In-depth study of tomato and weed viromes reveals undiscovered plant virus diversity in an agroecosystem

Abstract

Viruses influence plants in agroecosystems, where their pathogenic nature in crops is primarily studied. Within the same systems, their diversity in non-crop plants and role outside the disease perspective is less known. To better understand their diversity and ecology, we performed an extensive virome exploration focusing on tomatoes and diverse weed species within or surrounding tomato farms. We detected 126 viruses, wherein 80 were novel (70 exclusively from weeds), and demonstrated infectivity of a novel tobamovirus in solanaceous hosts. Diversities of predominantly detected tomato viruses were variable, in some cases, with patterns comparable to global isolates of same species. We phylogenetically analyzed and taxonomically classified novel viruses and showed links between a subgroup of phylogenetically-related rhabdoviruses and a group of taxonomically-related host plants. Around one-third of viruses (n=10) detected in tomatoes were also detected in weeds, which might indicate possible role of weeds as inoculum reservoirs. We showed that even in a relatively well studied agroecosystem, a large part of plant viromes can still be unknown. Extensive biological and ecological insights generated from such holistic agroecosystem virome exploration will aid in anticipating possible emergences of plant virus diseases, and would serve as baseline for post-discovery characterization studies.

Introduction

The awareness on the importance of virus diseases, especially amid an on-going COVID-19 pandemic[1], increased research interest on the exploration of virus diversity across ecosystems, assisted by high-throughput sequencing (HTS)[2–4], and by exploring global nucleotide databases[5–7]. In agroecosystems, viruses are ubiquitous microbes associated with eukaryotic hosts including crop and weed plants, fungi, oomycetes, arthropods, and nematodes, as well as prokaryotes such as bacteria[8,9]. Thus, viruses could influence dynamics of plant populations and individual phytobiomes, directly, or through modulation of other ecological or environmental factors[10]. Due to the parasitic nature, high transmissibility and adaptability of plant pathogenic viruses[11], it was estimated that they account for half of emerging diseases in plants[12], and losses equivalent to around a quarter of expected crop yield[13]. Tomato (*Solanum lycopersicum* L.), which has the highest volume of vegetable production globally[14], is associated with more than 300 viruses including several that are frequently associated with

disease symptoms and yield losses[15]. In recent years plant virologists have witnessed a spread of emerging tomato viruses, such as tomato brown rugose fruit virus (ToBRFV)[16] and tomato leaf curl New Delhi virus (ToLCNDV)[17]. To study such a high diversity of viruses, HTS has become the tool of choice. HTS-based viromics, coupled with bioinformatics tools, enable inference of biological, evolutionary, and ecological insights[18], which impact also fields of virus diagnostics and epidemiology[19].

With HTS, plant virology has greatly shifted from traditional focus on disease-associated relationships[18], to a scalable and unbiased ecosystems-level approach[3]. The study of plant virus pathogenesis and emergence with an ecological approach has also integrated the modulation of various environmental factors. Specifically, studies, which have been collectively reviewed in recent literature[20–22], demonstrated the importance of unmanaged weed plants surrounding cultivated areas in the distribution, ecology and emergence of viruses[23,24]. In tomato, HTS has significantly advanced virus discovery, diversity, ecological and epidemiological studies[15]. Several HTS-based studies in the recent decade have contributed to the current known set of tomato viruses. A survey of tomatoes showing virus disease-like symptoms in China uncovered the presence of 21 known and one novel viruses[25]. Similar studies reported known and novel viruses associated with individual tomato plants or a collection of tomato samples using HTS[26–28]. Findings from these studies significantly contributed to the characterization of the global tomato virome and presented several insights that an HTS-based viromics survey can provide regarding the diversity, ecology and evolution of tomato viruses. In a survey of viruses in tomato and in surrounding *Solanum nigrum* from different locations in France possible exchanges of viruses were observed between wild and cultivated plant species[23]. This suggests that more extensive studies exploring the viromes of the vast diversity of weeds surrounding crop farming areas would be needed to better understand diversity and dynamics of plant viromes in an agroecosystem.

Studies that uncovered plant viromes at the ecosystem scale have focused mainly on discovery, and its epidemiological or ecological implications[23,25,29]. Knowing which species of viruses are present in an agroecosystem and to what extent they influence the host fitness landscape will greatly aid prediction of emergence[30]. To better understand plant virus diversity, ecology and possible emergence of plant virus diseases in an agroecosystem, we selected tomato as a model crop, and investigated a very diverse set of mostly broadleaf dicotyledonous volunteer plants, including weeds (designated simply 'weeds' hereafter) surrounding selected tomato production sites in Slovenia. We examined their viromes using an HTS-based approach, followed by virus characterization, to answer the following questions: (1) How diverse and prevalent are plant

viruses and other virus-/viroid-like agents in tomatoes compared to that of surrounding weeds? (2) Are there overlaps of viruses detected in tomatoes and weeds? (3) Can we identify potential weed reservoirs of known crop viruses, and do weeds harbor an under-sampled virus diversity? (4) Can we identify some viruses in weeds, or those shared between tomatoes and weeds, which could potentially emerge in tomato? (5) What are the phylogenetic relationships of known and novel viruses to known taxa?

This study represented the largest simultaneous survey of viromes of diverse weed species and a crop (tomato) within a cropping system. Our approach can be used to gain insights on plant virus' diversity and dynamics in the wild-cultivated agroecological interface, a known zone for virus spillovers[11,22,31]. The virome dataset could also aid in future tomato virus disease monitoring, thus possibly contributing to the prevention of future virus epidemics in tomato.

Results

Overview of viruses detected in tomatoes and weeds and observed patterns of viral presence and diversity

Tomato (n=293), and weed (n=143, 59 species in 19 families) plant samples, were collected from six localities in Slovenia, wherein 14 farms were visited in 2019 and/or 2020 (Fig. 1a-b, for details see Supplementary Tables 1-3). Sequencing of pooled ribosomal (r)RNA-depleted total RNAs (*i.e.*, composite samples) from these samples, and subsequent bioinformatics analyses revealed 126 viruses, of which 37 are known and 56 are novel classified viruses that could be classified up to known species taxa (49 are exclusively from weeds). The remaining 33 viruses are either isolates of known viruses, which were not yet classified under established taxa in *Riboviria* (n=9), or unclassified putative novel *Riboviria*, satellite RNAs, and viroid-like circular RNAs (n=24, 21 are exclusively from weeds). Thus, the total of novel viruses discovered is 80, of which 70 were found exclusively in weed plants (Fig. 1c, for details see Supplementary Tables 4-6). Farms from the coastal region of SW Slovenia (Piran and Koper), where 51% of the samples were collected, have the highest count of virus species detected (*i.e.*, 57 viruses in Koper and 84 viruses in Piran) (Fig. 1d). The viruses were classified in 21 known plant virus taxa, and four other families that are known to be associated with other eukaryotic hosts (*Dicistroviridae*, *Iflaviridae*, *Lispiviridae*, and *Picornaviridae*) (Fig. 1e). Majority of the detected viruses were from 15 taxa of positive sense (+) single-stranded (ss) RNA viruses (n=61), followed by four families of negative sense (-) ssRNA viruses (n=17) (Fig. 1f). Ten viruses were found both in tomato and weed composite samples from several localities, some

of which are known to have wide host range, and are endemic in tomato (*i.e.*, tomato spotted wilt orthotospovirus (TSWV), cucumber mosaic virus (CMV), tomato mosaic virus (ToMV), and potato virus Y (PVY))[15] (Fig. 1g). Viruses recently detected in tomato (*i.e.*, Solanum nigrum ilarvirus 1 (SnIV1), Ranunculus white mottle ophiovirus (RWMV), tomato matilda virus (TMaV))[23,32–34], a known insect (Aphis glycines virus 1 (AGV1)) and a known fungal (Leveillula taurica associated rhabdo-like virus 1 (LtaRLV1)) virus, and a novel virus (plant associated tobamo-like virus 1 (PaToLV1)) were also detected in both sample types. Among the viruses detected both in tomatoes and weeds, there were several cases in which we could detect the same virus within the same location in both sample types: seven viruses (TSWV, CMV, ToMV, PaToLV1, LtaRLV1, PVY, and TMaV) with such pattern were observed.

Three known viruses were detected for the first time both in tomatoes and in Slovenia, and five others were detected for the first time in the country, (*i.e.*, were not reported before in local comprehensive records)[35] (Fig. 1h). Lastly, other known crop viruses were detected in composite weed samples, including two potyviruses (watermelon mosaic virus (WMV) and carrot thin leaf virus (CTLV)), ilarvirus (Prunus virus I (PrVI)), closterovirus (beet yellows virus), luteovirus (soybean dwarf virus), cucumovirus (peanut stunt virus), fabavirus (broad bean wilt virus 1), polerovirus (barley virus G), and potexvirus (white clover mosaic virus).

To get general insights into the association of detected viruses with observed disease symptoms, we have compared the number and overlap of detected viruses in sampled symptomatic and asymptomatic tomatoes. Out of the 45 viruses detected in tomato composite samples, only four (8.9%) were exclusively detected in asymptomatic tomatoes, and 23 (51.1%) exclusively in symptomatic tomatoes (Fig. 2a). Eighteen (40.0%) viruses were detected in both types of tomato samples, of which, six (*i.e.*, southern tomato virus (STV), CMV, PVY, olive latent virus 1 (OLV1), TMaV, and ToMV) were detected in at least six composite samples. A total of seven new viruses, and eight known but still unclassified arthropod, fungal and oomycete viruses were detected in tomatoes. Using RT-PCR assays (for details, see Methods and Supplementary Table 7), we confirmed the presence of a subset of mostly novel viruses in weeds and tomatoes, and identified key hosts that could be potential transmission hubs, or alternate hosts (Fig. 2b, for details see Supplementary Table 9).Some examples include: SnIV1 detected in tomatoes and in another weed host, *Physalis* sp., from a single farm, PaToLV1 detected in tomatoes and in *Convolvulus arvensis* also in a single farm, and TMaV detected in tomatoes and in three other weed species (*i.e.*, *Chenopodium* sp., *Ranunculus repens*, and *Erigeron annuus*) that span five localities. RWMV was detected both in *Solanum nigrum*, and in four pools of tomato samples from three different localities. Twelve novel viruses (discussed in succeeding sections),

including six rhabdoviruses and three tombusviruses, were detected in four Asteraceae species (*i.e., Picris echoides, Cichorium intybus, Taraxacum officinale,* and *Cirsium arvense*).

To gain further insights on the population genomic diversity of the most prevalent tomato viruses, we examined populations of 12 viruses (viruses for which we were able to assemble at least three full genomes). STV, ToMV, TMaV and TSWV showed the narrowest range of pairwise nucleotide identities, lowest nucleotide diversity and number of polymorphic sites, while Physostegia chlorotic mottle alphanucleorhabdovirus (PhCMoV), RWMV, and potato virus S (PVS) have a moderate level of diversity (Fig 3a, Supplementary Tables 11-22). PVY, CMV, potato virus M (PVM), tomato betanucleorhabdovirus 2 (TBRV2), and tomato vitivirus 1 (TomV1) showed wide range of pairwise nucleotide identities and high number of polymorphic sites (Fig 3a, Supplementary Tables 11-22). Sliding window analysis of nucleotide diversity for two novel tomato viruses found in Slovenia (TBRV2 and TomV1), did not pinpoint specific regions of their genomes that might be more variable than others (Fig. 3b-c). Divergent lineages detected for both TBRV2 and TomV1, resulted in a high genome-wide variability depicted as fluctuations of nucleotide diversity along the genome (Fig. 3b-c).

(-)ssRNA viruses in tomatoes and weeds: discovery of novel orthotospovirus, an ophiovirus, and a divergent bunya-like virus

Several (-)ssRNA viruses were detected in both, tomatoes and weeds. A novel orthotospovirus, Mercurialis orthotospovirus 1 (MerV1), was detected in *Mercurialis annua* (Fig. 4a) in four composite samples from three localities. Pairwise comparison of RNA-dependent RNA polymerase (RdRp) amino acid (aa) sequences showed 72.4-76.0% identity of MerV1 to related *Tospoviridae* species. MerV1 has genome segments similar to plant orthotospoviruses (Fig. 4b,e). Phylogenetic analyses based on conserved RdRp aa sequences of orthotospoviruses revealed that MerV1 is related to viruses in phylogroup C (*i.e.*, a clade of phylogenetically-related orthotospoviruses)[36] (Fig. 4i).

A novel fimovirus, Artemisia fimovirus 1 (ArtV1) was detected in *Artemisia verlotiorum*. It is 30.2-46.1% identical to related *Fimoviridae* species based on RdRp aa comparison, and has genome organization similar to plant fimoviruses (Fig. 4a,c,f). Phylogenetic analyses with known fimoviruses revealed that ArtV1 is related to *Perilla mosaic virus*, making it the fourth member of the divergent phylogroup IV of *Fimoviridae* (*i.e.*, a clade of phylogenetically-related fimoviruses)[37,38] (Fig. 4i).

A novel bunya-like virus, closely related to *Fimoviridae* and *Tospoviridae*, was detected in seven symptomatic tomatoes using RT-PCR assays, thus named tomato associated bunya-like virus 1 (TaBLV1). Pairwise comparison of RdRp aa sequences showed that TaBLV1 is only 20.8-22.4% identical to closely related bunyaviruses, however, only L- and M-like genome segments were found for TaBLV1 (L, M and S segments are characteristic of plant-infecting orthotospoviruses) (Fig. 4a,g). TaBLV1 is more closely related to orthotospoviruses based on RdRp phylogenetic analyses (Fig. 4i).

We assembled for the first time, the full genome of RWMV, which was previously detected in tomatoes and peppers in Slovenia[32] (Fig. 4a,d,h). Using RT-PCR assays, RWMV was detected in three different localities, in eight tomatoes, and one *S. nigrum*, which is a newly identified associated host. All RWMV isolates form a single clade (100% bootstrap support (BS)), but Slovenian isolates cluster separately (98.9% BS) from the Australian and Italian subclade (96.6% BS) (Fig. 4j). For details of the pairwise comparisons, see Supplementary Table 23-25. Phylogenetic trees where the subtrees in Fig. 4 were derived depicting *Tospoviridae* and *Fimoviridae* families in one tree, and *Aspiviridae* with other related viruses in one tree, are shown in Supplementary Fig. 4-01 and 4-02, where the GenBank accession numbers of the sequences used in the analyses are also indicated.

