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Viral Diversity in Mixed Tree Fruit Production Systems Determined through Bee-Mediated Pollen Collection

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Abstract: Commercially cultivated *Prunus* species are commonly grown in adjacent or mixed orchards and can be infected with unique or commonly shared viruses. Apple (*Malus domestica*), another member of the *Rosacea* and distantly related to *Prunus*, can share the same growing regions and common pathogens. Pollen can be a major route for virus transmission, and analysis of the pollen virome in tree fruit orchards can provide insights into these virus pathogen complexes from mixed production sites. Commercial honey bee (*Apis mellifera*) pollination is essential for improved fruit sets and yields in tree fruit production systems. To better understand the pollen-associated virome in tree fruits, metagenomics-based detection of plant viruses was employed on bee and pollen samples collected at four time points during the peak bloom period of apricot, cherry, peach, and apple trees at one orchard site. Twenty-one unique viruses were detected in samples collected during tree fruit blooms, including prune dwarf virus (PDV) and prunus necrotic ringspot virus (PNRSV) (Genus *Ilarvirus*, family *Bromoviridae*), *Secoviridae* family members tomato ringspot virus (genus *Nepovirus*), tobacco ringspot virus (genus *Nepovirus*), prunus virus F (genus *Fabavirus*), and *Betaflexiviridae* family member cherry virus A (CVA; genus *Capillovirus*). Viruses were also identified in composite leaf and flower samples to compare the pollen virome with the virome associated with vegetative tissues. At all four time points, a greater diversity of viruses was detected in the bee and pollen samples. Finally, the nucleotide sequence diversity of the coat protein regions of CVA, PDV, and PNRSV was profiled from this site, demonstrating a wide range of sequence diversity in pollen samples from this site. These results demonstrate the benefits of area-wide monitoring through bee pollination activities and provide new insights into the diversity of viruses in tree fruit pollination ecosystems.

Keywords: metagenomic; virus; pollen transmission; *Prunus*; honey bee; apricot; cherry; peach; apple; virus diversity

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1. Introduction

The *Rosaceae* family contains over 100 genera and 3000 individual species, including many important fruit tree crops [1]. *Prunus* species including apricots (*Prunus armeniaca*), sweet cherry [*Prunus avium* (L.)], and peach (*Prunus persica*), are often grown in close proximity in commercial farming operations. Apples (*Malus domestica*) are commonly

grown near *Prunus* orchards but have a wider habitat and production regions than *Prunus* spp. Tree fruit production is a major sector in Canada, with apples accounting for 20.1% of total fruit farm gate values, followed by sweet cherries (5.7%), peaches (3.4%), and apricots (0.2%), together accounting for over 350 million Canadian (CDN) dollars in farm gate values across Canada in 2021 [2]. Viruses are a major threat to tree fruit production and can result in severe losses [3]. The diversity, distribution, transmission, and host range of many of these viruses are poorly understood, especially at a whole-farm ecosystem level [4,5]. Effective monitoring approaches are required to fully appreciate the distribution of viruses in these systems, and the potential for virus transmission between tree fruit species and varieties.

Over 163 viruses have been reported to infect major woody fruit trees, of which at least six infect both *Prunus* species and apples, including apple mosaic virus (ApMV, genus *Ilarvirus*), apple chlorotic leaf spot virus (ACLSV; genus *Trichovirus*), cherry leaf roll virus (CLRV, genus *Nepovirus*), tomato ringspot virus (ToRSV, genus *Nepovirus*), and prunus necrotic ringspot virus (PNRSV, genus *Ilarvirus*) [3,6–10]. Common viruses of *Prunus* trees include prune dwarf virus (PDV; genus *Ilarvirus*) and cherry virus A (CVA; genus *Capillovirus*), which are not known to infect apples [11,12]. Other viruses have a more restricted *Prunus* host range, such as nectarine stem pitting virus (NSPaV, genus *Luteovirus*), which has only been reported in peach and nectarine [13,14]. At least twenty-one viruses are known to infect apple trees, including apple stem grooving virus (ASGV, genus *Capillovirus*), ACLSV, and apple stem pitting virus (ASPV, genus *Foveavirus*) [3]. Citrus concave gum-associated virus (CCGaV; genus *Phlebovirus*) is a recently described virus infecting apples and citrus but is not known to infect *Prunus* [3,10,15]. Viral host ranges can be quite complex and are often not well defined [16,17].

Transmission pathways can impose restraints on plant virus evolution and play a role in determining the natural host range [17–20]. Plant viruses can be transmitted mechanically, through vectors, seeds, or vegetative propagation. Pollen is another major route of transmission in tree fruits for some viruses, both horizontal and vertical [21,22]. Of the ~46 known pollen-transmitted viruses, only 18 are horizontally transmitted, suggesting different mechanisms and restrictions to pollen-mediated virus transmission [21,22]. Viruses can also have direct negative effects on flowering and fruit production. PNRSV, for example, has been observed on the surface and within apricot pollen grains and can negatively affect pollen germination [23,24]. In some cases, pollen germination and stigma penetration are essential for horizontal transmission, as demonstrated for raspberry bushy dwarf virus [25]. Other viruses can be potentially transmitted by pollinators like bees during foraging activities [26,27]. However, the relationship between the pollen virome and the orchard-wide plant virome is poorly understood [5,28,29]. Fruit tree orchards can be complex mixtures of closely related fruit tree species infected by multiple virus species with overlapping viral host ranges, but horizontal transmission could be restrained through pollen–pistil compatibility factors.

Some tree fruit species require pollen donors for successful fruit production. While peaches and many apricot varieties are self-compatible, many cherry varieties are self-incompatible, further complicating aspects of pollen transmission of viruses [30,31]. The risk of virus transmission between *Prunus* species through pollen is generally regarded to be low, while transmission between *Prunus* and *Malus* species would be even less likely. In addition to pollen–pistil compatibility, *Prunus* species and varieties do not necessarily flower at the same time, creating additional temporal barriers to pollen-associated virus transmission. Commercial honey bee (*Apis mellifera*) pollination services are widely used in tree fruit production systems to improve fruit set and fruit quality [32]. Honey bees forage intensively within a 1.5 km radius of their hives in agricultural settings, depending on flower availability [33,34]. Bees can also passively transmit plant viruses associated with pollen or through direct contact with flowers [21,26–28].

Bees and pollen can be useful in monitoring agricultural systems for pathogen presence or ecosystem health [4,5,35–37]. Foraging honey bees are potentially carrying pathogen-contaminated pollen and nectar, which could provide information on the presence of these pathogens in nearby flowering plants [5,36,37]. Here, we take advantage of bee-based area-wide metagenomics monitoring approaches to better understand the viral species and variant diversity in tree fruit production systems, with a specific focus on apricots, cherries, peaches, and apples.

2. Materials and Methods

2.1. Farm Site and Sampling Details

A long-established research farm containing multiple different tree fruit species in Jordan, Ontario (Jordan Farm) was selected as a study site because of the diversity and ages of tree fruit crops present. Three honey bee colonies were placed at various locations on the farm during the tree fruit bloom period in spring 2020, with each colony sampled independently at each time point, representing biological replicates for the site (Figure 1A). Three types of pooled samples were collected, including ~25 forager bees (collected outside of colonies returning from foraging trips, with visible signs of pollen on their corbiculae), ~25 hive bees (collected from the brood nest of each hive, with no visible signs of pollen), and ~5 mL of pollen collected using pollen traps (ApiHex, Guelph, Canada) installed on the front of each hive (Figure 1B–D). Samples were collected at four time points during the spring of 2020, corresponding to the peak bloom levels for apricots (23 April), cherries (4 May), peaches (12 May), and apples (26 May) (Figure 1E). In addition, two plant sample replicates consisting of leaf and flower tissue were collected from 10 random individuals located near the bee colony. One plot was sampled for leaf/flower tissue from each representative crop species examined (apricot, cherry, peach, and apple; Figure 1A). The most prominent tree fruit species grown on Jordan Farm at the time of sampling were peaches and apples, followed by cherries and apricots (Figure 1A; Table 1). Trees were of mixed age, variety, and rootstock combinations (Table 1). One plot each of nectarines (*Prunus persica v. nucipersica*) and plums (*Prunus domestica*) were also grown on-site and in flower during the sample collection period but were not analysed as a part of this study. Other crops grown at this site included garlic (*Allium sativum*), strawberries (*Fragaria x ananassa*), grapes (*Vitis vinifera*), and hops (*Humulus lupulus*) but were not flowering during the sample collection period.

Table 1. Summary of varieties, rootstocks, total number, and age of *Prunus* and *Malus* trees grown on Jordan Farm.

Crop	Number of Plots	Total Area (ha)	Variety	Rootstock	Total Number of Trees	Age of Trees (Years)
Apricot	1	0.57	Haroblush, Harlayne	Krymsk 1, Krymsk 86, Krymsk 99	208	2
Cherry	3	0.96	Vista, Vogue, Hedelfingen, Tehranivee, Stella, Vandalay, Montmorency	Mazzard, Mahaleb, Gisela 5	317	14–50
Peach	10	3.849	Redhaven, Vivid, Nectarine, Harrow Diamond, Cresthaven	Halford, Bailey, TLC, Bailey Field	2832	2–17
Apple	11	3.83	Brookfield Gala, Empire, Red Fuji, Ambrosia, Honey Crisp, Silken, Morspur McIntosh, Golden Delicious, Royal Gala, Courtland, Delicious Red Chief, McIntosh Marshall, Jonagold, McIntosh	G41, M26, BUD 9, M9, Mark	3123	3–26

