Figure 4-3 Phylogenetic relationship of the ApNMV hawthorn isolate with other isolates from apples and crabapples. The tree was constructed using the neighbor-joining method with 1000 bootstrap replications based on the amino acids of A, methyltransferase/NTP-binding helicase (MET/HEL); B, RNA polymerase (POL); C, movement protein (MP); and D, coat protein (CP). PNRSV (accession number: NC004362, NC004363 and NC004364) was chosen as an outgroup. Only bootstrap values $\geqslant$ 60% were shown.

3.5 Transmission test of ApNMV by grafting

Four one-year-old and one three-year-old apple plantlets, which had been confirmed as virus-free plantlets via tissue culture with meristem, were grafted using the

hawthorn buds infected by ApNMV. At 24 days post-grafting, new leaves on the top of the five tested apple plantlets were found to present symptoms of chlorosis, yellowing, mosaic, curling and necrosis (Fig.4-4 A). All of the five grafted plantlets were positive for ApNMV confirmed by RT-PCR (Fig.4-4 B). It suggested that ApNMV could be easily transmitted from hawthorn to apple by grafting.

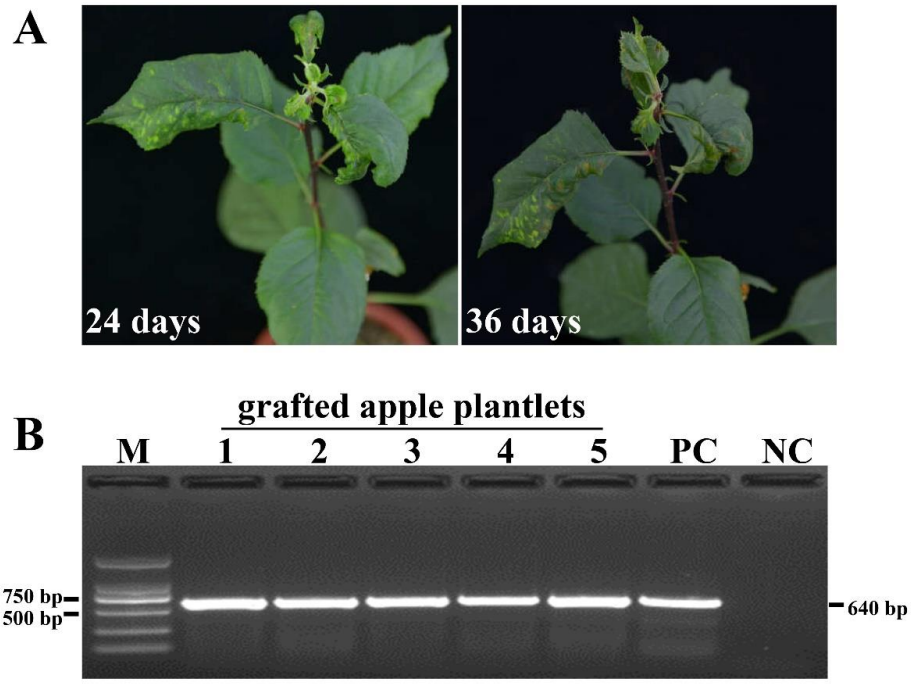

Figure 4-4 Transmission test of ApNMV by grafting. The new leaves on the top of apple plantlets presented obvious symptoms with chlorosis, yellowing, mosaic, curling and necrosis at 24, and 36 days post-grafting (A); and ApNMV infection was confirmed at 24 days post-grafting by RT-PCR (B). M: DNA marker 2000; PC: an apple tree infected by ApNMV as the positive control; NC: the double-distilled water as the negative control.