Discovery of novel rhabdoviruses and their links to Solanaceae and Asteraceae hosts

In this study, nine novel rhabdoviruses were discovered in tomatoes and weeds. Using RT-PCR assays, tomato alphanucleorhabdovirus 1 (TARV1), tomato betanucleorhabdovirus 1 (TBRV1) and TBRV2 were confirmed in tomatoes. Five were detected in Asteraceae weeds: *P. echoides* (Picris betanucleorhabdovirus 1 (PBRV1), Picris cytorhabdovirus 1 (PiCRV1)), *T. officinale* (Taraxacum betanucleorhabdovirus 1 (TarBRV1) and Taraxacum cytorhabdovirus 1 (TCRV1)), *Cirsium arvense* (Cirsium cytorhabdovirus 1 (CCRV1)). One cytorhabdovirus was detected in *Pastinaca sativa* (Apiaceae) (Pastinaca cytorhabdovirus 1 (PaCRV1)). TarBRV1 and TCRV1 were found to be co-infecting a single *T. officinale* sample, and PBRV1 and PiCRV1 co-infecting one *P. echoides* sample (Fig. 5a). Viruses were distinguished based on pairwise comparison of full genome nucleotide (nt) sequences (Fig. 5b-d). All novel rhabdoviruses have genomes typical of their genus, except for the highly divergent PiCRV1, which might have a putative bipartite genome, or the two contigs were not assembled together in our analyses (Fig. 5e). PiCRV1 is only 29.1-33.5% identical to closely related cytorhabdoviruses, based on comparison of RdRp aa sequences (Supplementary Table 26-B).

Investigation of virus-host relationships was done by drawing links between rhabdovirus phylogram and plant host cladogram (Fig. 5f). This revealed that rhabdoviruses from certain clades are often associated with Solanaceae and Asteraceae plant hosts. In particular, a clade of closely related alphanucleorhabdoviruses, including one discovered from this study (potato yellow dwarf virus, joa yellow blotch virus, eggplant mottled dwarf virus, PhCMoV, TARV1), was associated with eight plant species from family Solanaceae, both experimentally and as natural hosts. A few associations of betanucleorhabdoviruses discovered in weeds (PBRV1, TarBV1), and in tomatoes (TBRV1, TBRV2) with solanaceous plants were also found. Two novel betanucleorhabdoviruses (PBRV1, TarBV1) from this study and one known virus (Sonchus yellow net virus) were associated with three Asteraceae plants. For details of the pairwise comparisons, see Supplementary Tables 26-28. An identical rhabdovirus phylogenetic is shown in Supplementary Fig. 4-03, where the GenBank accession numbers of the sequences used in the analyses are indicated.

Detection of a kitavirus in tomatoes and other known and novel *Martellivirales* species

The recently described tomato fruit blotch virus (ToFBV, *Kitaviridae*)[28,39,40], was detected in two symptomatic tomato leaf samples from Slovenia (Fig. 6a). Infectivity and possible vector of ToFBV were not reported yet, and it is currently classified in the genus *Blunervirus*. Pairwise comparison of conserved RdRp aa sequences of blunerviruses (Fig. 6b), revealed high molecular divergence among members, with only 30.9-41.3% pairwise identity between species. Phylogenetic analyses using conserved RdRp aa sequences showed that all known ToFBV isolates from a single clade (100% BS) (Fig. 6k).

SnIV1, a recently described ilarvirus, was detected in *Physalis* sp., which is the sixth distinct source association with the virus, aside from three other plant species (*S. lycopersicum, S. nigrum*, and an unidentified legume plant)[23,29], and grapevines infected with a fungus (*Erysiphe necator*) and an oomycete (*Plasmopara viticola*)[41,42]. PrVI, recently discovered in sweet cherry[43], was detected in our study in *P. echoides*, co-infected with PiCRV1. Pairwise comparison of RdRp aa sequences of the isolates of SnIV1 and PrVI from this study showed that they are >98% identical to other isolates of each species (Fig. 6c). A novel subgroup II ilarvirus, tomato ilarvirus 1 (TIV1), was detected in an asymptomatic tomato sample, and is at most 83.3% identical to closely related ilarviruses based on RdRp aa sequence comparison (Fig. 6d,h).

Three new *Closteroviridae* species were detected in weeds through HTS (Fig. 6e,f,i).Two new alphaendornaviruses with their typical protein domains were also discovered, one of which is from a pool of symptomatic tomatoes (Fig. 6g,j). Novel ilarvirus, closterovirus and alphaendornavirus all form distinct phylogenetic clades with related viruses (>60% BS) (Fig. 6l-n). For details of the pairwise comparisons, see Supplementary Tables 29-33. Expanded versions of the collapsed phylogenetic trees in Fig. 6 are shown in Supplementary Fig. 4-04, 05, 06, and 07, where the GenBank accession numbers of the sequences used in the analyses are indicated. Details of InterPro domain search are in Supplementary Tables 43-44.

Characteristics of a new tobamovirus, and a divergent tobamo-like virus species

A new tobamovirus, Plantago tobamovirus 1 (PTV1), was discovered in *Plantago major* (Fig. 7a), and percent pairwise identities based on whole genome nt sequences revealed that PVT1 is 80.4-85.7% identical to closely related tobamoviruses, which is below the current species demarcation at <90% (Fig. 7b). PTV1 has genome composition and organization typical of tobamoviruses (Fig. 7c). Maximum likelihood phylogenetic analyses using conserved methyl transferase-helicase aa sequences showed that PTV1 is closely related to ribgrass mosaic virus (RMV), turnip vein clearing virus (TVCV), youcai mosaic virus (YoMV), and wasabi mottle virus (WMoV) (Fig. 7h, highlighted in yellow). Phylogenetic analyses using full nt genome sequences of all known isolates from this tobamovirus clade including RMV, TVCV, YoMV, WMoV revealed relatedness but distinct divergence of PTV1 from the RMV clade (Fig. 7i). By superimposing information on host and country of origin (Fig. 7l), we found that aside from PTV1, *P. major* (Plantaginaceae) is a common host for RMV from Germany, and for RMV and TVCV in another eco-geographically distinct country, New Zealand[44,45]. Systemic infection with disease symptoms, which was confirmed by RT-PCR, was observed in *Nicotiana benthamiana* and *Nicotiana clevelandii* that were mechanically-inoculated with PTV1-infected *P. major* leaf tissues. Inoculated tomato plants remained asymptomatic, however, PTV1 was detected by RT-PCR in systemic leaves until 28 dpi but not at 35 dpi and beyond (Fig. 7e-g, Supplementary Fig. 5), which might be a consequence of either transient infection or cross-contamination during the experiment. Transmission electron microscopy of a *N. clevelandii* infected plant revealed PTV1 virions that are rigid rods, typical for tobamoviruses (Fig. 7f). HTS of the same plant confirmed the presence of PTV1 in systemic tissues (Supplementary Fig. 5).

A tobamo-like virus, PaToLV1, was detected in symptomatic tomatoes and in *Convolvulus arvensis* (Fig. 7a). Pairwise identity analysis using RdRp aa sequences showed that PaToLV1

is <31% identical to Plumeria mosaic virus (*Tobamovirus*), tobacco rattle virus (*Tobravirus*) and Beihai chrarybdis crab virus 1 (BCCV1, unclassified *Riboviria*) (Supplementary Table 35). PaToLV1 has genome structure similar to BCCV1, except for an additional unknown open reading frame (ORF) (Fig. 7d). Phylogenetic analyses based on the RdRp and CP aa sequences both revealed that PaToLV1 isolates form a monophyletic clade, which is clustered together with BCCV1 (Fig. 7h). For details of the pairwise comparisons, see Supplementary Table 34-35. Detailed version of the *Virgaviridae* phylogenetic trees in Fig. 7 are shown in Supplementary Fig. 4-08-A (methyl transferase, helicase-based) and Supplementary Fig. 4-08-B (coat protein-based), where the GenBank accession numbers of the sequences used in the analyses are indicated. Details of InterPro domain search for virus genomes are in Supplementary Tables 45-46.

Characteristics of novel *Potyviridae* species, and crop-infecting potyviruses found in weeds

Aside from PVY, two known potyviruses (WMV and CTLV), previously detected in Slovenian crops and ornamentals[46,47], were detected in pools of weed plants from this study. Full genomes of these viruses were detected in composite samples from five different tomato farms, encompassing two localities in the Primorska region (SW Slovenia: Koper, Piran). Henbane mosaic virus, previously found in tomatoes from Slovenia[48], was detected in a composite sample of open field-grown symptomatic tomatoes. Three novel members of *Potyviridae* (Mentha macluravirus 1 (MenMV1), Plantago potyvirus 1 (PlaPV1) and Rumex potyvirus 1 (RumPV1)) were discovered in weeds showing virus disease-like symptoms (Fig. 8a). Pairwise comparison of full-length (nt, aa) polyprotein ORF distinguished the new species from the known ones based on molecular demarcation criteria for *Potyviridae* (<82% aa, <76% nt)[49] (Fig. 8b-e). The genome and polyprotein ORF of novel potyviruses were characterized, and all protein domains, typical of macluraviruses and potyviruses were found (Fig. 8f). Phylogenetic analyses based on conserved RdRp aa sequence placed MenMV1 in a clade with artichoke latent virus and Narcissus latent virus (100% BS) (Fig. 8g). PlaPV1 was placed in a clade with cucurbit vein banding virus (83.1% BS), and RumPV1 was placed in a clade with lotus latent virus and Calystegia hederacea virus (100% BS).

Two different lineages of broad-leafed dock virus A (BDVA), were also detected. A lineage with a considerably low pairwise identity compared with original isolate (86.6% aa, 76.0% nt) was found in *Rumex crispus*. For details of the pairwise comparisons, see Supplementary Tables 36-37. An expanded version of the collapsed *Potyviridae* phylogenetic tree in Fig. 8, is shown in Supplementary Fig. 4-09, where the GenBank accession numbers of the sequences used in

the analyses are indicated. Details of protein domain and cleavage site search for virus genomes are in Supplementary Table 47.

Known tomato tombusviruses, and discovery of new *Tombusviridae* species and tombusvirus-like associated RNAs in weeds

Genomes of three known members of *Tombusviridae* (tomato bushy stunt virus (TBSV), tobacco necrosis virus A (TNVA), olive latent virus 1 (OLV1)), and partial genome of olive mild mosaic virus (OMMV), were assembled from sequences derived from composite tomato samples. Five new viruses classified in four different *Tombusviridae* genera were discovered in weeds showing virus disease-like symptoms (Fig. 9a). These include two new umbraviruses (Pastinaca umbravirus 1 (PasUV1) and Picris umbravirus 1 (PicUV1)), one new aureusvirus (Convolvulus aureusvirus 1 (ConAV1)), one new pelarspovirus (Calystegia pelarspovirus 1 (CalPV1)), and one new alphacarmovirus (Cichorium alphacarmovirus 1 (CicAV1)). Three sequences, with similarity to self-replicating, coat-dependent RNA replicons, called tombusvirus-like associated RNAs[50], were also detected in composite weed samples. Pairwise comparisons of full genome nt or RdRp (replicase) aa sequences were done, whichever is appropriate for species demarcation (Fig. 9b-f). Genome organization typical for the genera was determined for the new *Tombusviridae* members. Novel unknown ORFs were detected in Picris umbravirus 1 and CalPV1 (Fig. 9g). Genome compositions and organizations of new tombusvirus-like associated RNAs were similar to those previously described[50], except for the two plant associated tombusvirus-like RNA 1 isolates, in which ambisense ORFs were detected. Phylogenetic analyses placed the novel tombusvirus-like associated RNAs in a clade with Arracacha latent virus E associated RNA, a member of subgroup III tombusvirus-like associated RNAs (78.7% BS)[50]. Novel tombusviruses were likewise placed in clades with known species of their respective genera (>60% BS) (Fig. 9h). For details of the pairwise comparisons, see Supplementary Tables 38-42. An expanded version of the collapsed *Tombusviridae* phylogenetic tree in Fig. 9, with providence virus (*Carmotetraviridae*) as outgroup, is shown in Supplementary Fig. 4-10, where the GenBank accession numbers of the sequences used in the analyses are also indicated.

Discovery of other numerous viruses, satellite RNAs, and viroid-like circular RNAs

Aside from viruses mentioned above, a diverse set of 37 classified known and novel viruses, 21 putative virus-like *Riboviria* species (*i.e.*, cannot be classified in known virus taxa), and four known and new satellite RNAs were also detected (Supplementary Table 5). Genome organization of these viruses and phylogenetic trees including the viruses are shown in

Supplementary Fig. 1 and 4. Aside from STV, other dsRNA viruses were discovered, including six new *Partitiviridae* members from weeds, two of which were detected in 2019 and 2020 at the same farm. Three new *Totiviridae* members were discovered, two of which are phylogenetically related to plant-associated members of the family. Circular DNA viruses were discovered, including two new caulimoviruses found in weeds, and partial genome of a new geminivirus (Calystegia geminivirus 1), from a symptomatic *Calystegia* sp. sample co-infected with CalPV1. To our knowledge, this could be one of the few geminiviruses in wild plants from Europe, since the majority of geminiviruses were found in Asia and the Americas[51]. Several other (+)ssRNA viruses were also discovered and classified under *Alphaflexiviridae* (n=1), *Dicistroviridae* (n=3), *Iflaviridae* (n=1), *Secoviridae* (n=5), and *Solemoviridae* (n=3). The new alphaflexivirus (Pastinaca potexvirus 1) was detected in a *P. sativa* sample co-infected with PasUV1 and PaCRV1. One new satellite virus (tomato albetovirus 1) and new satellite RNA (TBSV satellite RNA C), associated with *Tombusviridae* species[52,53], were detected in tomatoes. Six putative virus-like sequences detected were previously associated with arthropod[4], fungi[41], or oomycete[42] hosts, while other 15 are highly divergent ones with unidentified hosts. Using a custom discovery pipeline based on SLS-PFOR2[54], we assembled and screened 4249 circular RNAs, and identified 344 viroid-like candidates (Supplementary Table 6). Five viroid-like circular RNAs were preliminarily selected, based on predicted secondary structure, %GC content, and degree of branching. Genome circularity was confirmed for Taraxacum viroid-like circular RNA 1 (Supplementary Fig. 2), which was detected in a *T. officinale* sample co-infected with TarBV1.