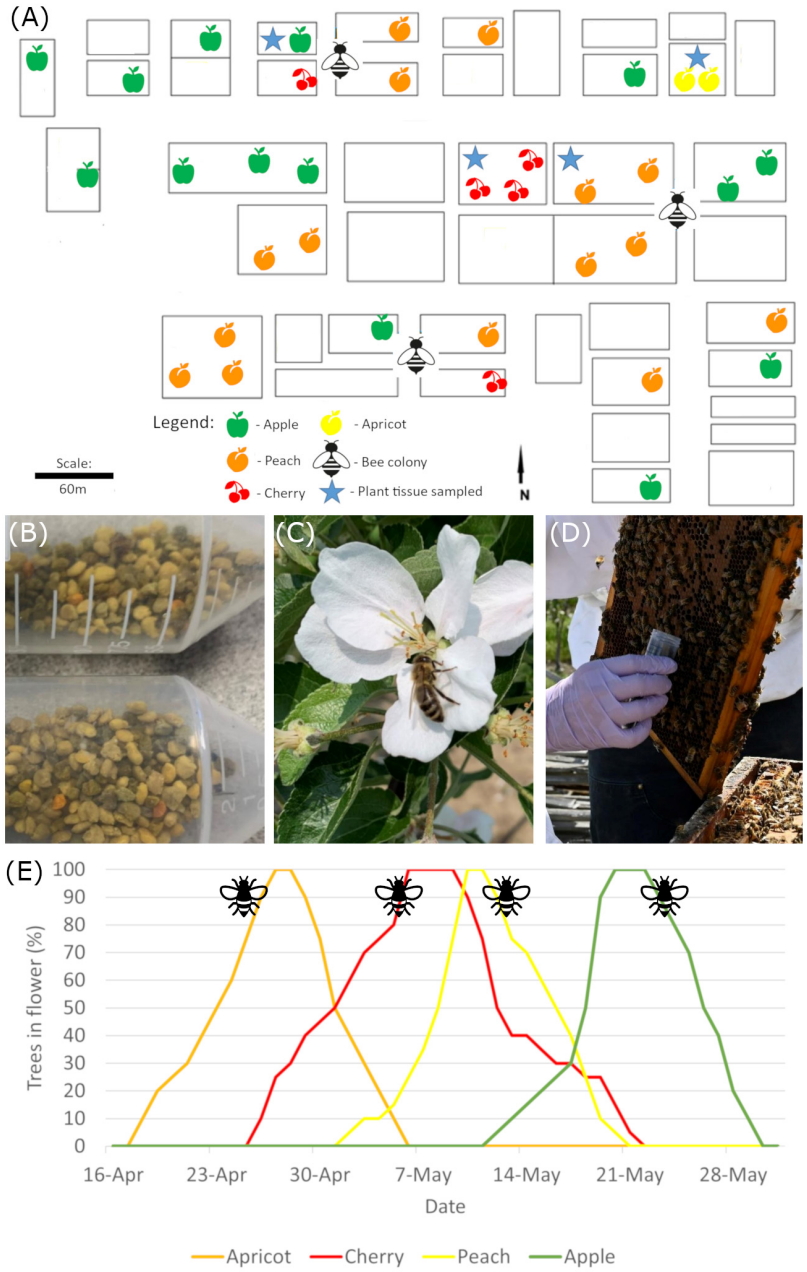

Figure 1. Map diagram of tree fruits grown on AAFC Jordan Farm and sampling details. (**A**) Map diagram of AAFC Jordan Farm. Honey bee icons indicate the location of colonies used for sampling. Fruit diagrams indicate the location of plots used to grow apricots (yellow), cherries (red), peaches (orange), and apples (green). Blue stars indicate which plots were sampled for leaf and flower tissue. (**B**) Collected pollen. (**C**) Forager bee on an apple flower. (**D**) Hive bees collected from inside the colony. (**E**) Approximate bloom levels of apricot, cherry, peach, and apple trees over time. Bee icons indicate approximate sampling times.

2.2. RNA Extraction and Sequencing

Sample processing, extraction, and sequencing were performed as in Smadi et al., 2024 [5,36]. Briefly, total RNA (totRNA) was extracted using the spectrum total plant RNA extraction kit (Sigma Aldrich, ON, Canada), while dsRNA was extracted from the composite plant samples following Kesanakurti et al. (2016) [12]. Extracted totRNA was treated with a rRNA depletion step using the RiboMinus^{TM} Plant Kit for RNA-Seq (Invitrogen, Waltham, MA, USA) as per the manufacturer's instructions. Ribo-depleted totRNA and dsRNA HTS libraries were generated using the Illumina TruSeq Stranded mRNA Library Prep kit,

following the manufacturer's protocol, starting after the mRNA selection steps [12]. The libraries were dual indexed using the IDT for Illumina TruSeq RNA UD Indexes (Illumina, San Diego, CA, USA), normalised and sequenced using a NextSeq500 high output kit v2.5, 75 cycles (Illumina), which generated between 10 and 16 million reads, on average for each sample. The DsRNA samples were also sequenced using a NextSeq500, but with approximately four to six million reads on average for each sample. The RNAseq files were uploaded to the Sequence Read Archive (SRA) under bioproject ID PRJNA1025014, and individual virus CP sequences were uploaded to GenBank with accession numbers PP930272-PP930329 (Supplemental Table S1; Supplemental File S1).

2.3. Bioinformatics and Phylogenetics

The HTS sample files were imported into Virtool (www.virtool.ca; accessed on 20 September 2021) for sample management, quality control (QC), and data analysis. Reads passing QC using FASTQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/, accessed on 20 September 2021) were mapped to the plant virus databases that were updated in December 2021 using the Pathoscope 2 pipeline [38]. The reads were aligned to the representative isolates of all known plant viruses pulled from GenBank on 20 September 2021. Viruses with a representative isolate receiving at least one mapped read then had their reads mapped against all of their known isolates. Bowtie2 2.3.2 [39] was used in the local mode for both rounds of read mapping with the minimum score (–min-score) set to "L, 20, 1.0", seed length (-L) to 15, and mismatches per seed (-N) set to 0. In the second round, the maximum number of alignments returned (-k) was set to 100. The reads matching the viruses were also mapped to a host reference genome. Except for a 0 value for mismatches per seed (-N), default Bowtie2 parameters were used for mapping. Reads were eliminated from the analysis if they had a greater or equal alignment score to the host versus the virus. Multi-mapping reads were handled using a refactored derivative of the Pathoscope2 identification module, which exactly matches the output of the published module. The read values were fractionally reassigned from the least likely source genomes to the most likely [40]. Virus identification, based on Virtool, was used to create sample-specific pathogen profiles. A minimum of 10% genome coverage was required for a virus species to be considered a positive detection from both the totRNA and dsRNA samples. Sample profiles were combined to create a site-specific profile, which included calculating the average frequency of detection for each sample type, the average frequency of detection across all samples, average genome coverage, and viral reads per million (VRPM) for each virus detected across all samples (Supplemental File S1). VRPM is similar to transcripts per million and was calculated from the total number of reads mapped to each individual virus, divided by the genome length of the virus in kilobase pairs (Kbps), and then normalised for the total number of reads in the sequencing run, per million. Due to inconsistencies in viroid detections, the read counts and VRPM were manually annotated using Geneious Prime, version 11.0.14.1 (Biomatters Inc., Auckland, New Zealand).

Using host genome-subtracted de novo assembled contigs for each sample, sequences were aligned to the PDV, PNRSV, and CVA reference sequences using Geneious Prime (Supplemental Table S1). Samples with full coat protein nucleotide sequence coverage were used to create a consensus sequence for pairwise and phylogenetic analyses, along with the GenBank reference sequences. Pairwise nucleotide distance comparisons were constructed using Geneious Prime. Maximum likelihood phylogenetic trees were constructed using MEGA 11 with 1000 bootstrap replications [41].

3. Results

3.1. Virus Detection during Tree Fruit Bloom

Viral profiles from each sample were used to calculate the average frequency of detection in each sample type replicated at each time point. The total average genome coverage and average VRPM across all samples were also calculated (Table 2; Supplemental File S1). A total of 21 virus species were identified from the bee and pollen samples: PDV

was most commonly detected (64%), followed by CVA (58%), PNRSV (58%), prunus virus F (44%; PVF; genus *Fabavirus*), ToRSV (44%), and tobacco ringspot virus (44%; TRSV; genus *Nepovirus*) (Table 2). The *Ilarvirus* genus was best represented, including PDV, PNRSV, apple mosaic virus (11%), blackberry chlorotic ringspot virus (8%), tobacco streak virus (8%), and tomato necrotic shock virus (ToNSV; 6%; Table 2). The six most frequently detected viruses (PDV, CVA, PNRSV, PVF, TRSV, and ToRSV) were detected at all four sampling time points and in all sample types (Figure 2). CVA had the highest average genome coverage (70%), while PDV had the highest normalised read counts (VRPM; Table 2).

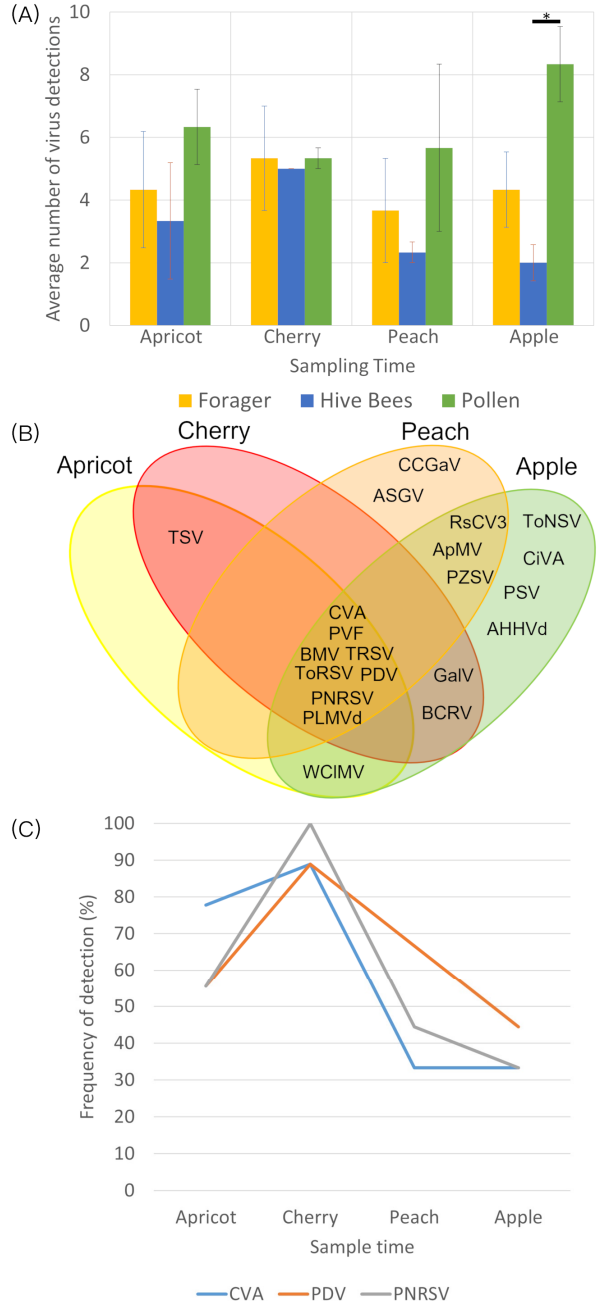

Figure 2. Virus species detection frequency and diversity. (**A**) Average number of virus species detected in forager, hive bees, or pollen samples collected during apricot, cherry, peach, and apple blooms. Error bars indicate standard error. The asterisk denotes a significant difference (one-way ANOVA, Tukey-HSD, $p < 0.05$). (**B**) Venn diagram of virus species detected during apricot, cherry, peach, and apple blooms. (**C**) Frequency of detection of CVA, PDV, and PNRSV during apricot, cherry, peach, and apple blooms.