3.6 Occurrence and distribution of ApNMV and ASPV in hawthorn trees in China.

In the present study, we confirmed hawthorn trees as a new natural host for ApNMV. To understand the occurrence and distribution of ApNMV in the main hawthorn-producing provinces and regions in China, total 145 leaf samples were collected from provinces or regions of Hebei, Jilin, Liaoning, Shandong and Xinjiang. ApNMV was detected by RT-PCR and nested PCR, as described before (Xing et al. 2018). A total of 20 samples (13.8%) tested positive for ApNMV (Table 4-2). Six samples showing

typically mosaic symptoms were found infected by ApNMV, indicating a correlation with the presence of ApNMV and the mosaic symptoms on hawthorn trees. ApNMV also tested positive with a low percentage in asymptomatic (i.e., 5.9%, 4 out of 68, Fig.1 H) and other-symptomatic (i.e., 14.1%, 10 out of 71, Fig.4-1 E-G) hawthorn samples. In addition, ASPV was found positive with a low frequency [i.e., 2.1%, 3 (2 mosaic leaf samples from Xinjiang and 1 asymptomatic leaf sample from Shenyang) out of 145], suggesting a lower relationship with mosaic disease. Taken together, the data suggested that ApNMV might be the causative agent of the hawthorn mosaic disease other than ASPV.

Table 4-2 Geographical distribution and incidence of ApNMV from hawthorn trees in the major hawthorn-producing areas in China

Location	asymptomatic		typically mosaic		other symptoms	
	No. of samples	positive (rate %)	No. of samples	positive (rate %)	No. of samples	positive (rate %)
Hebei	6	1 (16.7)	2	2 (100)	28	7 (25)
Jilin	16	1 (6.3)	1	1 (100)	19	1 (5.3)
Liaoning	11	0 (0)	1	1 (100)	24	2 (8.3)
Shandong	20	0 (0)	-	-	-	-
Xinjiang	15	2 (13.3)	2	2 (100)	-	-
Total	68	4 (5.9)	6	6 (100)	71	10 (14.1)

Note: typically mosaic: the mosaic symptoms in hawthorn trees varied from mild, moderate to severe chlorosis, yellowing, pale yellow mosaic to bright cream-colored irregular spots, rings, or irregular bands and/or line patterns on hawthorn leaves; other symptoms included chlorosis, yellowing or necrosis on the leaf margins, which might be caused by low temperature, bacterial or fungal diseases and were significantly different from the typically mosaic.

4 Discussion

The viral diseases of hawthorns are not well characterized (Wang and Dai, 2014; Guo et al. 2016), most probably due to its lower economic importance compared to apple or pear. In a previous study, we identified the association between ApNMV presence and apple mosaic disease (Xing et al. 2018). In the present study, we collected hawthorn leaves with mosaic symptoms similar to those observed on apple trees. By HTS, ApNMV was detected for the first time on hawthorns and might be the causing pathogen of hawthorn mosaic disease. The results, confirmed by RT-PCR, demonstrate that hawthorn is a new natural host for ApNMV. Nevertheless, to further confirm ApNMV as the causal agent of hawthorn mosaic disease, inoculation of virus-free hawthorn plantlets with infectious clone of ApNMV hawthorn isolate to display the resulting mosaic symptom is necessary in future.

The complete genome of ApNMV isolate from a hawthorn tree was cloned and

sequenced. Multiple sequence alignment and phylogenetic analysis demonstrated the genome of ApNMV isolated from the hawthorn had a relatively high sequence identity to those from apple and crabapple trees at both nucleotide and amino acid levels. However, two hypervariable regions were found at N- and C-terminals in the POL protein encoded by RNA2, respectively. It is possible the hypervariable regions are associated with adaptability to the different hosts but this hypothesis still remains to be further studied. Simultaneously, the high sequence similarity among the isolates might support transmission of ApNMV between hawthorn and apple trees. Our grafting test proved that ApNMV could be easily transmitted from hawthorn trees to apple trees and then caused severe mosaic, distortions and necrosis on apple trees.