Discussion

Virus diversity in specific crops is relatively well-studied compared to weed plants or crop's wild relatives[20,21]. A few studies used HTS to uncover virus diversity and possible exchanges in the cultivated and wild compartments of agroecosystems[23,29]. Here, we detected 37 known and 56 novel viruses, which were classified in established virus taxa, and at least 33 unclassified *Riboviria* members. This indicates a highly understudied diversity of novel viruses in an agroecosystem, especially in weeds that might serve as reservoirs of viruses that could infect crops. This study represents the largest survey of viromes of diverse weed species within tomato agroecosystems or any cropping system. 49 novel viruses classified in known taxa, and additional 21 novel unclassified viruses, were detected in weeds, some of which are also present in tomatoes (*e.g.*, PaToLV1, LtaRLV1). Overall, the viromes were dominated by 61 (+)ssRNA viruses (under *Martellivirales*, *Potyviridae*, and *Tombusviridae*) and 17 (-)ssRNA viruses (under *Mononegavirales*). Another study looked at the exchanges of viruses between tomato

and a wild relative, *S. nigrum*, in France, wherein PVY and SnIV1 were found to be present in both, cultivated and wild compartments[23]. Here we observed the same pattern, finding PVY and SnIV1 in tomato and weed samples (*Physalis* sp.), and in addition, we identified new weed hosts of viruses found in tomatoes, such as RWMV found in *S. nigrum.* We also detected in weed samples known viruses of other crops, which were previously detected in the country (*e.g.*, WMV, CTLV). The detection of crop-infecting viruses in weed plants implies the possible role of weeds as wild reservoirs and alternate hosts for such viruses[55].

In this study, around two-thirds (61/93) of the known and novel classified viruses detected were found exclusively in weeds surrounding tomato farms, 22 were found exclusively in tomatoes, and 10 viruses were found both in weed and tomato samples. The shared viruses include both generalist viruses (*i.e.*, viruses with known wide host range that includes wild plants) some of which are also important pathogens of tomatoes[15] (*e.g.*, TSWV, CMV, PVY). Overall, a small proportion of viruses was associated with plants from different families, which might suggest a wide host range for these viruses. Associated host plants were further confirmed using RT-PCR for 36 out of all 126 viruses detected, of which, only three (*i.e.*, TMaV, PaToLV, and Rumex potyvirus 1) were associated with at least two plants from different botanical families. Such small proportion of generalist viruses is consistent with previous hypotheses that most viruses evolved and settled as specialists that are only adapted to a few hosts (*i.e.*, have narrow host ranges), because of the fitness cost and evolutionary constraints associated to a generalist lifestyle[56]. This also implies presence of possible barriers for host-switching that could be established in the course of virus-host co-evolution[11]. Nevertheless, in this study, we collected just a portion of larger plant diversity and did not confirm plant infectivity of these viruses. Thus, post-discovery characterization and extending sampling and analysis to other crops and weeds, will help confirm these hypotheses.

Viruses infecting plants may not cause any disease symptoms, because of a rather neutral or beneficial interaction with its host, latent infection, host resistance, or modulation of environmental factors[10,57]. A previous study of tomato viruses in China focused only on symptomatic samples[25]. In our study, HTS and RT-PCR assays further revealed viruses that are shared between tomatoes and weeds, and those that were detected in both asymptomatic and symptomatic tomatoes. In tomatoes, 45 different viruses were detected, 18 of which were found in both asymptomatic and symptomatic tomatoes. This observation has an important implication when aiming to capture a significant breadth of virus diversity through sampling across ecosystem scales. This observation also gave important insights on possible symptom

masking due to virus-tomato-environment interactions leading to latency, or due to host resistance or tolerance.

Monitoring virus diversity and evolutionary dynamics of economically important and widespread viruses could help anticipate emergence of new variants, thus preventing disease outbreaks[30]. Here, we investigated the molecular diversity of viruses based on full genome nucleotide identities, and variability among virus isolates based on genome-wide polymorphic sites and nucleotide diversity scanning. These were determined on the most prevalent viruses in tomatoes, wherein some were detected for the first time in Slovenian tomatoes and are present in several localities, such as TMaV. Low diversity was observed among the isolates of ToMV and the persistent STV, which is consistent with previous reports on their global diversity[58,59]. TSWV, in contrast to its considerable diversity at the global scale[60], showed low diversity in the samples analyzed here.. Sequenced genomes of PVY, CMV, PVM, PhCMoV, RWMV showed moderate levels of variability. PhCMoV is a recently detected, and widespread pathogen of tomato and other crops in Europe, where considerable level of diversity was observed among the isolates[61,62]. RWMV was recently detected in Slovenian peppers and tomatoes, where isolates showed moderate diversity as well[32]. Moreover, two new viruses (TBRV2, TomV1) associated with tomatoes, showed high level of variability, which might imply that these viruses are circulating in the area for a long time already and/or are undergoing diversifying evolution. Further diversity and evolution studies of viruses found in tomatoes in our virome survey, such as ToFBV, PhCMoV, TMaV, SnIV1, RWMV, TBRV2, and TomV1 would bring insightful information about the nature of the observed variations, *e.g.*, if they are the result of neutral processes or are subjected to different kind of selection pressures and/or possible ongoing adaptation to tomato.

We observed high diversity of novel viruses at higher taxonomical levels. The most illustrative example is detection of 11 rhabdoviruses, of which 9 are newly discovered species. Three new rhabdoviruses phylogenetically-related to plant rhabdoviruses were associated with tomato, which increases the count of known tomato rhabdoviruses from two to five. In weeds, six new rhabdoviruses were detected, primarily associated with Asteraceae species. Our observations added to the discoveries made in other studies, *e.g.*, a recent discovery of 27 new viruses that are phylogenetically-related to plant rhabdoviruses through homology searches in the transcriptome shotgun assembly database of NCBI[63]. Some rhabdoviruses are known to replicate in its arthropod vector as well[64], thus, further infectivity tests are needed to confirm their infectivity and pathogenicity in associated plant hosts.

Among the most economically important viruses of tomato, tobamoviruses are the most problematic in recent years, due to the emergence of ToBRFV[16]. We detected a novel tobamovirus (PTV1) in *Plantago major* surrounding a greenhouse farm in Slovenia. We demonstrated the systemic infection of PTV1 in various Solanaceae hosts, but more extensive biological and genetic characterization is needed to understand its biological characteristics. Nevertheless, such discovery calls for further research on risks associated with tobamoviruses as one of the major causes of disease outbreaks in tomato and other solanaceous crops[15]. A novel tobamo-like virus (PaToLV1) was also detected in tomatoes and in *Convolvulus arvensis*. A previous study showed the evolutionary relationship of the most similar known virus, BCCV1, to *Virgaviridae* and *Martellivirales* families[4], which could also be the case for PaToLV1.

Moreover, besides viruses, we also attempted to uncover new viroid-like agents in our dataset, with the aim to contribute to their possibly still undiscovered diversity[54]. We obtained five candidate viroids, wherein one was experimentally confirmed to be circular and structurally similar to members of *Avsunviroidae* due to the presence of a hammerhead ribozyme motif[65]. These results suggest that further research in this direction and development of new bioinformatics tools for viroid discovery within sequencing datasets could uncover even larger hidden diversity of these non-protein coding pathogenic agents.

Aside from studying virus diversity and evolution, understanding of host-virus co-evolutionary relationships is also essential in predicting possible virus emergence through host-switching[66]. We observed an association of a clade of closely related alphanucleorhabdoviruses with plant species from family Solanaceae. However, the general topology of tanglegram suggested discordant evolutionary historic events between plant rhabdoviruses and their hosts, which is similar to what was previously shown in animal/insect rhabdoviruses[66]. Likewise, we found frequent association of different but related viruses with a single plant host. This is the case of *Plantago major*, which was associated with PTV1, RMV, and TVCV isolates from four different countries from two very distinct eco-geographical regions. This subgroup was originally associated with brassica hosts[67] and recently, as reported in this study, with several Plantaginaceae and Actinidiaceae plants. This observation indicates a possibility of virus spread through introduction of plants, followed by diversifying evolution through a mix of confounding environmental and host-related factors[68].

The present study uncovered the diversity of viromes of plant species in defined agroecosystems linked with tomato production. Similar systematic surveys in various crop

agroecosystems, which could also include fungal, oomycete, arthropod or nematode vectors and environmental media, such as soil and water, should in future provide more virome data from still undescribed compartments of agroecosystems. Collectively, this will help to a progressive uncovering of the fraction of unknown viruses in the global virosphere[69], and will contribute to prediction of possible virus emergence in the wild-cultivated agroecological interface, a zone of possible virus spillovers and disease outbreaks[11,22,31].

Methods

Sample collection and processing

Tomato and weed plants, within or in the immediate surroundings of tomato farming fields or greenhouses were collected in Slovenia during summer of 2019 and 2020. Fourteen farms at six different localities were visited, spanning central (Dol pri Ljubljani, 1 farm), west (Miren-Kostanjevica, 2 farms; Nova Gorica, 1 farm), southwest (Koper, 4 farms; Piran, 3 farms), and southeast (Novo Mesto, 3 farms) regions of Slovenia. A total of 436 samples were collected, full details of which are in Supplementary Table 1. Asymptomatic tomatoes were randomly sampled within an area, while symptomatic tomatoes and weeds were selectively sampled based on appearance of virus disease-like symptoms most commonly shown as foliar discoloration (*e.g.*, chlorosis, mosaic, yellowing), leaf deformation (*e.g.*, folding, curling), fruit deformation (*e.g.*, mottling, marbling), and systemic symptoms (*e.g.*, general stunting, rosetting), among others. Selected photographs of collected plants, with a notion of associated detected viruses, are shown in Supplementary Fig. 3 and Supplementary Table 9. Most tomato samples collected were in the early fruiting or fruit harvesting stage, while the most weed plants were sampled at the mature or flowering stage. To aliquot tissues for RNA extraction, leaves or fruits were obtained equally from different parts of the plant to account for possible uneven distribution of viruses within the plants. Leaf and fruit tissues were separately aliquoted in cases where these were collected from a single plant. Collected tissues were stored at -80°C.

RNA extraction and sequencing

Total RNAs were extracted from individual plant samples using RNeasy Plant Mini Kit (Qiagen, USA). RNA quality and quantity were checked using QuBit fluorometer (Thermo Fisher Scientific, USA), and NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). RNAs were then pooled equimolarly into composite samples based on plant type (*i.e.*, tomato or weed), health status (*i.e.*, symptomatic or asymptomatic), and the sampling location (Supplementary Table 2). A total of 67 composite samples were cleaned, concentrated and

treated with DNase (RNA Clean and Concentrator, Zymo Research, USA). Alien controls from total RNAs extracted from *Phaseolus vulgaris* infected with Phaseolus vulgaris alphaendornavirus 1, Phaseolus vulgaris alphaendornavirus 2 and Phaseolus vulgaris alphaendornavirus 3 were included into each sequencing run. The 2019 and 2020 sample set were separately sent to Macrogen, Inc. (South Korea), for library preparation and high-throughput sequencing. Sequencing libraries, including depletion of ribosomal RNA using Ribo-Zero rRNA Removal Kit (Plant) (Illumina, USA), were prepared suitable for 150 bp paired-end sequencing using TruSeq RNA Library Prep Kit (Illumina, USA) and sequenced using Illumina HiSeq 2500 platform.

Total RNAs from inoculated plants (discussed below) were extracted as mentioned above and depleted of ribosomal RNA using RiboMinus™ Plant Kit (Invitrogen, USA), then ligated with poly-A sequences using E. coli Poly(A) polymerase (NEB #M0276, UK). Library preparation was done using the PCR-cDNA Barcoding kit (SQK-PCB109, version PCB_9092_v109_revC_10Oct2019, Oxford Nanopore Technologies, UK), prior to sequencing using Oxford Nanopore Technologies MinION platform (Oxford Nanopore Technologies, UK) and base-calling following a previously described workflow[70].

Sequence quality screening, trimming and virus genome assembly

Raw reads were trimmed, screened for quality and analyzed following a previously described pipeline for plant virus detection using HTS[71]. Contigs were primarily assembled from the filtered reads using CLC Genomics Workbench (GWB) v. 20[71] (Qiagen, USA). Within the used pipeline, virus and virus-like reads and contigs were initially identified by mapping trimmed reads/contigs to virus RefSeq database (version from Jul. 2020)[72] and viral domains searches in contigs against pFam database v. 33. Candidate viral contigs were later confirmed by homology search using BLASTn against the NCBI nucleotide (nt) and BLASTx[73] against NCBI non-redundant protein (nr) databases from Dec. 2020. Assembly in metaSPAdes v. 3.14[74] was also implemented to recover longer contigs in some cases, where these were not assembled in CLC-GWB. Consensus genome sequences of detected viruses were reconstructed using de-novo assembled contigs and/or iterative reads mapping to the most similar reference sequences obtained from NCBI GenBank. Since datasets were derived from pooled samples, consensus viral genomes were reconstructed only for the viruses with observed low to moderate population diversity (determined after manual inspection of the mapping files), indicative of infection with a single viral lineage. As a final checkup, mapping of reads of corresponding datasets to reconstructed consensus virus genomes was implemented (with 95% identity and

read length fraction). Overall sequencing results and statistics, and information on sequencing read archive (SRA) metadata are given in Supplementary Table 3. The internal controls were used to check the prevalence of sequencing cross-talks. A threshold of <0.00001% of total reads in the library was set, based on the general virome composition of the internal controls, to classify sequences as either a possible contamination, crosstalk, or low titer virus infection.