Table 2. Plant virus detections in bee and pollen samples from AAFC Jordan Farm during apricot, cherry, peach, and apple blooms.

Virus	Abbreviation	Genus	Frequency of Detection (%)												Total Detections (n)	Average Frequency of Detection (%)	Average Genome Coverage (%)	Average VRPM
			Apricot			Cherry			Peach			Apple							
			Forager n = 3	Hive n = 3	Pollen n = 3	Forager n = 3	Hive n = 3	Pollen n = 3	Forager n = 3	Hive n = 3	Pollen n = 3	Forager n = 3	Hive n = 3	Pollen n = 3				
Prune dwarf virus	PDV	Ilarvirus	33	33	100	67	100	100	33	100	67	33		100	23	64	62	32,548
Cherry virus A	CVA	Capillovirus	67	67	100	67	100	100	33	33	33			100	21	58	70	5042
Prunus necrotic ringspot virus	PNRSV	Ilarvirus	67	33	67	100	100	100	67	33	33			100	21	58	54	26,401
Prunus virus F	PVF	Fabavirus	33	33	67	67	100	100	33					100	16	44	63	2920
Tobacco ringspot virus	TRSV	Nepovirus		33	33		67			67	67	100	100	67	16	44	47	11,657
Tomato ringspot virus	ToRSV	Nepovirus	67	67	100	33		33	33		100		67	33	16	44	36	4000
Brome mosaic virus	BMV	Bromovirus	67	67	100	33		33	67		33	67		33	15	42	29	1743
Peach latent mosaic viroid	PLMVd	Pelamoviroid			67	67		67	100					67	11	31	37	0
Apple mosaic virus	ApMV	Ilarvirus									67			67	4	11	33	135
White clover mosaic virus	WCMV	Potexvirus	33									33		67	4	11	13	343
Blackberry chlorotic ringspot virus	BCRV	Ilarvirus				33	33					33			3	8	34	5762
Pelargonium zonate spot virus	PZSV	Anulavirus									33	67			3	8	23	1449
Tobacco streak virus	TSV	Ilarvirus	67			33									3	8	16	117
Gaillardia latent virus	GLV	Carlavirus				33								33	2	6	18	4
Raphanus sativus cryptic virus 3	RSCV3	Unclassified Partitiviridae									33		33		2	6	47	53
Tomato necrotic spot virus	ToNSV	Ilarvirus										33		33	2	6	40	296
Apple hammerhead viroid	AHHVd	Pelamoviroid										33			1	3	20	0
Apple stem grooving virus	ASGV	Capillovirus									33				1	3	66	279
Citrus concave gum-associated virus	CCGaV	Coguvirus									33				1	3	67	1408
Citrus virus A	CiVA	Coguvirus												33	1	3	28	39
Peanut stunt virus	PSV	Cucumovirus												33	1	3	10	0

3.2. Virus Species Diversity in Different Sample Types and Sampling Times

To understand the distribution of viruses across the sample types and time points, the average number of virus species identified in each sample type was calculated (Figure 2A). Four viruses were identified in the majority of samples, with a range of two to eight viruses identified per sample (Figure 2A). When examining the number of viruses detected among the sample types irrespective of the time point, a significant main effect was detected (one-way ANOVA, $p = 0.007$, F = 5.80, df = 2), with differences observed between the number of virus types identified in the pollen and hive bee samples (Tukey's HSD, $p = 0.005$). When comparing among sampling times, no significant difference was observed between the number of viruses identified (one-way ANOVA, $p = 0.71$, F = 0.40, df = 3; Figure 2A). CVA, PVF, BMV, TRSV, ToRSV, PDV, PNRSV, and PLMVd were detected at all time points. TSV was associated with the apricot and cherry time points, CCGaV and ASGV were unique to the peach time point, ToNSV, AHHVd, peanut stunt virus (PSV; genus *Cucumovirus*), and citrus virus A (CiVA; genus *Coguvirus*) to the apple time point, and raphanus sativus cryptic virus 3 (RSCV3), ApMV, and PZSV with both the peach and apple time points (Figure 2B). PDV, PNRSV, and CVA were the three most prominent viruses detected overall, and they were most frequently detected during the cherry time point (Figure 2C). The greatest number of virus species were identified in the pollen and forager bee samples (Figure 3A). No unique virus species could be associated with hive bees, with the viruses identified in hive bees also identified in the pollen and forager bee samples (n = 7), including CVA, PDV, PNRSV, TRSV, ToRSV, PVF, and BMV (Figure 3A).

To correlate the viruses identified in the bee-collected samples with viruses infecting the targeted tree fruit species, two replicates of composite leaf and flower samples were collected from ~10 random individuals from one apricot, cherry, peach, and apple plot located near the bee colonies at the same time as when the bee samples were collected (Figure 1A). As opposed to the totRNA-extracted bee/pollen samples, dsRNA was extracted from the leaf/flower samples to preferentially isolate the replicating viruses (Table 3). During the apricot time point, fewer viruses were detected in the leaf/flower (n = 3) compared to bee/pollen samples (n = 10), with only PNRSV identified in both sample sets from this time point (Figure 3B). In cherries, seven viruses were unique to the bee/pollen samples [BMV, ToRSV, PLMVd, TSV, TRSV, BCRV, and Gaillardia latent virus (GaiLV; genus *Carlavirus*)], four viruses (PDV, CVA, PNRSV, and PVF) were identified in both the bee/pollen and leaf/flower samples (Figure 3C), while four viruses were unique to the leaf/flower samples (ApMV, BlShV, LCV1, and WCMV). In peaches, 13 viruses were identified in the bee/pollen samples compared with 3 from the leaf/flower samples; the nectarine stem pitting-associated virus (genus *Luteovirus*) and cherry necrotic rusty mottle virus (genus *Robigovirus*) were detected only in the leaf/flower samples, while PLMVd was detected in both sample types (Table 3; Figure 3D). Many viruses were identified in the apple leaf/flower samples (n = 8) but not in the bee/pollen samples, including ACLSV, apple flat limb virus (AFLV; genus *Rubodvirus*), apple rubbery wood virus 2 (ARWV2; genus *Rubodvirus*), ASPV, ASGV, and CCGaV (Figure 3E). The viruses identified in both bee/pollen and leaf/flower samples from the apple time point included ApMV, AHHVd, and RSCV3 (unclassified *Partitiviridae* family) (Figure 3D).

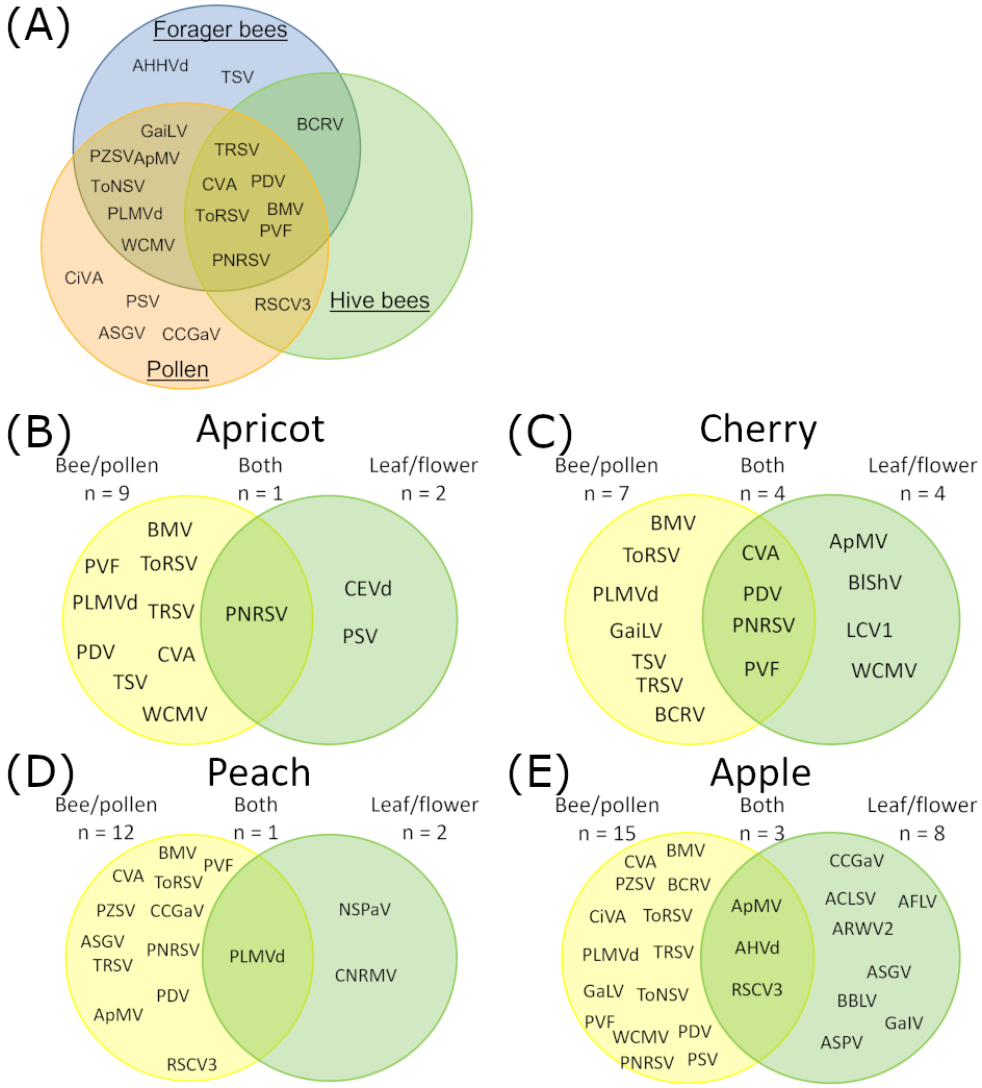

Figure 3. Venn diagrams of crop and sample type viral diversity. (**A**) Viral diversity in apricot, cherry, peach, and apple samples. (**B**) Viral species diversity in different sample types. (**B**–**E**) Viruses detected in bee-collected samples and plant samples for (**B**) apricots, (**C**) cherries, (**D**) peaches, and (**E**) apples.