In addition, geographical distribution and incidence of ApNMV was also conducted from hawthorn trees in some major hawthorn-producing areas in China. The presence of ApNMV in all six samples with mosaic symptoms indicated that ApNMV was associated with hawthorn mosaic disease. ApNMV was also detected in a low incidence rate of the hawthorn trees with other symptoms (14.1%) or without symptoms (5.9%). As for other fruit tree viruses (Pallás et al. 2012), it might suggest that symptoms development is a complex phenomenon influenced by the cultivar, the growth vigor, the season (temperature, light intensity), the virus isolate and titer, etc. This is consistent with our survey that only 6 out of 11,685 were observed with mosaic symptoms on hawthorn trees. These results indicated ApNMV might not be widespread on hawthorn trees in China. It might be related to low growing areas and low frequency of resource exchange in hawthorn industry. Indeed, ApNMV infection does not seem to spread so much in the hawthorn population. However, the hawthorn planting areas are usually close to the apple-planting areas in northern China according to our surveys, so there is a non-negligible risk to transmit ApNMV from hawthorn to apple trees when using the same pruning tools. In this situation, it will be a potential threat to affecting the economic cultivation of apples.

In our study, ASPV, a latent virus in most commercial cultivars (Jelkmann and Paunovic, 2011), was also identified and confirmed using HTS together with RT-PCR. However, only 3 (2 mosaic and 1 asymptomatic) out of 145 leaf samples were tested positive for ASPV, indicating a lower incidence and lower association with mosaic disease. Although apple stem pitting disease (ASPD) was reported in Crataegus monogyna about 40 years ago when the causal agent of ASPD was not yet identified as ASPV (Sutic et al. 1999; Sweet and Campbell, 1976), our results provide, for the first time, a definite evidence that hawthorn trees could be infected with ASPV in natural condition.

In summary, a new natural host, hawthorn, has been discovered for ApNMV, highly associated with hawthorn mosaic disease. High genomic similarity of the identified genomes with ApNMV genomes from apple and apple crab, suggested potential transmission of ApNMV among hawthorns and apples although this hypothesis warrants further investigation. However, ApNMV was detected with a low incidence from hawthorn trees in the major hawthorn-producing areas in China. This was the first report of ApNMV from hawthorn trees. It will be helpful to develop the diagnostic protocol and disease-control measures of ApNMV in hawthorn trees and

lay a foundation for the in-depth unraveling of the molecular mechanism of mosaic disease caused by ApNMV.

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Supplementary Materials

Table S4-1 The primers used in this study

Primer	Sequence (5′- 3′)	Reference
ApnS1	GTTTTATTTTCGTGGTTGA	Xing et al. 2018
fullRNA1-R1	TGCTCTAGAGCTTCCCTAACGGGGCATCCAAT	This study
RNA3-mcF	GTCGAAGTACTTTTCTGCGAAATG	This study
5R1GSP1	ATCGGAAGGTCACTCGTA	Xing et al. 2018
5R1GSP2	AAGTGGACAACACGAGTG	Xing et al. 2018
5R2GSP1	ACAATCTCCTTCACCAACAT	Xing et al. 2018
5R2GSP2	GTTGTTGGTAGTGGTGTCA	Xing et al. 2018
5R3GSP1	CGGCTTCTTGATATAAGTCATC	Xing et al. 2018
5R3GSP2	ATCCTGTCCGCTACCTTG	Xing et al. 2018
3R1GSP1	CCAATAGAGTGATGCGAAGT	Xing et al. 2018
3RACE-R2-Ft	GCTTGGTATGAATCCTGGTT	This study
3ApN-RACE2	GCACATAGCAACAGAGAAGT	This study
ApNMV-F	ATGGTGTGCAATCGCTGTCA	Noda et al. 2017
ApNMV-R	CATCGACCATAAGGATATCA	Noda et al. 2017
ApN-F2	AATCGCTGTCATCACACTCAC	Xing et al. 2018
ApN-R2	CCAACCATTGCCAACCTCTC	Xing et al. 2018
szASPV-F	CGATATTATAGACCTGCTTCCT	This study
szASPV-R	TCACTCATCATCTCCTCATTG	This study
szASPV-F2	CAGCAGAGGACTGGTTCAA	This study
szASPV-R2	ATCTCTTCTTCACAGCCATTAG	This study