For the sequences obtained from inoculated plants (discussed below) using MinION platform, quality screening was done following a customized workflow[70], that uses the programs from the NanoPack program[75] for quality screening and visualization. Reads were assembled using Racon[76]. The mapping program Minimap2[77] run in Geneious Prime software v. 2022.1.1 (Dotmatics, USA), was used for mapping reads to viral RefSeq (version from May 2022) and consensus genomes of viruses detected in this study.

Virus genome annotation and classification

Genomes of detected viruses were annotated using the ORF prediction tool in CLC-GWB v. 20, and checked based on known genome organization and open reading frame composition reported in the ICTV website[78], and in peer-reviewed publications. InterProScan was used to identify known and unknown protein domains[79], and in the case of potyviruses, homology alignment in BLASTp (E-val<10^{-4}) with well-characterized known species was implemented to detect gene products and their start and cleavage sites[80]. Viruses were taxonomically classified based on the percent pairwise identity obtained in SDT v1.2[81], and other genomic criteria imposed by the ICTV[78], as of Oct. 31, 2021. Multiple sequence alignments were made for each genus or family based on the recommended gene or genome segment by the ICTV. Unaligned ends were trimmed, and trimmed alignments were used for pairwise identity comparisons. Details of the genes or genome segments used, and the results of the pairwise identity comparisons are indicated in Supplementary Tables 23-42. Classifications were then confirmed by analyses of phylogenetic relationships with known virus taxa (described below).

Genome assembly and screening for putative viroids

Viroid-like circular RNAs were assembled using the SLS-PFOR2 program[54]. BLASTn searches[73] against the NCBI nt database and BLASTx searches against NCBI nr database, from Dec. 2020, were implemented to filter out sequences from known organisms, and sequences that code for proteins (E-val<10^{-4}). Filtered contigs were re-examined by remapping (95% identity and read length fraction) virtually-diced reads (generated as a part of SLS-PFOR2 pipeline) and trimmed reads to the assembled circular RNAs. Contigs with average mapping

depth below 10 were manually discarded. Contigs were again filtered based on the presence of two or more rotationally identical contigs of the same length (*i.e.*, indicating the presence of (+) and (-) strands). After selecting for rotational identical contigs, presence of viroid-like secondary structure motifs (*e.g.*, avsunviroid hammerhead ribozyme and rod-like pospiviroid structures) were predicted using mFold[82] and forna[83]. Low structural Gibbs free energy and visual inspections such as high degree of base paring, or degree of branching were the criteria used to preliminarily select for putative novel viroids.

RT-PCR assays and Sanger sequencing

PCR primers were designed using Primer3Plus[84], and RT-PCR assays were designed to identify individual plant host(s) of selected viruses detected using HTS of pooled samples (Supplementary Table 7). RT-PCRs were performed using OneStep RT-PCR kit (Qiagen, USA) with thermocycling program conditions given in Supplementary Table 8. To confirm genome circularity of viroid-like circular RNAs, abutting primers for inverse RT-PCR were manually designed as previously described[54] (Supplementary Fig. 2). Amplicons were visualized in 1% agarose gel, stained with ethidium bromide. When PCR amplicons need to be sequenced, to confirm sequence identity or circularity in the viroid-like sequences, they were purified using QIAquick PCR purification kit (Qiagen, USA), and sent at Eurofins Genomics, Germany. Amplicon sequences were visualized and trimmed in CLC-GWB v. 20. Final sequences were remapped (95% identity and read length fraction) to the target virus genome to confirm its identity, or to putative viroid genome to confirm its circularity (Supplementary Fig. 2). Photos of identified plant hosts are in Supplementary Fig. 3.

Phylogenetic analyses

Phylogenetic analyses were performed to investigate taxonomic positions of detected newly discovered and known viruses from different taxonomic groups (Supplementary Table 10). Conserved amino acid sequences of viruses (mainly conserved parts of RNA-dependent RNA polymerase (RdRp) (methyl transferase, helicase or CP were used in some cases) for RNA viruses, replicase (C1) protein for geminivirus and reverse transcriptase (RT) for caulimovirus) detected in this study were aligned with corresponding viral RefSeq amino acid sequences of viruses within a recognized virus family, in addition to selected BLASTp hits (E-val<10^{-4}). For multiple sequence alignment, ClustalW[85] was used as implemented in CLC-GWB v. 20. Non-aligned sequences at both ends were trimmed after alignment. Realignment of trimmed sequences was done using MAFFT[86] and further processed with trimAI[87].

In the case of tobamoviruses, a nucleotide-based phylogenetic analyses were done on full genomes. Genomes of different isolates gathered from GenBank and viral RefSeq database, were aligned using MUSCLE[88], and checked for possible recombinants in RDP v. 4[89]. All recombination events with p-val<10^{-4} reported by at least four methods were considered significant. Phylogenetic analyses were performed on alignments free of recombinants.

For both, protein and nucleotide alignments, maximum likelihood phylogenetic analyses were inferred in MEGA X[90], after the selection of the most suitable substitution models. Details on the sequence sets for each analysis, and the parameters used in the analyses are in Supplementary Table 10. The phylogenetic trees were visualized and edited in iToL v. 6.4[91]. To investigate possible structuring of virus species in phylogenetic trees by associated host plants, plant host cladograms were generated in phyloT v. 2 (https://phylot.biobyte.de/)[91], based on NCBI Taxonomy. Host associations were based on information on the specific accession in NCBI GenBank of the virus isolate of tobamoviruses in Fig. 7, and all isolates of species for rhabdoviruses in Fig. 5. Connections were manually inferred between viral and plant phylogram and cladogram and visually inspected.

Genome-wide molecular diversity analyses

Tomato viruses which have at least three isolates detected with full genomes reconstructed, including their isolates from weed samples, were included in the calculation of percent pairwise identities and polymorphisms. Full genome nucleotide sequences of selected viruses were aligned using MUSCLE[88)] in MEGA X[90], and unaligned ends were trimmed thereafter. Genome segments of multi-segmented virus genomes were concatenated after trimming. Percent pairwise identities and number of polymorphic sites were calculated using SDT v. 1.2[81]. For two newly discovered viruses, the aligned sequences were used to calculate genome-wide polymorphisms and nucleotide diversities (π) in DnaSP v. 6[92] in sliding windows of 100 bases and step size of 5.

Infectivity tests and transmission electron microscopy (TEM)

Further characterization studies were performed to test the infectivity and visualize the virions of newly discovered Plantago tobamovirus 1 (PTV1). Five plants each of *Solanum lycopersicum, Nicotiana benthamiana,* and *Nicotiana clevelandii,* were used in the inoculation experiments, with additional two plants each as mock inoculated controls. Plants were inoculated at the four-leaf stage. The source of inoculum for PTV1 was the original *Plantago major* infected sample from the field. Mechanical inoculation of infected plant sap, diluted 1:10

in phosphate buffer, was done on two leaves (second and third youngest), dusted with carborundum, as previously described[48]. The plants were kept in a controlled environment at 20-24°C, with 16 h daylight and 8 h darkness. Inoculated leaves were tested in pools for the presence of the target virus on 7th and 14th day post inoculation (dpi) in RT-PCR assays. Systemic leaves of inoculated plants were tested for the presence of the PTV1 using RT-PCR assays, wherein pooled newly grown (uninoculated) leaves at 14, 21, 28 and 35 dpi were used. TEM was done to confirm the presence of the virus particles in inoculated *N. clevelandii* plants. Tissue homogenates were applied to Formvar-coated, carbon-stabilized copper grids, and negatively stained with 1% uranyl acetate (SPI Supplies, PA, USA), before inspection in TEM. To associate the observed disease symptoms with the presence of PTV1, pool of five inoculated *N. clevelandii* plant showing virus disease-like symptoms and tested positive for PTV1 was collected at 21 dpi and analyzed using nanopore sequencing as described in section ' RNA extraction and sequencing' above.

Reporting summary

Further information on research design is available in the 'Reporting summary' file linked to this article.

Figures

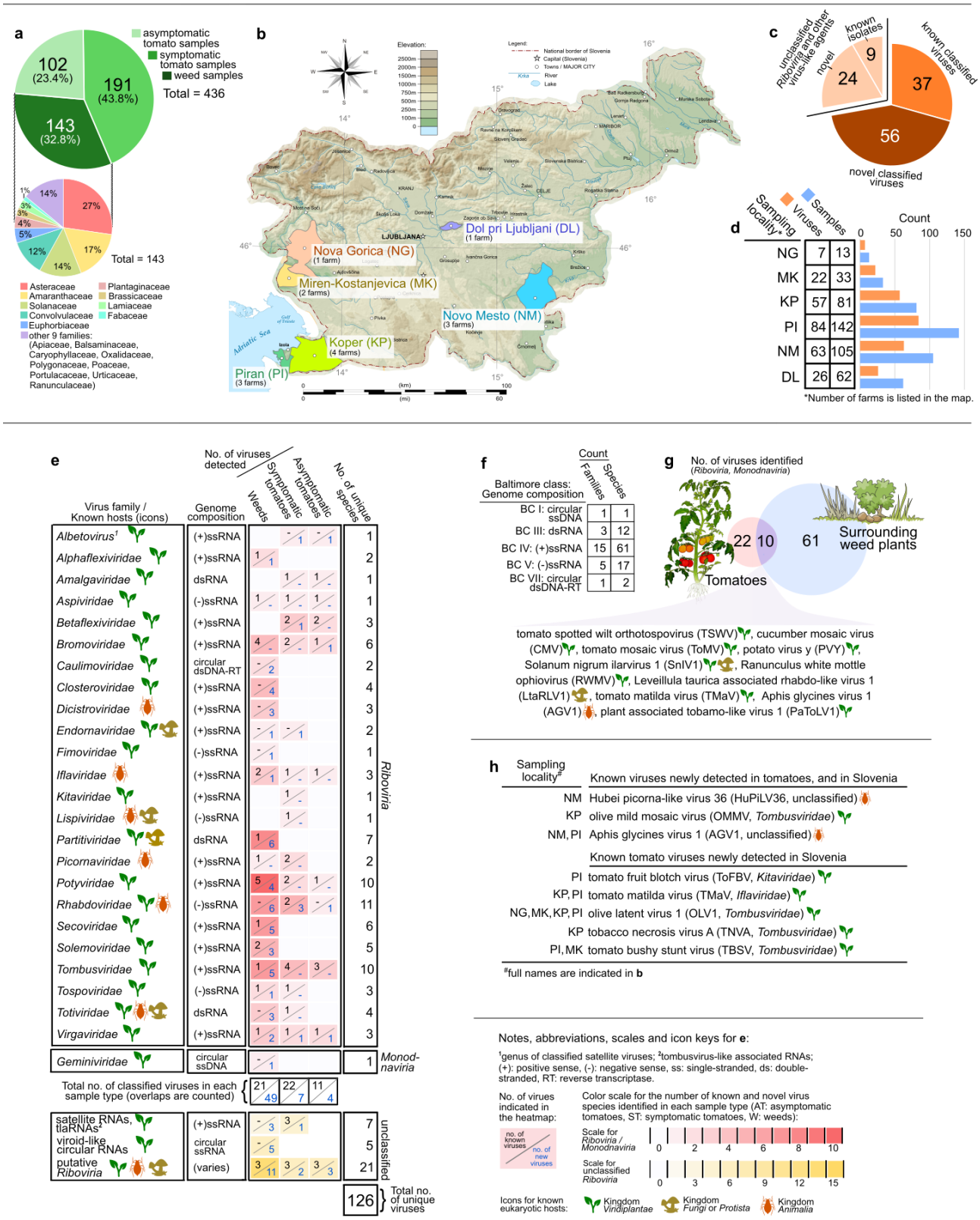
a
asymptomatic tomato samples
symptomatic tomato samples
weed samples
Total = 436
102 (23.4%)
191 (43.8%)
143 (32.8%)
Total = 143
Asteraceae
Amaranthaceae
Solanaceae
Convolvulaceae
Euphorbiaceae
Plantaginaceae
Brassicaceae
Lamiaceae
Fabaceae
other 9 families: (Apiaceae, Balsaminaceae, Caryophyllaceae, Oxalidaceae, Polygonaceae, Poaceae, Portulacaceae, Urticaceae, Ranunculaceae)
b
Elevation:
Legend:
National border of Slovenia
Capital (Slovenia)
Towns / MAJOR CITY
River
Lake
Dol pri Ljubljani (DL) (1 farm)
Nova Gorica (NG) (1 farm)
Miren-Kostanjevica (MK) (2 farms)
Novo Mesto (NM) (3 farms)
Koper (KP) (4 farms)
Piran (PI) (3 farms)
Adriatic Sea
c
unclassified Riboviria and other virus-like agents
known isolates
known classified viruses
novel
9
24
37
56
novel classified viruses
d
Sampling locality*
Viruses
Samples
Count
NG 7 13
MK 22 33
KP 57 81
PI 84 142
NM 63 105
DL 26 62
*Number of farms is listed in the map.
e
No. of viruses detected
Virus family / Known hosts (icons)
Genome composition
Weeds
Symptomatic tomatoes
Asymptomatic tomatoes
No. of unique species
Albetovirus[1] (+)ssRNA 1
Alphaflexiviridae (+)ssRNA 1/1 2
Amalgaviridae dsRNA 1/- 1/- 1
Aspiviridae (-)ssRNA 1/- 1/- 1/- 1
Betaflexiviridae (+)ssRNA 2/1 2/- 3
Bromoviridae (+)ssRNA 4/- 2/- 1/1 6
Caulimoviridae circular dsDNA-RT -/2 2
Closteroviridae (+)ssRNA -/4 4
Dicistroviridae (+)ssRNA -/3 3
Endornaviridae (+)ssRNA -/1 -/1 2
Fimoviridae (-)ssRNA -/1 1
Iflaviridae (+)ssRNA 2/1 1/- 1/- 3
Kitaviridae (+)ssRNA 1/- 1
Lispiviridae (-)ssRNA 1/- 1
Partitiviridae dsRNA 1/6 7
Picornaviridae (+)ssRNA 1/- 2/- 2
Potyviridae (+)ssRNA 5/4 2/- 1/- 10
Rhabdoviridae (-)ssRNA -/6 2/3 -/1 11
Secoviridae (+)ssRNA 1/5 6
Solemoviridae (+)ssRNA 2/3 5
Tombusviridae (+)ssRNA 1/5 4/- 3/- 10
Tospoviridae (-)ssRNA 1/1 1/- 3
Totiviridae dsRNA -/3 1/- 4
Virgaviridae (+)ssRNA 1/2 1/1 1/1 3
Riboviria
Geminiviridae circular ssDNA -/1 1
Monodnaviria
Total no. of classified viruses in each sample type (overlaps are counted) 21/49 22/7 11/4
satellite RNAs, tlaRNAs[2] (+)ssRNA -/3 3/1 7
viroid-like circular RNAs circular ssRNA -/5 5
putative Riboviria (varies) 3/11 3/2 3/3 21
unclassified
126 Total no. of unique viruses
f
Count
Baltimore class: Genome composition
Families
Species
BC I: circular ssDNA 1 1
BC III: dsRNA 3 12
BC IV: (+)ssRNA 15 61
BC V: (-)ssRNA 5 17
BC VII: circular dsDNA-RT 1 2
g
No. of viruses identified (Riboviria, Monodnaviria)
22 10 61
Tomatoes
Surrounding weed plants
tomato spotted wilt orthotospovirus (TSWV), cucumber mosaic virus (CMV), tomato mosaic virus (ToMV), potato virus y (PVY), Solanum nigrum ilarvirus 1 (SnIV1), Ranunculus white mottle ophiovirus (RWMV), Leveillula taurica associated rhabdo-like virus 1 (LtaRLV1), tomato matilda virus (TMaV), Aphis glycines virus 1 (AGV1), plant associated tobamo-like virus 1 (PaToLV1)
h
Sampling locality#
Known viruses newly detected in tomatoes, and in Slovenia
NM Hubei picorna-like virus 36 (HuPiLV36, unclassified)
KP olive mild mosaic virus (OMMV, Tombusviridae)
NM, PI Aphis glycines virus 1 (AGV1, unclassified)
Known tomato viruses newly detected in Slovenia
PI tomato fruit blotch virus (ToFBV, Kitaviridae)
KP, PI tomato matilda virus (TMaV, Iflaviridae)
NG, MK, KP, PI olive latent virus 1 (OLV1, Tombusviridae)
KP tobacco necrosis virus A (TNVA, Tombusviridae)
PI, MK tomato bushy stunt virus (TBSV, Tombusviridae)
#full names are indicated in b
Notes, abbreviations, scales and icon keys for e:
[1]genus of classified satellite viruses; [2]tombusvirus-like associated RNAs; (+): positive sense, (-): negative sense, ss: single-stranded, ds: double-stranded, RT: reverse transcriptase.
No. of viruses indicated in the heatmap:
no. of known viruses
no. of new viruses
Color scale for the number of known and novel virus species identified in each sample type (AT: asymptomatic tomatoes, ST: symptomatic tomatoes, W: weeds):
Scale for Riboviria / Monodnaviria 0 2 4 6 8 10
Scale for unclassified Riboviria 0 3 6 9 12 15
Icons for known eukaryotic hosts:
Kingdom Viridiplantae
Kingdom Fungi or Protista
Kingdom Animalia