Table 3. Plant virus detection from apricot, cherry, peach, and apple leaf/flower tissue.

Virus Species	Abbreviation	Family	Genus	Frequency (%)				Total Detections (n)	Average Frequency of Detection (%)	Average Genome Coverage (%)	Average VRPM
				Apricot	Cherry	Peach	Apple				
				Plant Tissue							
				n = 2	n = 2	n = 2	n = 2				
Prunus necrotic ringspot virus	PNRSV	*Bromoviridae*	Ilarvirus	100	50			3	38	70.8	2041
Apple mosaic virus	ApMV	*Bromoviridae*	Ilarvirus		50		50	2	25	20.6	0
Cherry virus A	CVA	*Betaflexiviridae*	Capillovirus		100			2	25	99.6	1181
Prune dwarf virus	PDV	*Bromoviridae*	Ilarvirus		100			2	25	15.7	1
Apple chlorotic leaf spot virus	ACLSV	*Betaflexiviridae*	Trichovirus				100	2	25	78.7	500
Apple flat limb virus	AFLV	*Phenuiviridae*	Rubodvirus				100	2	25	39.65	4
Apple rubbery wood virus 2	ARWV2	*Phenuiviridae*	Rubodvirus				100	2	25	91.5	107
Apple stem pitting virus	ASPV	*Betaflexiviridae*	Foveavirus				100	2	25	67.6	225
Prunus virus F	PVF	*Secoviridae*	Fabavirus		100			2	25	97.1	67
Little cherry virus 1	LCV1	*Closteroviridae*	Velarivirus		100			2	25	99.95	1314
Nectarine stem pitting-associated virus	NSPaV	*Tombusviridae*	Luteovirus			100		2	25	82.2	223
Cherry necrotic rusty mottle virus	CNRMV	*Betaflexiviridae*	Robigovirus			100		2	25	95.05	58
Raphanus sativus cryptic virus 3	RSCV3	*Partitiviridae*	Unclassified				50	1	13	18.9	0
Apple hammerhead viroid	AHHVd	*Avsunviroidae*	Pelamoviroid				50	1	13	100	2410
Apple stem grooving virus	ASGV	*Betaflexiviridae*	Capillovirus				50	1	13	99.6	7528
Citrus concave gum-associated virus	CCGaV	*Phenuiviridae*	Coguvirus				50	1	13	99.2	417
Peach latent mosaic viroid	PLMVd	*Avsunviroidae*	Pelmaviroid			50		1	13	99.6	27
Blueberry latent virus	BLV	*Amalgaviridae*	Amalgavirus				50	1	13	13.2	4
Blueberry shock virus	BlScV	*Bromoviridae*	Ilarvirus		50			1	13	11.1	7
Citrus excordis viroid	CEVd	*Pospiviroidae*	Pospiviroid	50				1	13	96.4	169
Grapevine associated ilarvirus	GalV	*Bromoviridae*	Ilarvirus				50	1	13	24.6	1
Peanut stunt virus	PSV	*Bromoviridae*	Cucumovirus	50				1	13	16.9	0
White clover mosaic virus	WCMV	*Alphaflexiviridae*	Potexvirus		50			1	13	26.7	0

3.3. Coat Protein Sequence Diversity of CVA, PDV, and PNRSV

Pairwise and phylogenetic analyses of the PDV, CVA, and PNRSV coat protein (CP) nucleotide sequences were undertaken to investigate virus sequence diversity. Only sample data with full coverage of the CP open reading frame (ORF) for one or more of the three viruses were used. The PNRSV CP ORF sequences were the most diverse, ranging from 89–100% identity, while CVA and PDV CP sequences ranged from 95.3–98.5% and 96–100% identity, respectively (Figures 4A, 5A and 6A). In total, 11 samples had full CVA CP coverage, including 2 leaf/flower samples collected from cherries (ONJF1-CH-1T1 and ONJF1-CH-1T2; Figure 4; Supplemental Table S1). Six CP sequences were obtained from the cherry time point samples, three from apricots, and two from apples (Figure 4B). All CVA CP sequences clustered closely together with a previously reported CVA isolate from Jordan Farm (MF062118) and were grouped within phylogroup II, as defined in Gao et al., 2017 (Figure 4B) [42,43].

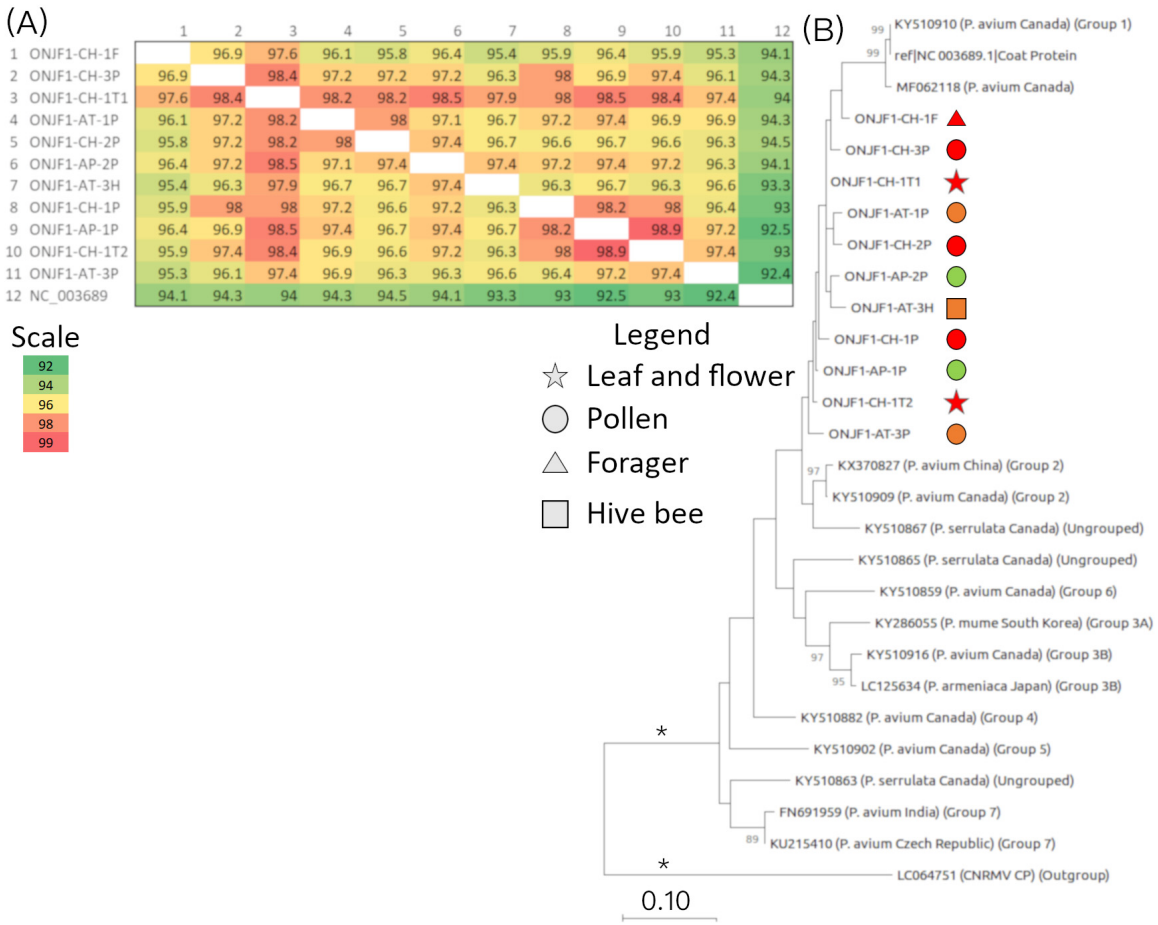

Figure 4. CVA CP nucleotide sequence diversity. (**A**) Pairwise identity of nucleotide CP sequences, with NC_003689 as a reference sequence. (**B**) Maximum likelihood phylogenetic tree of CVA CP sequences. Scale bars and corresponding numbers indicate the average number of mutations per base. The asterisk indicates these branches are not to scale. Numbers indicate support for branches; only branch points with over 70% support are shown. Orange indicates apricot samples, red for cherry, yellow for peach, and green for apple.