4. RNA-seq reveals hawthorn tree as a new natural host for apple necrotic mosaic virus, possibly associated with hawthorn mosaic disease

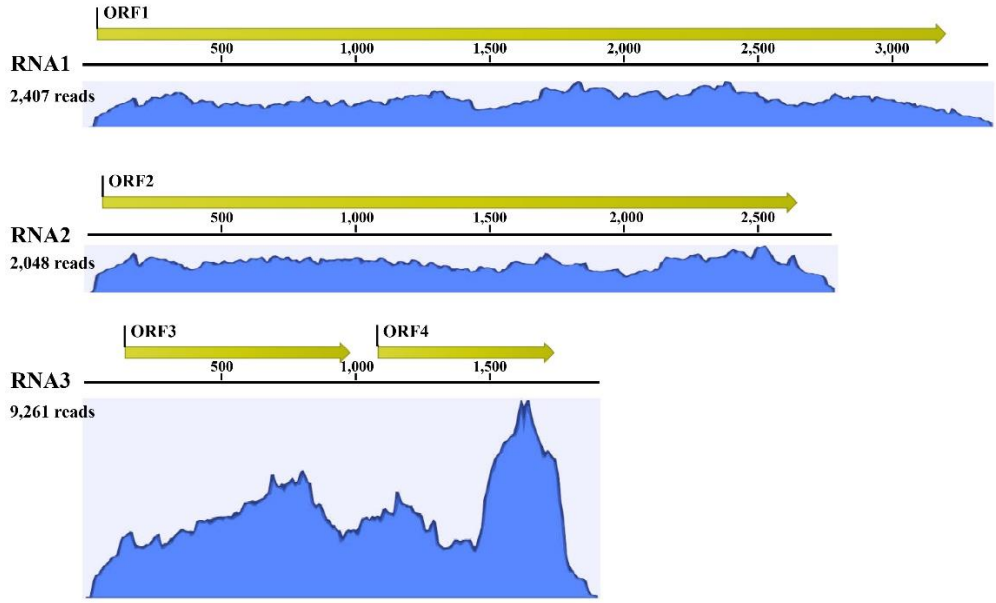

Figure S4-1 The mapping scheme of ApNMV genome (RNA1, RNA2 and RNA3) and reads obtained by RNA-seq in the hawthorn tree showing mosaic symptom.

5

Conclusion and future prospects

Conclusion

Fruit trees are high-value crops which viruses or viroids can infect. These pathogens tend to accumulate due to the vegetative propagation of fruit trees and grafting. Some of these pathogens cause severe crop losses and often reduce the productive life of the orchards. Therefore, the detection and identification of plant viruses are paramount for successfully managing a viral disease. Nevertheless, identifying these pathogens has been problematic due to their high genome variability. The advent of HTS technologies during the last years has dramatically changed research on viral and virus-like pathogens. These technologies have accelerated the identification of a distant variant of known viral species, unknown viruses or new host plants for known viral species. However, it also raises the critical question: can biological characterization of the new viruses follow the current pace of virus discovery? Indeed, it is a complex and lengthy process compared to applying HTS technologies on plant samples. So the first objective of this thesis is to critically analyze which biological information accompanied the publication of the genome sequence of a new fruit tree virus and, further on, what the scientific community carries out the downstream studies.

Retrospective analysis for new fruit tree viruses

The original and citing publications on 84 newly identified viral species in fruit tree samples since 2011 were reviewed. This review focused on the biological information accompanying the publication of the genome sequence of a new fruit tree virus and complementary reports on biological information carried out by the scientific community after the first publication. A published framework for the biological characterization of new viruses was used as the backbone reference of our analysis (Massart et al., 2017).