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Figure 1 | Diversity and distribution of samples, and viruses detected in tomatoes and weeds surrounding tomato farming areas from Slovenia. a Pie chart showing proportion of sample count per sample type, and separately for weed samples, proportion of sample count per plant family. **b** Map showing the geographical locations where sampling was done. Farm codes are indicated for each locality. **c** Overall count of viruses, with (near) complete genomes, identified from this study, distributed as proportion of known, novel and unclassified *Riboviria* species. **d** Bar plot summarizing the number of samples collected, and viruses detected in each sampling locality. **e** Heatmap showing the diversity of identified viruses by genome composition, their distribution in each known virus taxa by sample type, and the known eukaryotic host (Kingdom) of each virus taxa. **f** Summary of count of identified viruses based on Baltimore class or genome composition. **g** Venn diagram showing the number of identified known and novel viruses found exclusively in tomato or weed composite samples, and the viruses found in both sample types. **h** Known viruses newly detected in tomato and/or in Slovenia. Localities where the viruses were detected are designated by two-letter codes. Credit: Images or icons used in the figure were from https://freesvg.com/, and are under Public Domain (CC0 license, https://creativecommons.org/licenses/publicdomain/). The map of Slovenia was derived from work of Andrei Nacu uploaded in Wikimedia Commons, the free media repository, under the Public Domain (CC0 license).

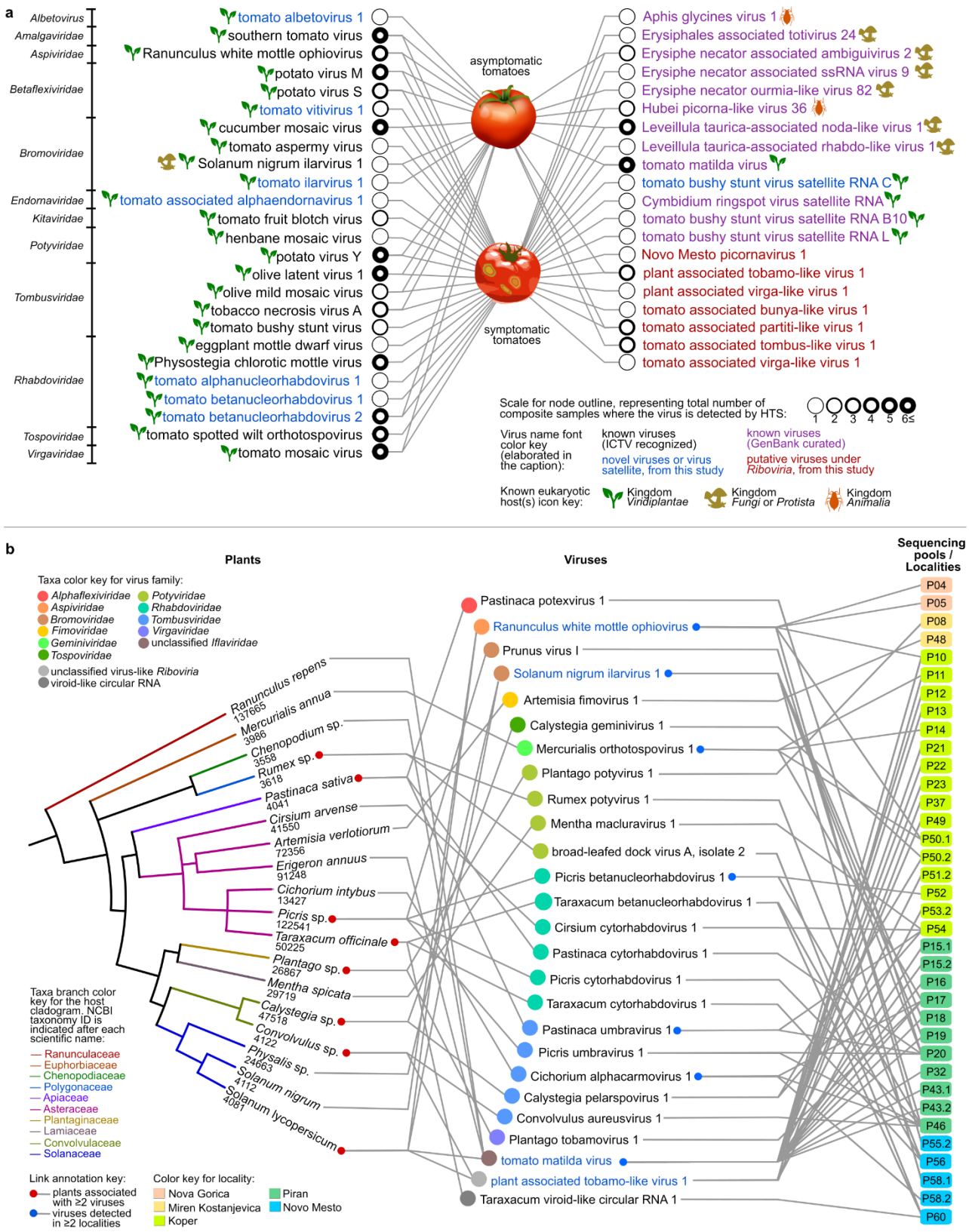
a
Albetovirus
Amalgaviridae
Aspiviridae
Betaflexiviridae
Bromoviridae
Endornaviridae
Kitaviridae
Potyviridae
Tombusviridae
Rhabdoviridae
Tospoviridae
Virgaviridae
tomato albetovirus 1
southern tomato virus
Ranunculus white mottle ophiovirus
potato virus M
potato virus S
tomato vitivirus 1
cucumber mosaic virus
tomato aspermy virus
Solanum nigrum ilarvirus 1
tomato ilarvirus 1
tomato associated alphaendornavirus 1
tomato fruit blotch virus
henbane mosaic virus
potato virus Y
olive latent virus 1
olive mild mosaic virus
tobacco necrosis virus A
tomato bushy stunt virus
eggplant mottle dwarf virus
Physostegia chlorotic mottle virus
tomato alphanucleorhabdovirus 1
tomato betanucleorhabdovirus 1
tomato betanucleorhabdovirus 2
tomato spotted wilt orthotospovirus
tomato mosaic virus
asymptomatic tomatoes
symptomatic tomatoes
Aphis glycines virus 1
Erysiphales associated totivirus 24
Erysiphe necator associated ambiguivirus 2
Erysiphe necator associated ssRNA virus 9
Erysiphe necator ourmia-like virus 82
Hubei picorna-like virus 36
Leveillula taurica-associated noda-like virus 1
Leveillula taurica-associated rhabdo-like virus 1
tomato matilda virus
tomato bushy stunt virus satellite RNA C
Cymbidium ringspot virus satellite RNA
tomato bushy stunt virus satellite RNA B10
tomato bushy stunt virus satellite RNA L
Novo Mesto picornavirus 1
plant associated tobamo-like virus 1
plant associated virga-like virus 1
tomato associated bunya-like virus 1
tomato associated partiti-like virus 1
tomato associated tombus-like virus 1
tomato associated virga-like virus 1
Scale for node outline, representing total number of composite samples where the virus is detected by HTS:
1 2 3 4 5 6≤
Virus name font color key (elaborated in the caption):
known viruses (ICTV recognized)
known viruses (GenBank curated)
novel viruses or virus satellite, from this study
putative viruses under Riboviria, from this study
Known eukaryotic host(s) icon key:
Kingdom Viridiplantae
Kingdom Fungi or Protista
Kingdom Animalia
b
Plants
Viruses
Sequencing pools / Localities
Taxa color key for virus family:
Alphaflexiviridae
Aspiviridae
Bromoviridae
Fimoviridae
Geminiviridae
Tospoviridae
Potyviridae
Rhabdoviridae
Tombusviridae
Virgaviridae
unclassified Iflaviridae
unclassified virus-like Riboviria
viroid-like circular RNA
Ranunculus repens 137665
Mercurialis annua 3986
Chenopodium sp. 3558
Rumex sp. 3618
Pastinaca sativa 4041
Cirsium arvense 41550
Artemisia verlotiorum 72356
Erigeron annuus 91248
Cichorium intybus 13427
Picris sp. 122541
Taraxacum officinale 50225
Plantago sp. 26867
Mentha spicata 29719
Calystegia sp. 47518
Convolvulus sp. 4122
Physalis sp. 24663
Solanum nigrum 4112
Solanum lycopersicum 4087
Taxa branch color key for the host cladogram. NCBI taxonomy ID is indicated after each scientific name:
Ranunculaceae
Euphorbiaceae
Chenopodiaceae
Polygonaceae
Apiaceae
Asteraceae
Plantaginaceae
Lamiaceae
Convolvulaceae
Solanaceae
Pastinaca potexvirus 1
Ranunculus white mottle ophiovirus
Prunus virus I
Solanum nigrum ilarvirus 1
Artemisia fimovirus 1
Calystegia geminivirus 1
Mercurialis orthotospovirus 1
Plantago potyvirus 1
Rumex potyvirus 1
Mentha macluravirus 1
broad-leafed dock virus A, isolate 2
Picris betanucleorhabdovirus 1
Taraxacum betanucleorhabdovirus 1
Cirsium cytorhabdovirus 1
Pastinaca cytorhabdovirus 1
Picris cytorhabdovirus 1
Taraxacum cytorhabdovirus 1
Pastinaca umbravirus 1
Picris umbravirus 1
Cichorium alphacarmovirus 1
Calystegia pelarspovirus 1
Convolvulus aureusvirus 1
Plantago tobamovirus 1
tomato matilda virus
plant associated tobamo-like virus 1
Taraxacum viroid-like circular RNA 1
P04
P05
P08
P48
P10
P11
P12
P13
P14
P21
P22
P23
P37
P49
P50.1
P50.2
P51.2
P52
P53.2
P54
P15.1
P15.2
P16
P17
P18
P19
P20
P32
P43.1
P43.2
P46
P55.2
P56
P58.1
P58.2
P60
Link annotation key:
plants associated with ≥2 viruses
viruses detected in ≥2 localities
Color key for locality:
Nova Gorica
Miren Kostanjevica
Koper
Piran
Novo Mesto

Figure 2

Figure 2 | Connectivity and overlaps of viruses detected in different sample types. a Bipartite network showing detection of viruses in composite symptomatic and asymptomatic tomato samples from the present study. The node represents the viruses, in which outline thickness is weighted based on the number of composite samples where the virus was detected by HTS. The lines connect viruses with a sample type in which they were detected. Virus names are color coded according to classification status: known viruses are either officially recognized ICTV species (black font), or viruses that are deposited and classified in GenBank, but not ICTV-recognized (purple). Novel viruses from this study, which are classified up to the species level, are in blue font, while putative viruses without any official classification yet are in red font. **b** Plant-virus-sample pool tripartite network, showing the connectivity of the subset of viruses detected in weeds with associated hosts. Shown in the network is the subset of novel viruses detected in weeds by HTS, which were further associated with specific host species (NCBI taxonomy IDs are shown) using RT-PCR assays or deduction from HTS data (for tomato, which was not pooled with other species). Virus names in blue font are those detected in both tomato and weed samples by HTS and RT-PCR. Credit: Images and icons used in the figure were from www.freesvg.com, and are under Public Domain (CC0 license, https://creativecommons.org/licenses/publicdomain/).