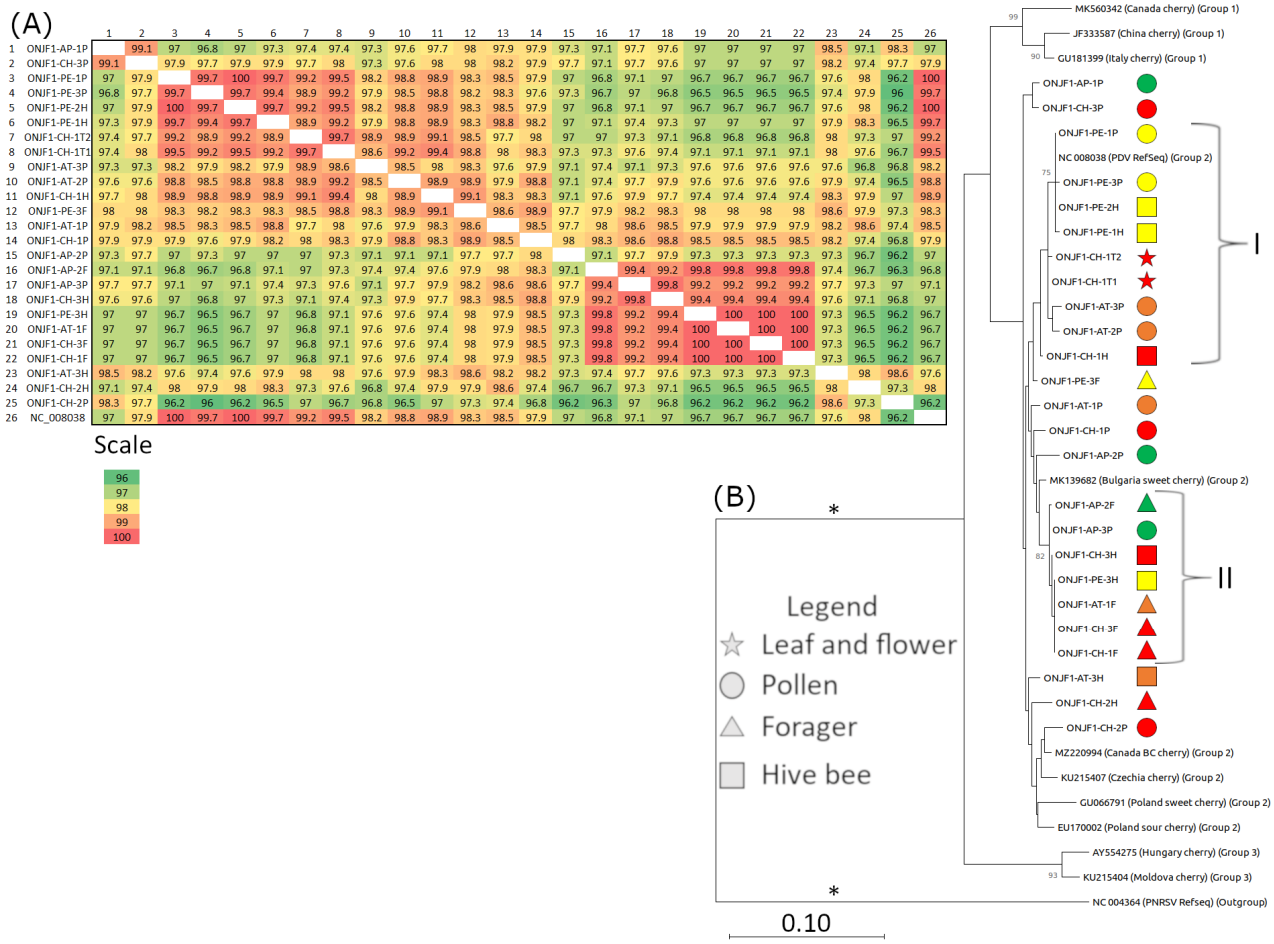

Figure 5. PDV CP nucleotide sequence diversity. (**A**) Pairwise identity of nucleotide CP sequences, with NC_008038 as a reference sequence. (**B**) Maximum likelihood phylogenetic tree of PDV CP sequences. Scale bars and corresponding numbers indicate the average number of mutations per base. The asterisk indicates these branches are not to scale. Numbers indicate support for branches; only branch points with over 70% support are shown. Orange indicates apricot samples, red for cherry, yellow for peach, and green for apple.

In total, 23 PNRSV CP sequences were recovered from the pollen, bee, and plant sample datasets (Figure 6A). Of these, 17 clustered within the PV32 phylogroup along with the reference sequence (NC_004363) and shared a high degree of identity (>98.5%) (Figure 6) [46]. These sequence data were derived from all four time points, including only one isolate from the apple time point (ONJF1-AP-2P; Figure 6B). Two isolates derived from the cherry and apricot leaf/flower samples were included in this group. One sequence, derived from an apricot hive bee sample, branched more closely with the PV96 phylogroup (ONJF1-AT-3H). One sequence, derived from pollen collected during apple bloom (ONJF1-AP-3P), was distantly related to all other sequences from this study (90.4–91.9%) and did not associate with any particular phylogroup.

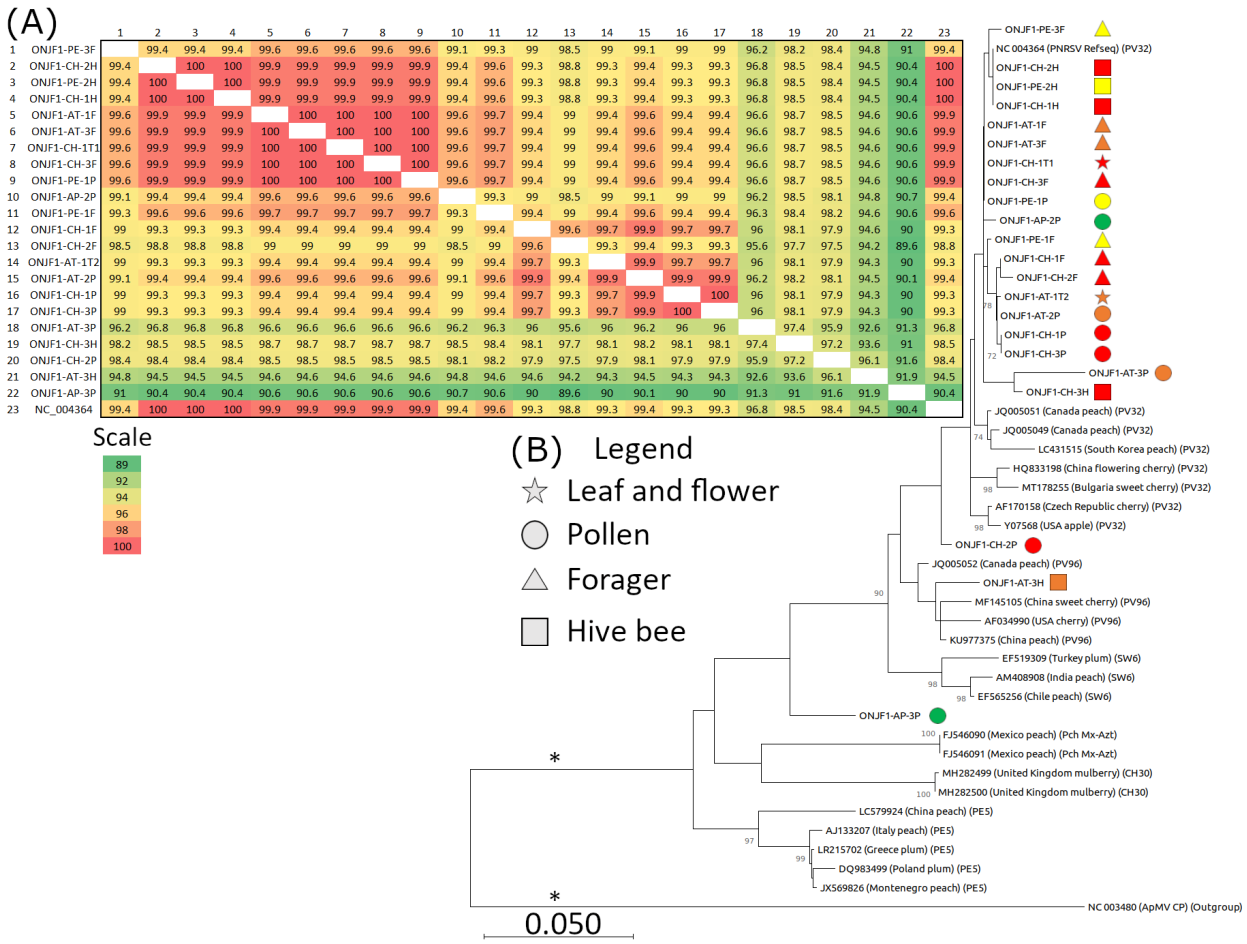

Figure 6. PNRSV CP nucleotide sequence diversity. (**A**) Pairwise identity of CP sequences, with NC_004364 as a reference sequence. (**B**) Maximum likelihood phylogenetic tree of PNRSV CP sequences. Scale bars and corresponding numbers indicate the average number of mutations per base. The asterisk indicates these branches are not to scale. Numbers indicate support for branches; only branch points with over 70% support are shown. Orange indicates apricot samples, red for cherry, yellow for peach, and green for apple. Complete PDV CP sequences were obtained from 26 sample datasets, including plant tissue samples from cherry (n = 2) (Figure 5A; Supplemental Table S1). Two branches of sequences derived from these samples were identified, all within PDV phylogroup II, as defined in Kinoti et al., 2018 (Figure 5B) [44]. The first branch contained nine sequences derived from peach (n = 4), cherry (n = 3, two from leaf/flower samples), and apricot (n = 2) and clustered closely with the reference sequence (NC_008038). Identities within this branch ranged from 97.9–100% (Figure 5B). The second branch contained eight sequences with identities ranging from 99.2–100% and clustered closely with an isolate from a Bulgarian sweet cherry sample (MK139682; Figure 5) [45]. Sequence identity was typically over 98% within each branch and less than 97.6% identical between branches (Figure 5A). Five other sequences derived from apricot and cherry samples branched more independently (Figure 5B).