Key trends were revealed. First, two categories of information are always or nearly always provided: complete or nearly complete genome, confirmation of detection by an independent technique, usually by (RT)-PCR using specific primers. Second, an epidemiological survey is carried out in more than two-thirds of the publications. Third, biological characterization experiments (transmission, host range and symptom) were only selectively carried out in the publications. Importantly, information on the biological characterization should have been brought up for 20% of the new species. In addition, the analysis of citing publications showed that biological characterization is rarely completed after the first publication, except for identifying a new host or country.

According to the current situation with fruit tree viruses, exhaustive knowledge on the aetiology of a disease or the epidemiology of a virus is complicated and time-consuming to achieve. Taking into account this constraint and the review carried out, it should be recommended to accompany the publication of the genome of a new fruit tree virus with the following information: (1) confirmation of the detection by an independent technique, taxonomic assignation, and sample documentation, (2) development of a diagnostic test and prevalence study locally (i.e. at the place where

the new virus has been identified or on the other plants of the intercept batch). These steps could become mandatory before publication.

Finally, the analysis of symptoms could include biological experiments in controlled conditions and association studies from field surveys. An epidemiological survey is strongly recommended since it will provide handy information with minimal resources and time compared to biological experiments. Another essential element must be addressed so far is scanning public databases of HTS raw data, as the virus could have been sequenced previously without being noticed. This task was very complex until Serratus was published in 2022, greatly facilitating such operation (Edgar et al., 2022). This can quickly give valuable information on the virus's host range and geographical spread, enabling better risk evaluation and limiting unnecessary regulatory action risks.

Identification of a new host for the apple necrotic mosaic virus

A novel *ilarvirus*, apple necrotic mosaic virus (ApNMV), was discovered by HTS technologies from apple leaves presenting mosaic disease in Japan. Epidemiological analyses showed a low prevalence in Japan, contrasting with the higher prevalence observed on apple trees in China (Cho et al., 2017; Xing et al., 2018a).

The suggested framework for efficient characterization of new phytoviruses (Massart et al., 2017) that was used as a backbone of the review analysis has been well fulfilled in the frame of this thesis for this virus. Its application was triggered by identifying ApNMV on a new natural host, hawthorn:

1.HTS technologies analyzed a hawthorn tree with mosaic symptoms in China, and ApNMV was detected.

2.Its genome was reconstructed, and RT-PCR further confirmed its presence.

3.Biological assays using grafting were carried out. These tests demonstrated that ApNMV was easily transmissible from hawthorns to apple trees.

4.According to the symptoms observed during grafting experiments, an epidemiological survey was carried out in main producing areas in China.

5.It revealed a wide dispersion of the virus and its association with mosaic symptoms on hawthorn.

Perspective

Identifying novel viruses was one of the first areas of plant health impacted by applying HTS technologies. A decade after the first discovered of a phytovirus by HTS technologies, tens of virus/viroid species have been identified from fruit trees. In our review of original and citing publications on newly identified new viral species affection fruit trees since 2011, epidemiology and disease aetiology studies were identified as clear bottlenecks for evaluating the biological significance and phytosanitary impact of novel viruses or complex of different viruses. This thesis allowed us to propose minimal steps for publishing a new virus.

Minimal steps of characterizing new viruses are recommended while applying HTS

High throughput sequencing technologies have shown great potential and are becoming a powerful tool that enables the simultaneous detection and potential identification of any organisms present in a sample. They have been applied in plant health, especially in plant pest routine diagnostics(Lebas et al., 2022; Massart et al., 2022) . However, HTS analysis provides only sequence data. It does not provide biological observations. Thus, it is not likely to completely replace molecular detection, such as PCR, to confirm detection and further biological characterization (pathogenicity, hosts, transmission, epidemiology...). Nevertheless, the massive application of HTS technologies has highlighted the increasing importance of understanding the biology of any new viral sequence for providing a basis for risk assessment and scientifically-based decisions.