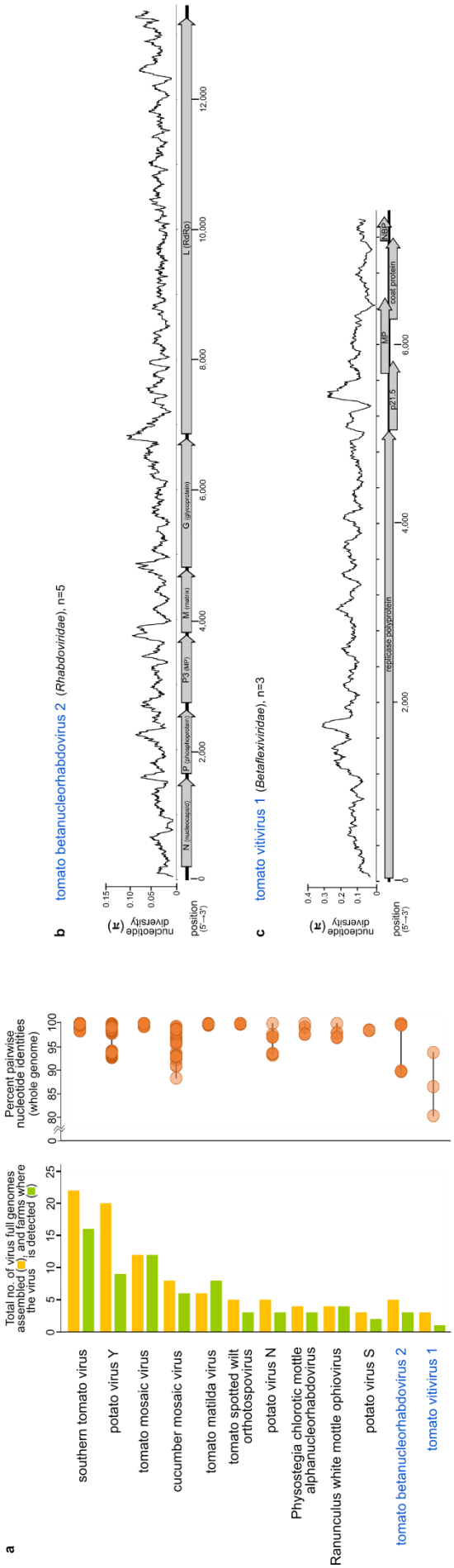
a
Total no. of virus full genomes assembled (■), and farms where the virus is detected (■)
Percent pairwise nucleotide identities (whole genome)
southern tomato virus
potato virus Y
tomato mosaic virus
cucumber mosaic virus
tomato matilda virus
tomato spotted wilt orthotospovirus
potato virus N
Physostegia chlorotic mottle alphanucleorhabdovirus
Ranunculus white mottle ophiovirus
potato virus S
tomato betanucleorhabdovirus 2
tomato vitivirus 1
b
tomato betanucleorhabdovirus 2 (Rhabdoviridae), n=5
nucleotide diversity (π)
position (5'→3')
N (nucleocapsid)
P (phosphoprotein)
P3 (MP)
M (matrix)
G (glycoprotein)
L (RdRp)
c
tomato vitivirus 1 (Betaflexiviridae), n=3
replicase polyprotein
p21.5
MP
coat protein
NBP

Figure 3

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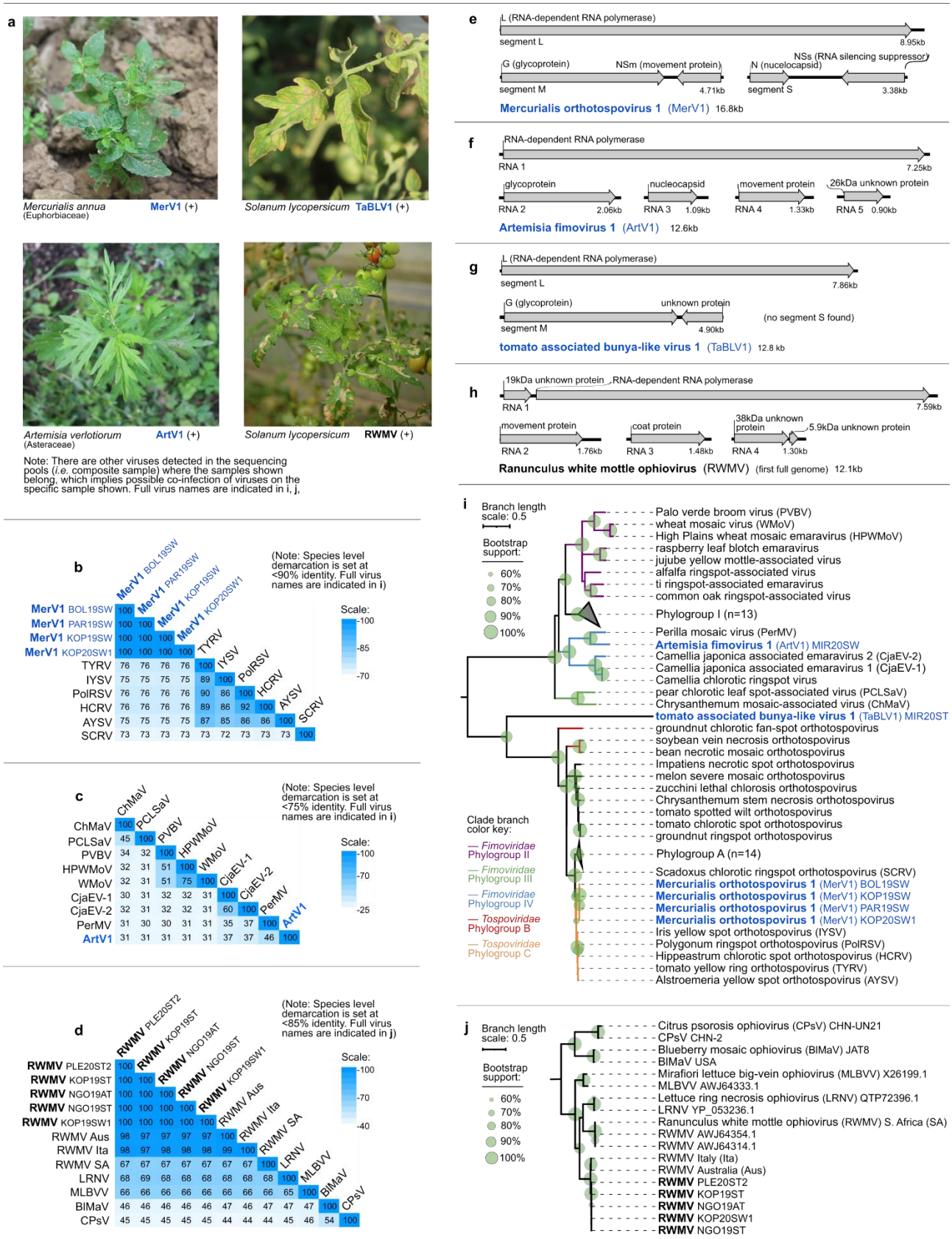
Figure 3 | Molecular diversity and genome-wide variability of most frequently detected tomato viruses in this study. a Pairwise nucleotide identities of populations of tomato viruses from Slovenia. Isolates of tomato viruses from weed samples, were included in the calculation of pairwise identities. **b, c** Genome-wide nucleotide diversity of novel viruses in tomatoes from Slovenia with at least three isolates. All open reading frames and protein domain cleavage sites are shown for each virus genome.


a
Mercurialis annua (Euphorbiaceae) MerV1 (+)
Solanum lycopersicum TaBLV1 (+)
Artemisia verlotiorum (Asteraceae) ArtV1 (+)
Solanum lycopersicum RWMV (+)
Note: There are other viruses detected in the sequencing pools (i.e. composite sample) where the samples shown belong, which implies possible co-infection of viruses on the specific sample shown. Full virus names are indicated in i, j,
b
(Note: Species level demarcation is set at <90% identity. Full virus names are indicated in i)
Scale: 100 85 70
MerV1 BOL19SW 100
MerV1 PAR19SW 100 100
MerV1 KOP19SW 100 100 100
MerV1 KOP20SW1 100 100 100 100
TYRV 76 76 76 76 100
IYSV 75 75 75 75 89 100
PolRSV 76 76 76 76 90 86 100
HCRV 76 76 76 76 89 86 92 100
AYSV 75 75 75 75 87 85 86 86 100
SCRV 73 73 73 73 73 72 73 73 73 100
c
(Note: Species level demarcation is set at <75% identity. Full virus names are indicated in i)
Scale: 100 70 25
ChMaV 100
PCLSaV 45 100
PVBV 34 32 100
HPWMoV 32 31 51 100
WMoV 32 31 51 75 100
CjaEV-1 30 31 32 32 31 100
CjaEV-2 32 31 32 32 31 60 100
PerMV 31 30 30 31 31 35 37 100
ArtV1 31 31 31 31 31 37 37 46 100
d
(Note: Species level demarcation is set at <85% identity. Full virus names are indicated in j)
Scale: 100 70 40
RWMV PLE20ST2 100
RWMV KOP19ST 100 100
RWMV NGO19AT 100 100 100
RWMV NGO19ST 100 100 100 100
RWMV KOP19SW1 100 100 100 100 100
RWMV Aus 98 97 97 97 97 100
RWMV Ita 98 97 98 98 98 99 100
RWMV SA 67 67 67 67 67 67 67 100
LRNV 68 69 68 68 68 68 68 68 100
MLBVV 66 66 66 66 66 66 66 66 65 100
BlMaV 46 46 46 46 46 47 46 47 47 47 100
CPsV 45 45 45 45 45 44 44 44 45 46 54 100
e
L (RNA-dependent RNA polymerase)
segment L 8.95kb
G (glycoprotein) NSm (movement protein) 4.71kb segment M
N (nucelocapsid) NSs (RNA silencing suppressor) segment S 3.38kb
Mercurialis orthotospovirus 1 (MerV1) 16.8kb
f
RNA-dependent RNA polymerase
RNA 1 7.25kb
glycoprotein RNA 2 2.06kb
nucleocapsid RNA 3 1.09kb
movement protein RNA 4 1.33kb
26kDa unknown protein RNA 5 0.90kb
Artemisia fimovirus 1 (ArtV1) 12.6kb
g
L (RNA-dependent RNA polymerase)
segment L 7.86kb
G (glycoprotein) unknown protein segment M 4.90kb
(no segment S found)
tomato associated bunya-like virus 1 (TaBLV1) 12.8 kb
h
19kDa unknown protein RNA-dependent RNA polymerase
RNA 1 7.59kb
movement protein RNA 2 1.76kb
coat protein RNA 3 1.48kb
38kDa unknown protein RNA 4 1.30kb
5.9kDa unknown protein
Ranunculus white mottle ophiovirus (RWMV) (first full genome) 12.1kb
i
Branch length scale: 0.5
Bootstrap support: 60% 70% 80% 90% 100%
Palo verde broom virus (PVBV)
wheat mosaic virus (WMoV)
High Plains wheat mosaic emaravirus (HPWMoV)
raspberry leaf blotch emaravirus
jujube yellow mottle-associated virus
alfalfa ringspot-associated virus
ti ringspot-associated emaravirus
common oak ringspot-associated virus
Phylogroup I (n=13)
Perilla mosaic virus (PerMV)
Artemisia fimovirus 1 (ArtV1) MIR20SW
Camellia japonica associated emaravirus 2 (CjaEV-2)
Camellia japonica associated emaravirus 1 (CjaEV-1)
Camellia chlorotic ringspot virus
pear chlorotic leaf spot-associated virus (PCLSaV)
Chrysanthemum mosaic-associated virus (ChMaV)
tomato associated bunya-like virus 1 (TaBLV1) MIR20ST
groundnut chlorotic fan-spot orthotospovirus
soybean vein necrosis orthotospovirus
bean necrotic mosaic orthotospovirus
Impatiens necrotic spot orthotospovirus
melon severe mosaic orthotospovirus
zucchini lethal chlorosis orthotospovirus
Chrysanthemum stem necrosis orthotospovirus
tomato spotted wilt orthotospovirus
tomato chlorotic spot orthotospovirus
groundnut ringspot orthotospovirus
Phylogroup A (n=14)
Scadoxus chlorotic ringspot orthotospovirus (SCRV)
Mercurialis orthotospovirus 1 (MerV1) BOL19SW
Mercurialis orthotospovirus 1 (MerV1) KOP19SW
Mercurialis orthotospovirus 1 (MerV1) PAR19SW
Mercurialis orthotospovirus 1 (MerV1) KOP20SW1
Iris yellow spot orthotospovirus (IYSV)
Polygonum ringspot orthotospovirus (PolRSV)
Hippeastrum chlorotic spot orthotospovirus (HCRV)
tomato yellow ring orthotospovirus (TYRV)
Alstroemeria yellow spot orthotospovirus (AYSV)
Clade branch color key:
Fimoviridae Phylogroup II
Fimoviridae Phylogroup III
Fimoviridae Phylogroup IV
Tospoviridae Phylogroup B
Tospoviridae Phylogroup C
j
Branch length scale: 0.5
Bootstrap support: 60% 70% 80% 90% 100%
Citrus psorosis ophiovirus (CPsV) CHN-UN21
CPsV CHN-2
Blueberry mosaic ophiovirus (BlMaV) JAT8
BlMaV USA
Mirafiori lettuce big-vein ophiovirus (MLBVV) X26199.1
MLBVV AWJ64333.1
Lettuce ring necrosis ophiovirus (LRNV) QTP72396.1
LRNV YP_053236.1
Ranunculus white mottle ophiovirus (RWMV) S. Africa (SA)
RWMV AWJ64354.1
RWMV AWJ64314.1
RWMV Italy (Ita)
RWMV Australia (Aus)
RWMV PLE20ST2
RWMV KOP19ST
RWMV NGO19AT
RWMV KOP20SW1
RWMV NGO19ST

683 **Figure 4**

Figure 4 | Characteristics of (-)ssRNA viruses under family *Tospoviridae, Fimoviridae*, and *Aspiviridae*, from the present study. a Selected photographs showing associated symptoms in field samples that tested positive for the respective viruses in RT-PCR assays. **b-d** Heatmaps showing the pairwise identities of selected viruses based on alignment and comparison of full length RdRp amino acid sequences. **e-h** Genome organization of the viruses presented herewith showing the predicted open reading frames and protein products. **i-j** Maximum likelihood phylogenetic trees based on the conserved RNA-dependent RNA polymerase (RdRp) amino acid sequences of *Tospoviridae* and *Fimoviridae* also showing tomato associated bunya-like virus 1 (**i**), and *Aspivirdae* (**j**). Branch length scale represents amino acid substitution per site. Virus names and acronyms in blue bold font are the novel viruses, while those in black bold font are known viruses from the present study. Indicated after the name or acronym are the isolate IDs of the viruses. Full virus names of those abbreviated in the pairwise identity matrices (**b-d**) can be found in the genome organization and phylogenetic trees (**e-j**).