4. Discussion

4.1. Metagenomics-Based Detection of Plant Viruses through Pollen

Honey bees are capable of foraging from a broad diversity of plants in various landscapes [47–49]. Individual honey bees choose floral resources based on exploiting profitable flowers in the vicinity of their hive and exhibit considerable fidelity to a crop or floral patch during successive flights, only switching when pollen becomes scarce or unattractive [47]. Colony-level pollen foraging decisions are affected by landscape and closely follow changes in the seasonal availability of floral resources [48–50]. Flowering plant species, including

those of agricultural importance, have overlapping bloom periods, which can result in a complex of pollens from multiple species being collected by honey bees at any given time. While each time point represented the peak bloom period for each species we examined, other tree fruit and naturally occurring plant species were in bloom, which could have contributed to the diversity of pollens collected, resulting in a complex mixture of pollens from multiple potential plant species (Figure 2B). Consistent with this, the CVA, PDV, and PNRSV coat protein sequences did not assort together based on the time of sampling, suggesting broad floral inputs into the bee and pollen samples. It is, therefore, difficult to accurately ascertain the origins of the viruses identified from pollen-based environmental sampling [50]. Viruses could be originating from the closely related *Prunus* and *Malus* species or potential weedy host reservoirs.

The identification of plant species from the pollen samples through DNA metabarcoding could help to define the limits of pollen-based virus identification and better understand the potential for alternate hosts for viruses of concern [51–54]. Combining work-flows to include multiple environmental health indicators, including bacterial and fungal pathogens, pesticide and chemical monitoring, invasive plant species, and even honey bee pathogens and parasites, could create a powerful one-health monitoring tool [5,37,51,52,54–58]. Regardless, the most frequent detections of plant viruses (ex., PDV and PNRSV) were viruses known to cause disease in these systems and identified a striking amount of nucleotide sequence diversity, demonstrating the accuracy and sensitivity of the pollen-mediated metagenomics-based detection of plant viruses in tree fruit orchard systems [5,59].

4.2. Viral Diversity in Bee-Collected Pollen Samples

Many viruses are associated with pollen transmission, including members of the *Alphacryptovirus*, *Ilarvirus*, *Nepovirus*, and *Potyvirus* genera [21,59,60]. *Ilarviruses,* in particular, were widely prevalent in this study, with representatives from three of the four *Ilarvirus* subgroups identified [60,61]. ToNSV from subgroup 1, BCRV, TSV, and PDV from subgroup 3, and PNRSV and ApMV from subgroup 4 were detected, demonstrating a large diversity of *Ilarviruses* at this site. BCRV and ToNSV are not normally associated with *Prunus* hosts, and while BCRV has been reported to infect apples, this virus was not detected in our survey of leaf/flower samples from apples [62,63]. BCRV is typically associated with cane fruits like blackberries (*Rubus allegheniensis*) or other *Rubus* plants [63,64]. Primary hosts for ToNSV are *Solanaceous* plants [65], yet no *Solanaceous* crops were grown at the time on Jordan Farm. Both viruses were detected in low frequencies, which highlights the sensitivity of this approach and the complexities of interpretation. Three other viruses in the *Bromoviridae* family were identified, including BMV, PSV, and pelargonium zonate spot virus (genus *Anulavirus*). BMV, PSV, BCRV, and many other viruses identified in this study were also identified in a recent survey of the pollen virome of wild plants, confirming a strong association with pollen and suggesting they could be widespread in the endemic plant pollen ecosystem near Jordan Farm [4]. Understanding the host origins of viruses in bee and pollen samples can be difficult, but it can inform targeted follow-up studies in commercial fruit crops [66].

Viruses in the *Secoviridae* family were also detected at all time points, including the nepoviruses ToRSV and TRSV and *Fabavirus* PVF [67,68]. Nepoviruses are commonly transmitted via nematodes, seeds, and pollen [67], while little is known regarding the transmission of PVF. Fabaviruses have a broad host range and are associated with aphid transmission, but no vector has been identified for PVF to date, while this and our previous study strongly associate PVF with pollen in tree fruit systems [5,69]. ToRSV and TRSV were not identified in the leaf/flower samples but were present in all other bee and pollen samples. ToRSV was more frequent during apricot and cherry blooms, while TRSV was more frequent during apple blooms. It is possible they could have been missed in our limited random sampling of leaf/flower tissue or are infecting other plant species in the area.

The number of viruses identified per sample was largely consistent from the apricot through to the peach blooms, with slightly more viruses detected in the apple pollen samples despite substantial increases in the number of individual trees in flower as the bloom period progressed (Table 1). However, the diversity of viral species increased during peach and apple blooms and was associated with increased numbers of individuals in flower. PDV, PNRSV, and CVA were the most frequently detected viruses, present across all time points and sample types, yet were more prevalent during the cherry time bloom. The virus profiles taken during the flowering of the three *Prunus* species were more similar, whereas the profiles obtained during the apple flowering time point included detections of apple-infecting viruses/viroids like CiVA, ApMV, and AHHVd. Apple-infecting viruses like ASGV and CCGaV were only identified in the peach time point, suggesting a potential crossover from apple or other pome pollen. Two apple-infecting coguviruses (CCGaV and CiVA) were detected in pollen samples from peach and apple, respectively. These two viruses were also prominent in the apple pollen samples from our previous study, which could suggest a previously unreported association with pollen [5]. Coguviruses can be graft-transmissible, and CCGaV was recently reported to be seed-borne [70,71].

4.3. Sequence Diversity of Identified Viruses

The known cherry-infecting viruses CVA, PDV, and PNRSV were the most common viruses detected during the cherry time point, each with a unique pattern of observed nucleotide sequence diversity. All CVA CP sequences clustered with a previously described cherry isolate from this site [45,72]. In this study, aside from detection in the bee/pollen samples from all time points, CVA was identified in cherry leaf/flower tissue with high read counts but not from other *Prunus* or apple leaf/flower samples, consistent with this virus originating primarily from cherry trees (Figure 4). Consistent with previous studies, the CVA nucleotide sequences were highly variable despite grouping together in the phylogenetic analysis [44,73]. CVA is transmitted via grafting, and no other major transmission route has been widely reported; further studies could investigate the high association of CVA with pollen [74].

PDV isolates cluster into three major phylogroups, with all isolates identified in this study belonging to phylogroup II [44]. The phylogenetic and pairwise sequence analyses identified two distinct sub-groupings within phylogroup II—one associated with the peach bee/pollen samples and two cherry leaf/flower samples, and another detected in all four sampling time points. Additional minor isolates were identified, including one closely related to an isolate from cherries in British Columbia (MZ220994), demonstrating multiple variants of PDV present at this site. The high variability and distinct groupings of isolates in this study could be due to the wide diversity of *Prunus* species, varieties, and the ages of plants. Further studies could investigate the host range and pathogenicity of these variants, which could help to identify priorities for management.

PNRSV variants present in the samples were more diverse than CVA or PDV, with the CP nucleotide sequence identities ranging from 89.6–99.7%. This was greater than previous reports from this site (95.1–100%, or in other related studies of PNRSV pathology, 94–100%) [43,75]. The PNRSV sequence data separated into two major clusters, one associated with the apricot leaf/flower samples and the second from the cherry leaf/flower samples, suggesting unique variants of PNRSV associated with these hosts. A third unique variant of PNRSV with the lowest identity to all other samples (89.6–91.9%) was detected from the apple time point, possibly arising from apple trees or other host flowering at this site at the same time. Data from this study demonstrate a broad diversity of sequences within the PV32 phylogroup—one isolate was associated with PV96 [29,75], and one isolate was from an apple pollen sample distantly related to other groups. PV32 and PV96 have been described as mild isolates of PNRSV but can still result in suppressed growth, necrotic spots, and chlorosis on *Prunus tomentosa* [76].

5. Conclusions

Bee-mediated plant virus monitoring can provide an overview of the pollen-associated virome and potential viral pathogens in the immediate area [5,36,37,48]. Bee-mediated sampling can greatly facilitate surveying multiple individuals at a commercial farm site, which can often have thousands of trees, but determining the exact origins of the viruses detected or understanding the dangers associated with transmission is still poorly understood. Compared to virus identification from plant samples based on visual symptoms and individual virus testing and sequencing, bee/pollen-mediated surveys of viral diversity could provide many advantages, including reduced sampling labour, capturing greater levels of viral diversity, and identifying emerging viruses before they become widespread. PDV, PNRSV, and CVA were widely prevalent in the bee and pollen samples from most time points, with the highest frequency of detection during cherry bloom. Viruses detected during the apple bloom were consistent with apple-infecting viruses like AHHVd, ApMV, and CiVA. A broad diversity of PDV and PNRSV variants were present at this site based on the CP nucleotide sequence comparisons, demonstrating many advantages to bee-mediated metagenomic-based virus monitoring.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v16101614/s1, Table S1: Viral coat protein sequences and GenBank accession numbers; File S1: RNAseq metadata.

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Data Availability Statement: All RNAseq files and CP consensus sequences are available on the Sequence Read Archive and GenBank, respectively. SRA accessions are provided in Supplemental File S1 and can be accessed online at the SRA under bioproject ID PRJNA1025014. GenBank accession numbers for coat protein sequences are provided in Supplemental Table S1.