The minimal steps for characterizing new viruses should include the confirmation of detection and test for infection activity. First is the ensure the reliability of detection, sample collection, and molecular detection should be done, generally for polymerase chain reaction (PCR). Then bioassay for the infection activity, fulfilling Koch's postulate, can be considered a cornerstone and is pivotal in biological characterization. Finally, further epidemiological surveys should be undertaken based on the diagnostic technique(s) developed for the new agent, which can be undertaken to evaluate its prevalence and distribution within a given ecosystem.

Grafting and epidemiological surveys are suggested to be used in the biological characterization of new viruses

The most difficult identified biological characteristic is symptoms for the fruit trees virology, since the occurrence of symptomless infection and the impact of the cultivar on the symptom development. Frequently symptomless infections or fluctuation of symptom intensity during the season and on different cultivars challenged further proved whether the pathogen can induce the disease when introduced to a previously unaffected host since it usually takes decades to characterize fruit trees ((Maliogka et al., 2018). Therefore, epidemiological surveys and grafting experiments were suggested to be used while biological characteristics of the new virus were applied with HTS. In the case of ApNMV, it could easily transmit from hawthorn to apple by grafting and cause severe mosaic, distortions and necrosis on apple trees. In addition, geographical distribution and incidence of ApNMV in some major hawthorn-producing areas in China proved it is possibly associated with hawthorn mosaic disease.

Grafting is a powerful and efficient tool to study symptoms and host range : The bioassay is the basis for any biological characterization. In bioassays, the detected virus can be inoculated to the test plants through grafting, mechanical rubbing of leaves, or the cutting or slashing of stems in laboratory/greenhouse conditions. Through grafting, the virus can be inoculated into its indicator plants, host plant candidates, or other cultivars of the same species. It is simpler and faster to prepare when a virus causes characteristic symptoms in a specific indicator or one of its host plants. In our review, 36% of new species tested their ability to infectivity. All the infectivity bioassays experiments have positive RT-PCR detection results, while 11% failed to inoculate onto the herbaceous indicators. Symptoms have been

observed in 19% of viral species, including symptoms observed on both grafted host plants and herbaceous indicators. Since the symptomatology is the most difficult for the fruit trees virology, grafting can be a powerful and efficient tool to study symptoms and host range. Although creating infectious clones requires time and resources, it is essential to note that this method is needed to verify the infectivity and biological properties of a new viroid (Owens et al).

An epidemiological survey is considered an essential step : An epidemiological survey was highlighted as an important biological characterization step for it will provide helpful information with minimal resources and time compared to biological experiments. However, the survey's low incidence or absence of virus impeded any association based on a sufficient number of samples that had happened in 8% of species. Therefore, the epidemiological survey should include healthy and symptomatic trees presenting the same symptoms as the original tree. In addition, testing other viruses during the survey is recommended, provided there is a risk of co-infection that could puzzle the analysis of the results. In addition, it can identify new hosts (Basso et al., 2015a) and study the association of the virus with symptoms (Fox, 2020) .

Suggestion for cooperation between different research groups

Besides the recovery of the complete genome, the biological characterizations, such as the incidence, vector (or other transmission methods), host range and symptoms, of the newly identified viruses need to be carried out to evaluate its agronomical, commercial, biosecurity impacts. A much richer biological characterization could be achieved when a virus is detected by several groups from different host plants presenting diverse symptoms in different countries using multiple tests and sequencing strategies. For example, the case of ApNMV was first isolated from apple leaves infected with the mosaic disease in Japan, but it occurs much less frequently in Japan (Noda et al., 2017). On the contrary, ApNMV is widespread in apple-producing areas in China through material sharing and collaborative research between scientists. Later epidemiology survey further proved that it is highly related to apple mosaic disease in China (Xing et al., 2018a).