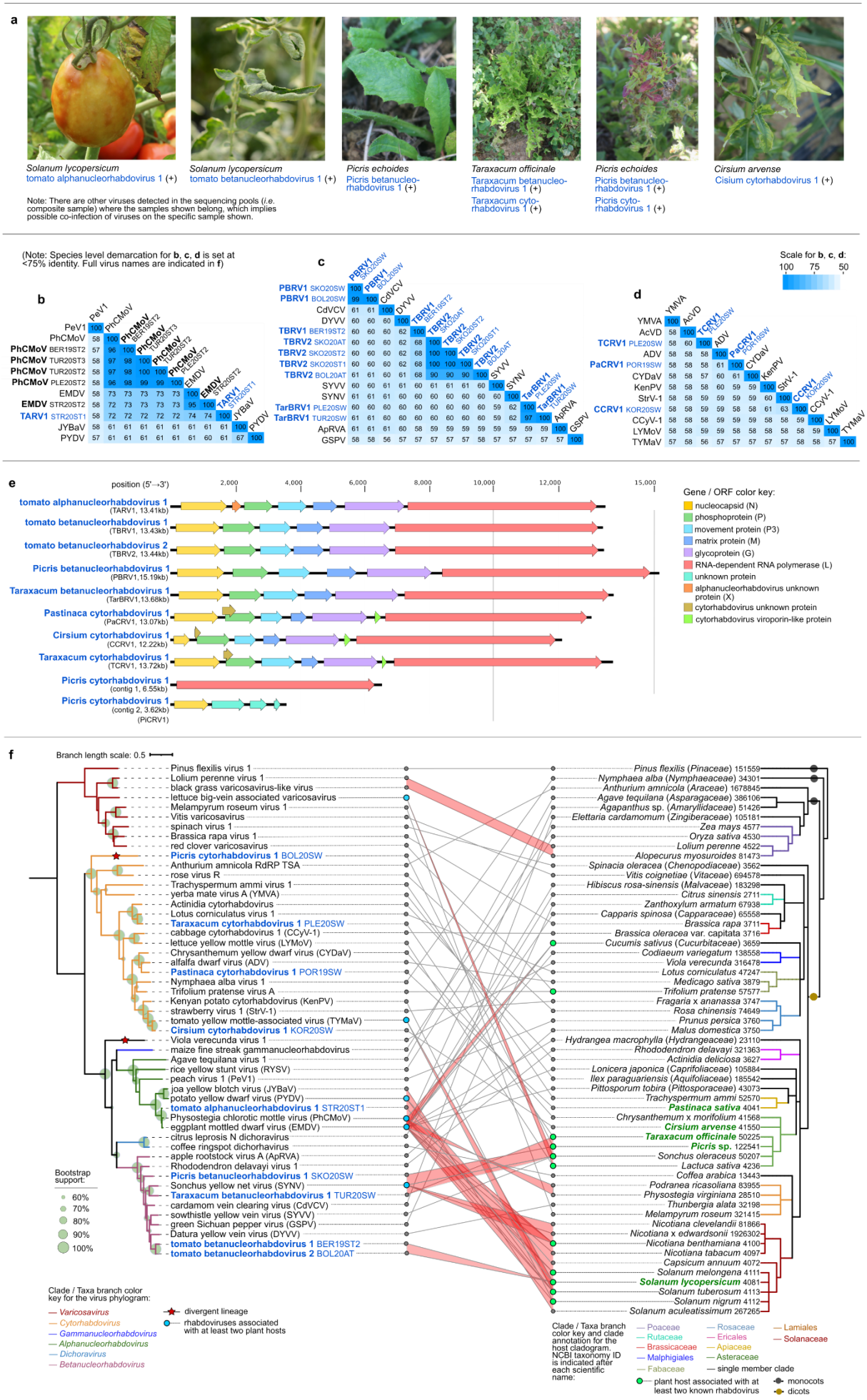

a
Solanum lycopersicum
tomato alphanucleorhabdovirus 1 (+)
Solanum lycopersicum
tomato betanucleorhabdovirus 1 (+)
Picris echoides
Picris betanucleo-rhabdovirus 1 (+)
Taraxacum officinale
Taraxacum betanucleo-rhabdovirus 1 (+)
Taraxacum cyto-rhabdovirus 1 (+)
Picris echoides
Picris betanucleo-rhabdovirus 1 (+)
Picris cyto-rhabdovirus 1 (+)
Cirsium arvense
Cisium cytorhabdovirus 1 (+)
Note: There are other viruses detected in the sequencing pools (i.e. composite sample) where the samples shown belong, which implies possible co-infection of viruses on the specific sample shown.
(Note: Species level demarcation for b, c, d is set at <75% identity. Full virus names are indicated in f)
Scale for b, c, d:
100 75 50
b
c
d
e
position (5'→3')
2,000 4,000 6,000 8,000 10,000 12,000 15,000
tomato alphanucleorhabdovirus 1 (TARV1, 13.41kb)
tomato betanucleorhabdovirus 1 (TBRV1, 13.43kb)
tomato betanucleorhabdovirus 2 (TBRV2, 13.44kb)
Picris betanucleorhabdovirus 1 (PBRV1,15.19kb)
Taraxacum betanucleorhabdovirus 1 (TarBRV1,13.68kb)
Pastinaca cytorhabdovirus 1 (PaCRV1, 13.07kb)
Cirsium cytorhabdovirus 1 (CCRV1, 12.22kb)
Taraxacum cytorhabdovirus 1 (TCRV1, 13.72kb)
Picris cytorhabdovirus 1 (contig 1, 6.55kb)
Picris cytorhabdovirus 1 (contig 2, 3.62kb) (PiCRV1)
Gene / ORF color key:
nucleocapsid (N)
phosphoprotein (P)
movement protein (P3)
matrix protein (M)
glycoprotein (G)
RNA-dependent RNA polymerase (L)
unknown protein
alphanucleorhabdovirus unknown protein (X)
cytorhabdovirus unknown protein
cytorhabdovirus viroporin-like protein
f
Branch length scale: 0.5
Bootstrap support:
60%
70%
80%
90%
100%
Clade / Taxa branch color key for the virus phylogram:
Varicosavirus
Cytorhabdovirus
Gammanucleorhabdovirus
Alphanucleorhabdovirus
Dichoravirus
Betanucleorhabdovirus
divergent lineage
rhabdoviruses associated with at least two plant hosts
Clade / Taxa branch color key and clade annotation for the host cladogram. NCBI taxonomy ID is indicated after each scientific name:
Poaceae
Rutaceae
Brassicaceae
Malphigiales
Fabaceae
Rosaceae
Ericales
Apiaceae
Asteraceae
single member clade
Lamiales
Solanaceae
plant host associated with at least two known rhabdovirus
monocots
dicots

Figure 5

Figure 5 | Characteristics of novel (-)ssRNA viruses classified in family *Rhabdoviridae* (order *Mononegavirales*) from the present study. a Photographs of samples that tested positive for selected novel rhabdoviruses in RT-PCR assays. **b-d** Heatmaps showing the pairwise identities of species in genus *Alphanucleorhabdovirus* (**b**), *Betanucleorhabdovirus* (**c**), and *Cytorhabdovirus* (**d**), based on full genome length nucleotide sequences. Virus acronyms in blue bold font are the novel rhabdoviruses, while those in black bold font are known rhabdoviruses from the present study. Indicated after virus names and acronyms are the isolate IDs. **e** Genome organization of novel rhabdoviruses discovered in the present study. Genome lengths are shown to scale. Open reading frames (ORFs) and the proteins they code for are color coded accordingly. **f** Co-phylogenetic tree (tanglegram) showing the phylogenetic relationships of novel rhabdoviruses among known species (left tree), which are linked with associated plant host(s) indicated in GenBank, shown on the right tree. Numerous links of well-supported clade of viruses to taxonomically-related plants are highlighted in red. Maximum likelihood phylogenetic tree of rhabdoviruses was constructed based on the conserved amino acid sequence of the RdRp. Branch length scale represents amino acid substitution per site. The host cladogram was made in phyloT (www.phylot.biobyte.de). In the virus tree, viruses associated with at least two plant hosts are indicated by a blue circle. In the host tree, green circles are used to indicate plants that are associated with two or more rhabdoviruses, and the host species names in bold green font are those that have representative samples in the present study. The clades are separately color coded and annotated for each tree. Full virus names of those abbreviated in the pairwise identity matrices (**b-d**) can be found in the genome organization and phylogenetic trees (**e, f**).

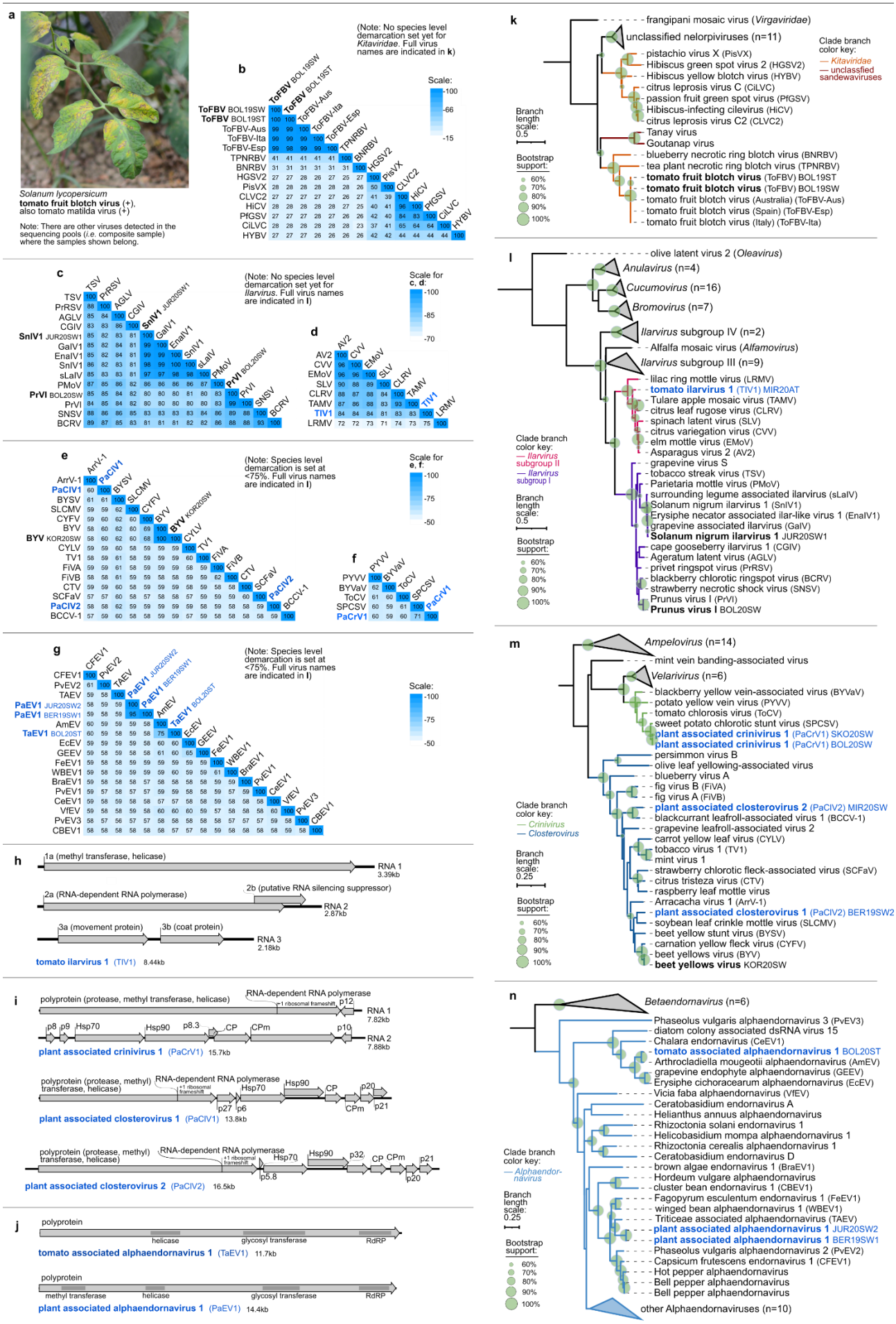
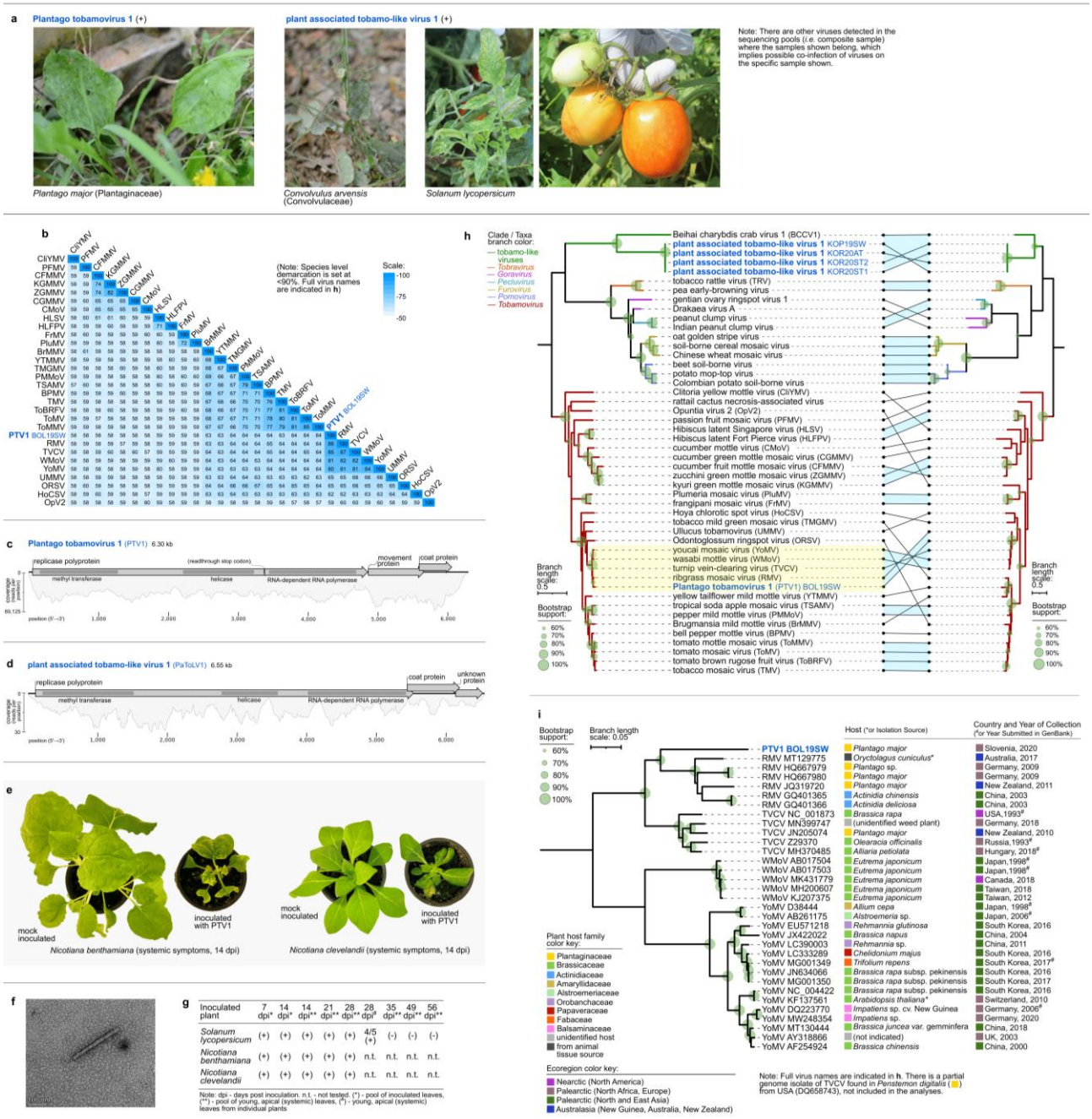