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References

1. Soundararajan, P.; Won, S.Y.; Kim, J.S. Insight on Rosacea family with genome sequencing and functional genomics perspective. *Biomed. Res. Int.* **2019**, *2019*, 7519687. [CrossRef] [PubMed]
2. AAFC-Agriculture and Agri-Food Canada. Statistical Overview of the Canadian Fruit Industry 2021. Horticulture Section, Crops and Horticulture Division. 2022. Available online: https://agriculture.canada.ca/sites/default/files/documents/2022-12/Fruit%20Report_2021_ENG.pdf (accessed on 2 November 2022).
3. Umer, M.; Liu, J.; You, H.; Xu, C.; Dong, K.; Luo, N.; Kong, L.; Li, X.; Hong, N.; Wang, G.; et al. Genomic, morphological and biological traits of viruses infecting major fruit trees. *Viruses* **2019**, *11*, 515. [CrossRef] [PubMed]
4. Fetters, A.M.; Cantalupo, P.G.; Wei, N.; Robles, M.T.S.; Stanley, A.; Stephens, J.D.; Pipas, J.M.; Ashman, T.L. The pollen virome of wild plants and its association with variation in floral traits and land use. *Nat. Commun.* **2022**, *13*, 523. [CrossRef]
5. Smadi, M.; Lee, E.; Phelan, J.; Wang, A.; Bilodeau, G.J.; Pernal, S.F.; Guarna, M.M.; Rott, M.; Griffiths, J.S. Plant virus diversity in bee and pollen samples from apple (*Malus domestica*) and cherry (*Prunus avium*) agroecosystems. *Front. Plant Sci.* **2024**, *15*, 1335281. [CrossRef]
6. Chandel, V.; Rana, T.; Handa, A.; Thakur, P.D.; Hallan, V.; Zaidi, A.A. Incidence of Prunus necrotic ringspot virus on Malus domestica in India. *J. Phytopath.* **2008**, *156*, 382–384. [CrossRef]
7. Hu, G.J.; Dong, Y.F.; Zhang, Z.P.; Fan, X.D.; Ren, F.; Li, Z.N.; Zhou, J. First report of Prunus necrotic ringspot virus infection of apple in China. *Plant Dis.* **2016**, *100*, 1955. [CrossRef]

8. Kinoti, W.M.; Constable, F.E.; Nancarrow, N.; Plummer, K.M.; Rodoni, B. Generic amplicon deep sequencing to determine ilarvirus species diversity in Australian Prunus. *Front. Microbio.* **2017**, *8*, 1219. [CrossRef]
9. Celik, A.; Ertunc, F. First report of prunus necrotic ringspot virus infecting apple in Turkey. *J. Plant Pathol.* **2019**, *101*, 1227. [CrossRef]
10. Xiao, H.; Hau, W.; Storoschuk, G.; MacDonald, J.L.; Sanfacon, H. Characterizing the virome of apple orchards affected by rapid decline in the Okanagan and Similkameen valleys of British Columbia (Canada). *Pathogens* **2022**, *11*, 1231. [CrossRef]
11. Koziel, E.; Bujarski, J.J.; Otulak, K. Molecular biology of prune dwarf virus—A lesser known member of the bromoviridae but a vital component in the dynamic virus-host cell interaction network. *Int. J. Mol. Sci.* **2017**, *18*, 2733. [CrossRef]
12. Kesanakurti, P.; Belton, M.; Saeed, H.; Rast, H.; Boyes, I.; Rott, M. Screening for plant viruses by next generation sequencing using a modified double strand RNA extraction protocol with an internal amplification control. *J. Virol. Meth.* **2016**, *236*, 35–40. [CrossRef] [PubMed]
13. Bag, S.; Rwahnih, M.A.; Li, A.; Gonzalez, A.; Rowhani, A.; Uyemoto, J.K.; Sudarsharna, M.R. Detection of a new luteovirus in imported nectarine trees: A case study to propose adoption of metagenomics in post-entry quarantine. *Phytopathology* **2015**, *105*, 840–846. [CrossRef] [PubMed]
14. Villamore, D.E.; Mekuria, T.A.; Pillai, S.S.; Eastwell, K.C. High-throughput sequencing identifies novel viruses in nectarines: Insights to the etiology of stem-pitting disease. *Phytopathology* **2016**, *106*, 519–527. [CrossRef] [PubMed]
15. Wright, A.A.; Cross, A.R.; Harper, S.J. A bushel of viruses: Identification of seventeen novel putative viruses by RNA-seq in six apple trees. *PLoS ONE* **2020**, *15*, e0227669. [CrossRef]
16. McLeish, M.J.; Fraile, A.; Farcia-Arsenal, F. Ecological complexity in plant virus host range evolution. *Adv. Virus Res.* **2018**, *101*, 293–339.
17. McLeish, M.J.; Fraile, A.; Farcia-Arsenal, F. Evolution of plant-virus interactions: Host range and virus emergence. *Curr. Opin. Virol.* **2019**, *34*, 50–55. [CrossRef]
18. Hamelin, F.M.; Allen, L.J.S.; Prendeville, H.R.; Hajimorad, M.R.; Jeger, M.J. The evolution of plant virus transmission pathways. *J. Theor. Biol.* **2016**, *396*, 75–89. [CrossRef]
19. Gallet, R.; Michalakis, Y.; Blanc, S. Vector-transmission of plant viruses and constraints imposed by virus-vector interactions. *Curr. Opin. Virol.* **2018**, *33*, 144–150. [CrossRef]
20. McLaughlin, A.A.; Hanley-Bowdoin, L.; Kennedy, G.G.; Jacobson, A.L. Vector acquisition and co-inoculation of two plant viruses influences transmission, infection, and replication in new hosts. *Sci. Rep.* **2022**, *12*, 20355. [CrossRef]
21. Card, S.D.; Pearson, M.N.; Clover, G.R.G. Plant pathogens transmitted by pollen. *Aust. Plant Pathol.* **2007**, *36*, 455–461. [CrossRef]
22. Jones, R.A.C. Plant and insect viruses in managed and natural environments: Novel and neglected transmission pathways. *Adv. Virus Res.* **2018**, *101*, 149–187.
23. Amari, K.; Burgos, L.; Pallas, V.; Sanchez-Pina, M.A. Prunus necrotic ringspot virus early invasion and its effects on apricot pollen grain performance. *Phytopathology* **2007**, *97*, 892–899. [CrossRef]
24. Amari, K.; Burgos, L.; Pallas, V.; Sanchez-Pina, M.A. Vertical transmission of Prunus necrotic ringspot virus: Hitch-hiking from gametes to seedling. *J. Gen. Virol.* **2009**, *90*, 1676–1774. [CrossRef]
25. Isogai, M.; Yoshida, T.; Nakanowatari, C.; Yoshikawa, N. Penetration of pollen tubes with accumulated Raspberry bushy dwarf virus into stigmas if involved in initial infection of maternal tissue and horizontal transmission. *Virology* **2014**, *452–453*, 247–253. [CrossRef]
26. Shipp, J.L.; Buitenhuis, R.; Stobbs, L.; Wang, K.; Kim, W.S.; Ferguson, G. Vectoring of Pepino mosaic virus by bumble-bees in tomato greenhouses. *Ann. Appl. Biol.* **2008**, *153*, 149–155. [CrossRef]
27. Levitzky, N.; Smith, E.; Lachman, O.; Luria, N.; Mizrahi, Y.; Bakelman, H.; Sela, N.; Laskar, O.; Milrot, E.; Dombrovsky, A. The bumblebee *Bombus terrestris* carries a primary inoculum of Tomato brown rugose fruit virus contributing to disease spread in tomatoes. *PLoS ONE* **2019**, *14*, e0210871. [CrossRef]
28. Tayal, M.; Wilson, C.; Cieniewicz, E. Bees and thrips carry virus-positive pollen in peach orchards in South Carolina, United States. *J. Econ. Entomol.* **2023**, *116*, 1091–1101. [CrossRef]
29. Oliver, J.E.; Freer, J.; Anderson, R.L.; Cox, K.D.; Robinson, T.L.; Fuchs, M. Genetic diversity of Prunus necrotic ringspot virus isolates within a cherry orchard in New York. *Plant Dis.* **2009**, *93*, 599–606. [CrossRef]
30. Abdallah, D.; Baraket, G.; Perez, V.; Hannachi, A.S.; Hormaza, J.I. Self-compatibility in peach [*Prunus persica* (L.) Batsch]: Patterns of diversity surrounding the S-locus and analysis of SFB alleles. *Hort. Res.* **2020**, *7*, 170. [CrossRef]
31. Piri, S.; Kiani, E.; Sedaghathoor, S. Study on fruitset and pollen-compatibility status in sweet cherry (*Prunus avium* L.) cultivars. *Erwebs-Obstbau* **2022**, *64*, 165–170. [CrossRef]
32. Delaplane, K.S.; Mayer, D.F. *Crop Pollination by Bees*; CABI Publishing: New York, NY, USA, 2000.
33. Beekman, M.; Ratnieks, F.L.W. Long-range foraging by the honey-bee, *Apis mellifera* L. *Func. Ecol.* **2000**, *14*, 490–496. [CrossRef]
34. Steffan-Dewenter, I.; Kuhn, A. Honeybee foraging in differentially structured landscapes. *Proc. R. Soc. B Biol. Sci.* **2003**, *270*, 569–575. [CrossRef]
35. Roberts, J.M.K.; Ireland, K.B.; Tay, W.; Paini, D. Honey bee-assisted surveillance for early plant virus detection. *Ann. Appl. Biol.* **2018**, *173*, 285–293. [CrossRef]