An neglected important element is the availability of the HTS raw data supporting the virus discovery in public databases to allow the further use of these data by the scientific community. Since a total of 79 scientific peer-review publications, only one publication (Jo et al., 2018) made the raw sequencing data available with the link in the publication while using high throughput sequencing. HTS data sharing allowed for a better understanding of the geographical spread of a new closterovirus (Koloniuk et al., 2018b). The pre-publication data sharing could also include biological experiments, as was shown for the European wheat striate mosaic virus (Sõmera et al., 2020). The lack of resources can be partially solved by improving pre-publication data sharing between research groups. Such cooperation is also optimizing the use of resources when the data are shared before publication. Such pre-publication data sharing allows sharing the burden of biological experiment between groups and leverage the useful information provided in the publication reporting the discovery or the characterization of a new plant virus. For the examples of data sharing have been

done with tomato and wheat (Sõmera et al., 2021; Temple et al., 2022).

The using of HTS will continue to rise, which will trigger the discovery of new viruses and complexifies still more the prioritization for biological confirmation. The publication of Serratus in 2022 can quickly give valuable information on the virus's host range and geographical spread (Edgar et al., 2022). It will expand the known sequence diversity of viruses and enable better risk evaluation and the anticipation and mitigation of future pandemics. To run Serratus, a target list of SRA run accessions is required. SRA mining through Serratus will give a handy information window on hidden viruses in the public database in the near 3 to 5 years.

Our suggested steps for the biological characterization of new plant viruses are essential for efficient biological characterization when a previously known or a new plant virus is identified, understanding the epidemiology of diseases, and enabling effective management decisions and risk assessment. The primary consideration of experimental demonstration of downstream analyses, and improved collaboration between scientists, including data sharing and will improve the situation and manage the potential risk more efficiently. Ultimately, we will have a global knowledge of the fruit tree virome and the virus's geographical spread and host range.

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Appendix – Publications

1. **Wanying Hou**, Shifang Li, Sebastien Massart. (2020) Is there a "biological desert" with the discovery of new plant viruses? A retrospective analysis for new fruit tree viruses. *Frontiers in Microbiology*, 11,1-15. doi: 10.3389/fmicb.2020.592816

2. Fei Xing [#], **Wanying Hou**[#], Sebastien Massart, Dehang Gao, Wenhui Li, Mengji Cao, Zhixiang Zhang, Hongqing Wang, Shifang Li.(2020). RNA-seq reveals hawthorn tree as a new natural host for apple necrotic mosaic virus, possibly associated with hawthorn mosaic disease. Plant Disease, Published Online:7 Apr 2020, doi:org/10.1094/PDIS-11-19-2455-RE

3. Dehang Gao, Chengyong He, Fei Xing, **Wanying Hou**, Shifang Li, Binhui Zhan, Hongqing Wang. (2020) Identification and sequence analysis of apple stem pox virus from hawthorn tree (*Crataegus* spp.). Acta Phytopathologica Sinica. doi:10.13926/j. cnki. apps.000551.

4. Jun Zhou, **Wanying Hou**, Zhixiang Zhang,Chenan Wang,Linnan Jie,Hongqing Wang, Shifang Li. (2019) First report of peach calico (PC) isolate of peach latent mosaic viroid from peach trees in China. *Plant Disease*,103(10)

5. Zhixiang Zhang, Akito Taneda, Changjian Xia, Takahiro Matsuda, Fumiko Murosaki, **Wanying Hou**, Robert Owens, Shifang Li1, Teruo Sano. (2020). Effects of host-adaptive mutations on hop stunt viroid pathogenicity and small RNA biogenesis. *Frontiers in Microbiology* (submitted)

6. Changjian Xia, Shifang Li, **Wanying Hou**, Zaifeng Fan, Hong Xiao, Meiguang Lu, Teruo Sano, Zhixiang Zhang. (2017). Global transcriptomic changes induced by infection of cucumber (*Cucumis sativus L*) with mild and severe variants of hop stunt viroid. *Frontiers in Microbiology*,8,1-16.