36. Lee, E.; Vansia, R.; Phelan, J.; Lofano, A.; Smith, A.; Wang, A.; Bilodeau, G.J.; Pernal, S.F.; Guarna, M.M.; Rott, M.; et al. Area wide monitoring of plant and honey bee (*Apis mellifera*) viruses in blueberry (*Vaccinium corymbosum*) agroecosystems facilitated by honey bee pollination. *Viruses* **2023**, *15*, 1209. [CrossRef]
37. Cunningham, M.M.; Tran, L.; McKee, C.G.; Polo, R.O.; Newman, T.; Lansing, L.; Griffiths, J.S.; Bilodeau, G.J.; Rott, M.; Guarna, M.M. Honey bees as biomonitors of environmental contaminants, pathogens, and climate change. *Ecol. Indic.* **2022**, *134*, 108457. [CrossRef]
38. Hong, C.; Manimaran, S.; Shen, Y.; Perez-Rogers, J.F.; Byrd, A.L.; Castro-Nallar Crandall, K.A.; Johnson, W.E. PathoScope 2.0: A complete computational framework for strain identification in environmental or clinical sequencing samples. *Microbiome* **2014**, *2*, 33. [CrossRef]
39. Langmead, B.; Salzberg, S.L. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **2012**, *9*, 357–359. [CrossRef]
40. Rott, M.; Xiang, Y.; Boyes, I.; Belton, M.; Saeed, H.; Kesanakurti, P.; Hayes, S.; Lawrence, T.; Birch, C.; Bhagwat, B.; et al. Application of next generation sequencing for diagnostic testing of tree fruit viruses and viroids. *Plant Dis.* **2017**, *101*, 1489–1499. [CrossRef]
41. Tamura, K.; Stecher, G.; Kumar, S. MEGA 11: Molecular evolutionary genetics analysis version 11. *Mol. Biol. Evol.* **2021**, *38*, 3022–3027. [CrossRef]
42. Gao, R.; Xu, Y.; Candresse, T.; He, Z.; Li, S.; Ma, Y.; Lu, M. Further insight into genetic variation and haplotype diversity of cherry virus A from China. *PLoS ONE* **2017**, *12*, e0186273. [CrossRef]
43. Simkovich, A.J.; Li, Y.; Kohalmi, S.E.; Griffiths, J.S.; Wang, A. Molecular identification of prune dwarf virus (PDV) infecting sweet cherry in Canada and development of a PDV full-length infectious cDNA clone. *Viruses* **2021**, *13*, 2025. [CrossRef]
44. Kinoti, W.M.; Constable, F.E.; Nancarrow, N.; Plummer, K.M.; Rodoni, B. The incidence and genetic diversity of apple mosaic virus (ApMV) and prune dwarf virus (PDV) in prunus species in Australia. *Viruses* **2018**, *10*, 136. [CrossRef]
45. Kamenova, I.; Borisova, A.; Popov, A. Incidence and genetic diversity of Prune dwarf virus in sweet and sour cherry in Bulgaria. *Biotech. Biotech. Equip.* **2019**, *33*, 980–987. [CrossRef]
46. Glassa, M.; Betinova, E.; Kudela, O.; Subr, Z. Biological and molecular characterization of Prunus necrotic ringspot virus isolates and possible approaches to their phylogenetic typing. *Ann. Appl. Biol.* **2021**, *140*, 215–329.
47. Free, J.B. The flower constancy of honeybees. *J. Anim. Ecol.* **2002**, *32*, 119–131. [CrossRef]
48. Danner, N.; Molitor, A.M.; Schiele, S.; Härtel, S.; Steffan-Dewenter, I. Season and landscape composition affect pollen foraging distances and habitat use of Honey bees. *Ecol. Appl.* **2016**, *26*, 1920–1929. [CrossRef]
49. Jones, L.; Lowe, A.; Ford, C.R.; Christie, L.; Creer, S.; de Vere, N. Temporal patterns of honeybee foraging in a diverse floral landscape revealed using pollen DNA metabarcoding of honey. *Integr. Comp. Biol.* **2022**, *62*, 199–210. [CrossRef]
50. Lowe, A.; Jones, L.; Brennan, G.; Creer, S.; Christie, L.; de Vere, N. Temporal change in floral availability leads to periods of resource limitation and affects diet specificity in a generalist pollinator. *Mol. Ecol.* **2022**, *32*, 6363–6376. [CrossRef]
51. Tremblay, E.D.; Duceppe, M.O.; Thurston, G.B.; Gagnon, M.C.; Cote, M.J.; Bilodeau, G.J. High-resolution biomonitoring of plant pathogens and plant species using metabarcoding of pollen pellet contents collected from a honey bee hive. *Environ. DNA* **2019**, *1*, 155–175. [CrossRef]
52. McKinnon, A.C.; Collins, L.; Wood, J.L.; Murphy, N.; Franks, A.E.; Steinbauer, M.J. Precision monitoring of Honey bee (Hymenoptera Apidae) activity and pollen diversity during pollination to evaluate colony health. *Insects* **2023**, *14*, 95. [CrossRef]
53. Leponiemi, M.; Freitak, D.; Monreno-Torres, M.; Pferschy-Wenzig, E.M.; Becker-Scarpitta, A.; Tiusanen, M.; Vesterienen, E.J.; Wirta, H. Honeybees' foraging choices for nectar and pollen revealed by DNA metabarcoding. *Sci. Rep.* **2023**, *13*, 14753. [CrossRef]
54. Leontidou, K.; Vokou, D.; Sandionigi, A.; Brune, A.; Lazarina, M.; De Groeve, J.; Li, M.; Varotto, C.; Girardi, M.; Casiraghi, M.; et al. Plant biodiversity assessment through pollen DNA metabarcoding in Natura 2000 habitats (Italian Alps). *Sci. Rep.* **2021**, *11*, 18226. [CrossRef]
55. Hasiow-Jaroszewska, B.; Boezen, D.; Zwart, M.P. Metagenomic studies of viruses in weeds and wild plants: A powerful approach to characterize variable virus communities. *Viruses* **2021**, *13*, 1939. [CrossRef]
56. Bell, K.L.; de Vere, N.; Keller, A.; Richardson, R.T.; Gous, A.; Burgess, K.S.; Brosi, B.J. Pollen DNA barcoding: Current applications and future prospects. *Genome* **2016**, *59*, 629–640. [CrossRef]
57. Milla, L.; Schmidt-Lebuhn, A.; Bovill, J.; Encinas-Viso, F. Monitoring of honey bee floral resources with pollen DNA metabarcoding as a complementary tool to vegetation surveys. *Ecol. Solut. Evid.* **2022**, *3*, e12120. [CrossRef]
58. Hoffman, V.; Paul, B.; Falade, T.; Moodley, A.; Ramankutty, N.; Olawoye, J.; Djouaka, R.; Lekei, E.; de Haan, N.; Ballantyne, P.; et al. A one health approach to plant health. *CABI Agric. Biosci.* **2022**, *3*, 62. [CrossRef]
59. Fetters, A.M.; Ashman, T.L. The pollen virome: A review of pollen-associated viruses and consequences for plants and their interactions with pollinators. *Am. J. Bot.* **2023**, *110*, e16144. [CrossRef]
60. Pallas, V.; Aparicio, F.; Herranz, M.C.; Sanches-Navarro, J.A.; Scott, S.W. The molecular biology of ilarviruses. *Adv. Virus Res.* **2013**, *87*, 139–181.
61. Pallas, V.; Aparicio, F.; Herranz, M.C.; Amari, K.; Sanchez-Pina, M.A.; Myrta, A.; Sanchez-Navarro, J.A. Ilarviruses of Prunus spp.: A continued concern for fruit trees. *Phytopathology* **2012**, *102*, 1108–1120. [CrossRef]
62. Tzanetakis, I.E.; Gergerich, R.C.; Martin, R.R. A new Ilarvirus found in rose. *Plant Pathol.* **2006**, *55*, 568. [CrossRef]
63. Tzanetakis, I.E.; Postman, J.D.; Martin, R.R. First report of blackberry chlorotic ringspot virus in *Rubus* sp. in the United States. *Plant Dis.* **2007**, *91*, 463. [CrossRef] [PubMed]

64. Poudel, B.; Ho, T.; Laney, A.; Khadgi, A.; Tzanetakis, I.E. Epidemiology of blackberry chlorotic ringspot virus. *Plant Dis.* **2014**, *98*, 547–550. [CrossRef]
65. Bratsch, S.A.; Grinstead, S.; Creswell, T.C.; Ruhl, G.E.; Mollov, D. Characterization of tomato necrotic spot virus, a subgroup 1 ilarvirus causing necrotic foliar, stem, and fruit symptoms in tomatoes in the United States. *Plant Dis.* **2019**, *103*, 1391–1396. [CrossRef]
66. Bonilla, F.R.; Cieniewicz, E. Distribution and diversity of prunus necrotic ringspot virus, prune dwarf virus, and peach latent mosaic viroid in wild *Prunus* spp. in South Carolina and Georgia. *Phytofrontiers* **2022**, *2*, 363–370. [CrossRef]
67. Fuchs, M.; Hily, J.M.; Petrzik, K.; Sanfacon, H.; Thompson, J.R.; van der Vlugt, R.; Wetzel, T.; ICTV Report Consortium. ICTV taxonomy profile: Secoviridae 2022. *J. Gen. Virol.* **2022**, *103*, 1807. [CrossRef]
68. Thompson, J.R.; Dasgupta, I.; Fuchs, M.; Iwanami, T.; Karasev, A.V.; Petrzik, K.; Sanfacon, H.; Tzanetakis, I.; van der Vlugt, R.; Wetzel, T.; et al. ICTV virus taxonomy profile: Secoviridae. *J. Gen. Virol.* **2017**, *98*, 529–531. [CrossRef]
69. Koloniuk, I.; Sarkisova, T.; Petrzik, K.; Lenz, O.; Pribylova, J.; Franova, J.; Spak, J.; Lotos, L.; Beta, C.; Katsiani, A.; et al. Variability studies of two Prunus-infecting fabaviruses with the aid of high-throughput sequencing. *Viruses* **2018**, *10*, 204. [CrossRef]
70. Sasaya, T.; Palacios, G.; Briese, T.; Di Serio, F.; Groschup, M.H.; Neriya, Y.; Song, J.W.; Tomitaka, Y. ICTV Virus taxonomy profile: Phenuiviridae. 2023. *J. Gen. Virol.* **2023**, *104*, 001893. [CrossRef] [PubMed]
71. Wunsch, A.; Hoff, B.; Sazo, M.M.; van Zoeren, J.; Lamour, K.H.; Hurtado-Gonzales, O.P.; Fuchs, M. Viruses of apple are seedborne but likely not vertically transmitted. *Viruses* **2024**, *16*, 95. [CrossRef]
72. Simkovich, A.; Kohalmi, S.E.; Wang, A. First report of little cherry virus 1 infecting sweet cherry in Ontario, Canada. *Plant Dis.* **2021**, *105*, 3780–4145. [CrossRef]
73. Kesanakurti, P.; Belton, M.; Saeed, H.; Rast, H.; Boyes, I.; Rott, M. Comparative analysis of cherry virus A genome sequences assembled from deep sequencing data. *Arch. Virol.* **2017**, *162*, 2821–2828. [CrossRef] [PubMed]
74. Beaver-Kanuya, E.; Harper, S.J. Detection and quantification of four viruses in Prunus pollen: Implications for biosecurity. *J. VIrol. Method* **2019**, *271*, 113673. [CrossRef] [PubMed]
75. Cui, H.; Hong, N.; Wang, G.; Wang, A. Detection and genetic diversity of Prunus necrotic ringspot virus in the Niagara fruit belt, Canada. *Can. J. Plant Pathol.* **2012**, *34*, 104–113. [CrossRef]
76. Kamenova, I.; Borisova, A. Biological and molecular characterization of *Prunus necrotic ringspot virus* isolates from sweet and sour cherry. *Biotechnol. Biotechnol. Equip.* **2021**, *35*, 567–575. [CrossRef]