a
Solanum lycopersicum
tomato fruit blotch virus (+),
also tomato matilda virus (+)
Note: There are other viruses detected in the sequencing pools (i.e. composite sample) where the samples shown belong.
b
(Note: No species level demarcation set yet for Kitaviridae. Full virus names are indicated in k)
Scale:
c
(Note: No species level demarcation set yet for Ilarvirus. Full virus names are indicated in l)
Scale for c, d:
d
e
(Note: Species level demarcation is set at <75%. Full virus names are indicated in l)
Scale for e, f:
f
g
(Note: Species level demarcation is set at <75%. Full virus names are indicated in l)
Scale:
h
1a (methyl transferase, helicase)
RNA 1 3.39kb
2a (RNA-dependent RNA polymerase)
2b (putative RNA silencing suppressor)
RNA 2 2.87kb
3a (movement protein)
3b (coat protein)
RNA 3 2.18kb
tomato ilarvirus 1 (TIV1) 8.44kb
i
polyprotein (protease, methyl transferase, helicase)
RNA-dependent RNA polymerase
+1 ribosomal frameshift
RNA 1 7.82kb
RNA 2 7.88kb
plant associated crinivirus 1 (PaCrV1) 15.7kb
plant associated closterovirus 1 (PaCIV1) 13.8kb
plant associated closterovirus 2 (PaCIV2) 16.5kb
j
polyprotein
helicase
glycosyl transferase
RdRP
tomato associated alphaendornavirus 1 (TaEV1) 11.7kb
methyl transferase
plant associated alphaendornavirus 1 (PaEV1) 14.4kb
k
frangipani mosaic virus (Virgaviridae)
unclassified nelorpiviruses (n=11)
pistachio virus X (PisVX)
Hibiscus green spot virus 2 (HGSV2)
Hibiscus yellow blotch virus (HYBV)
citrus leprosis virus C (CiLVC)
passion fruit green spot virus (PfGSV)
Hibiscus-infecting cilevirus (HiCV)
citrus leprosis virus C2 (CLVC2)
Tanay virus
Goutanap virus
blueberry necrotic ring blotch virus (BNRBV)
tea plant necrotic ring blotch virus (TPNRBV)
tomato fruit blotch virus (ToFBV) BOL19ST
tomato fruit blotch virus (ToFBV) BOL19SW
tomato fruit blotch virus (Australia) (ToFBV-Aus)
tomato fruit blotch virus (Spain) (ToFBV-Esp)
tomato fruit blotch virus (Italy) (ToFBV-Ita)
Clade branch color key:
Kitaviridae
unclassified sandewaviruses
Branch length scale: 0.5
Bootstrap support:
l
olive latent virus 2 (Oleavirus)
Anulavirus (n=4)
Cucumovirus (n=16)
Bromovirus (n=7)
Ilarvirus subgroup IV (n=2)
Alfalfa mosaic virus (Alfamovirus)
Ilarvirus subgroup III (n=9)
lilac ring mottle virus (LRMV)
tomato ilarvirus 1 (TIV1) MIR20AT
Tulare apple mosaic virus (TAMV)
citrus leaf rugose virus (CLRV)
spinach latent virus (SLV)
citrus variegation virus (CVV)
elm mottle virus (EMoV)
Asparagus virus 2 (AV2)
grapevine virus S
tobacco streak virus (TSV)
Parietaria mottle virus (PMoV)
surrounding legume associated ilarvirus (sLaIV)
Solanum nigrum ilarvirus 1 (SnIV1)
Erysiphe necator associated ilar-like virus 1 (EnaIV1)
grapevine associated ilarvirus (GaIV)
Solanum nigrum ilarvirus 1 JUR20SW1
cape gooseberry ilarvirus 1 (CGIV)
Ageratum latent virus (AGLV)
privet ringspot virus (PrRSV)
blackberry chlorotic ringspot virus (BCRV)
strawberry necrotic shock virus (SNSV)
Prunus virus I (PrVI)
Prunus virus I BOL20SW
Clade branch color key:
Ilarvirus subgroup II
Ilarvirus subgroup I
Branch length scale: 0.5
Bootstrap support:
m
Ampelovirus (n=14)
mint vein banding-associated virus
Velarivirus (n=6)
blackberry yellow vein-associated virus (BYVaV)
potato yellow vein virus (PYVV)
tomato chlorosis virus (ToCV)
sweet potato chlorotic stunt virus (SPCSV)
plant associated crinivirus 1 (PaCrV1) SKO20SW
plant associated crinivirus 1 (PaCrV1) BOL20SW
persimmon virus B
olive leaf yellowing-associated virus
blueberry virus A
fig virus B (FiVA)
fig virus A (FiVB)
plant associated closterovirus 2 (PaCIV2) MIR20SW
blackcurrant leafroll-associated virus 1 (BCCV-1)
grapevine leafroll-associated virus 2
carrot yellow leaf virus (CYLV)
tobacco virus 1 (TV1)
mint virus 1
strawberry chlorotic fleck-associated virus (SCFaV)
citrus tristeza virus (CTV)
raspberry leaf mottle virus
Arracacha virus 1 (ArrV-1)
plant associated closterovirus 1 (PaCIV2) BER19SW2
soybean leaf crinkle mottle virus (SLCMV)
beet yellow stunt virus (BYSV)
carnation yellow fleck virus (CYFV)
beet yellows virus (BYV)
beet yellows virus KOR20SW
Clade branch color key:
Crinivirus
Closterovirus
Branch length scale: 0.25
Bootstrap support:
n
Betaendornavirus (n=6)
Phaseolus vulgaris alphaendornavirus 3 (PvEV3)
diatom colony associated dsRNA virus 15
Chalara endornavirus (CeEV1)
tomato associated alphaendornavirus 1 BOL20ST
Arthrocladiella mougeotii alphaendornavirus (AmEV)
grapevine endophyte alphaendornavirus (GEEV)
Erysiphe cichoracearum alphaendornavirus (EcEV)
Vicia faba alphaendornavirus (VfEV)
Ceratobasidium endornavirus A
Helianthus annuus alphaendornavirus
Rhizoctonia solani endornavirus 1
Helicobasidium mompa alphaendornavirus 1
Rhizoctonia cerealis alphaendornavirus 1
Ceratobasidium endornavirus D
brown algae endornavirus 1 (BraEV1)
Hordeum vulgare alphaendornavirus
cluster bean endornavirus 1 (CBEV1)
Fagopyrum esculentum endornavirus 1 (FeEV1)
winged bean alphaendornavirus 1 (WBEV1)
Triticeae associated alphaendornavirus (TAEV)
plant associated alphaendornavirus 1 JUR20SW2
plant associated alphaendornavirus 1 BER19SW1
Phaseolus vulgaris alphaendornavirus 2 (PvEV2)
Capsicum frutescens endornavirus 1 (CFEV1)
Hot pepper alphaendornavirus
Bell pepper alphaendornavirus
Bell pepper alphaendornavirus
other Alphaendornaviruses (n=10)
Clade branch color key:
Alphaendornavirus
Branch length scale: 0.25
Bootstrap support:

Figure 6

Figure 6 | Characteristics of (+)ssRNA viruses in families *Kitaviridae*, *Bromoviridae*, *Closteroviridae*, and *Endornaviridae* (order *Martellivirales*) from the present study. a Photograph of one of the symptomatic tomato samples that tested positive for ToFBV in RT-PCR assays. **b-g** Heatmaps showing the pairwise identities to distinguish different *Kitaviridae* (**b**) and *Bromoviridae* (**c, d**) species based on alignment and comparison of RdRp amino acid sequences, members of *Closterovirus* genus (**e**) based on full genome nucleotide sequences, members of *Crinivirus* genus (**f**) based on full RNA1 segment nucleotide sequence, and members of *Alphaendornavirus* (**g**) based on full genome nucleotide sequences **h-j** Genomes of the novel viruses, with the predicted open reading frames, protein domains, and genome size. **k-n** Maximum likelihood phylogenetic trees constructed based on the conserved amino acid sequence of the RdRp of (**k**) *Kitaviridae*, (**l**) *Bromoviridae*, (**m**) *Closteroviridae*, and (**n**) *Endornaviridae*. Branch length scale represents amino acid substitution per site. Indicated after the acronym are the isolate IDs of the viruses. Virus names and acronyms in blue bold font are the novel viruses, while those in black bold font are known viruses from the present study. Full virus names of those abbreviated in the pairwise identity matrices (**b-g**) can be found in the genome organization and phylogenetic trees (**h-n**).



Inoculated plant	7 dpi*	14 dpi*	14 dpi**	21 dpi**	28 dpi**	28 dpi#	35 dpi**	49 dpi**	56 dpi**
Solanum lycopersicum	(+)	(+)	(+)	(+)	(+)	4/5 (+)	(-)	(-)	(-)
Nicotiana benthamiana	(+)	(+)	(+)	(+)	(+)	n.t.	n.t.	n.t.	n.t.
Nicotiana clevelandii	(+)	(+)	(+)	(+)	(+)	n.t.	n.t.	n.t.	n.t.

Note: dpi - days post inoculation. n.t. - not tested. (*) - pool of inoculated leaves, (**) - pool of young, apical (systemic) leaves, (#) - young, apical (systemic) leaves from individual plants

737 **Figure 7**

Figure 7 | Characteristics of (+)ssRNA viruses in family *Virgaviridae* (order *Martellivirales*) from the present study. a Selected photographs of samples that tested positive in RT-PCR assays for the viruses presented herewith. **b** Heatmap of pairwise identities of *Tobamovirus* (*Virgaviridae*) species based on full genome nucleotide sequences. **c, d** Genome organization and read coverage of novel viruses showing the open reading frames, and the proteins they code for. **e** Photographs of the PTV1 inoculated symptomatic plants and mock inoculated control plants. **f** Electronmicrograph showing particle morphology of PTV1. **g** RT-PCR assays of test plants inoculated with Plantago tobamovirus 1 (PTV1). **h** Maximum likelihood phylogenetic trees constructed based on the conserved amino acid sequence of methyl transferase-helicase (left) and coat protein (CP) (right). The phylogenetic congruence of the methyl transferase-helicase-based tree (left) is demonstrated by line connecting identical viruses to the CP-based tree (right). Congruent clades with >60% bootstrap support are highlighted (light blue). The clade of viruses closely related to PTV1 is highlighted in yellow. **i** Maximum likelihood phylogenetic tree, based on full genome nucleotide sequences, of all the isolates of a subgroup of tobamoviruses including PTV1. Virus names and acronyms in blue bold font are the novel viruses from the present study. Indicated after the acronym are the isolate IDs of the viruses. Branch length scale represents amino acid (**h**) or nucleotide substitution (**i**) per site. The hosts or isolation sources of the viruses as indicated in NCBI GenBank, and the countries and dates of collection are indicated. Full virus names of those abbreviated in (**b**) and (**i**) are indicated in (**c-d**) and (**h**).

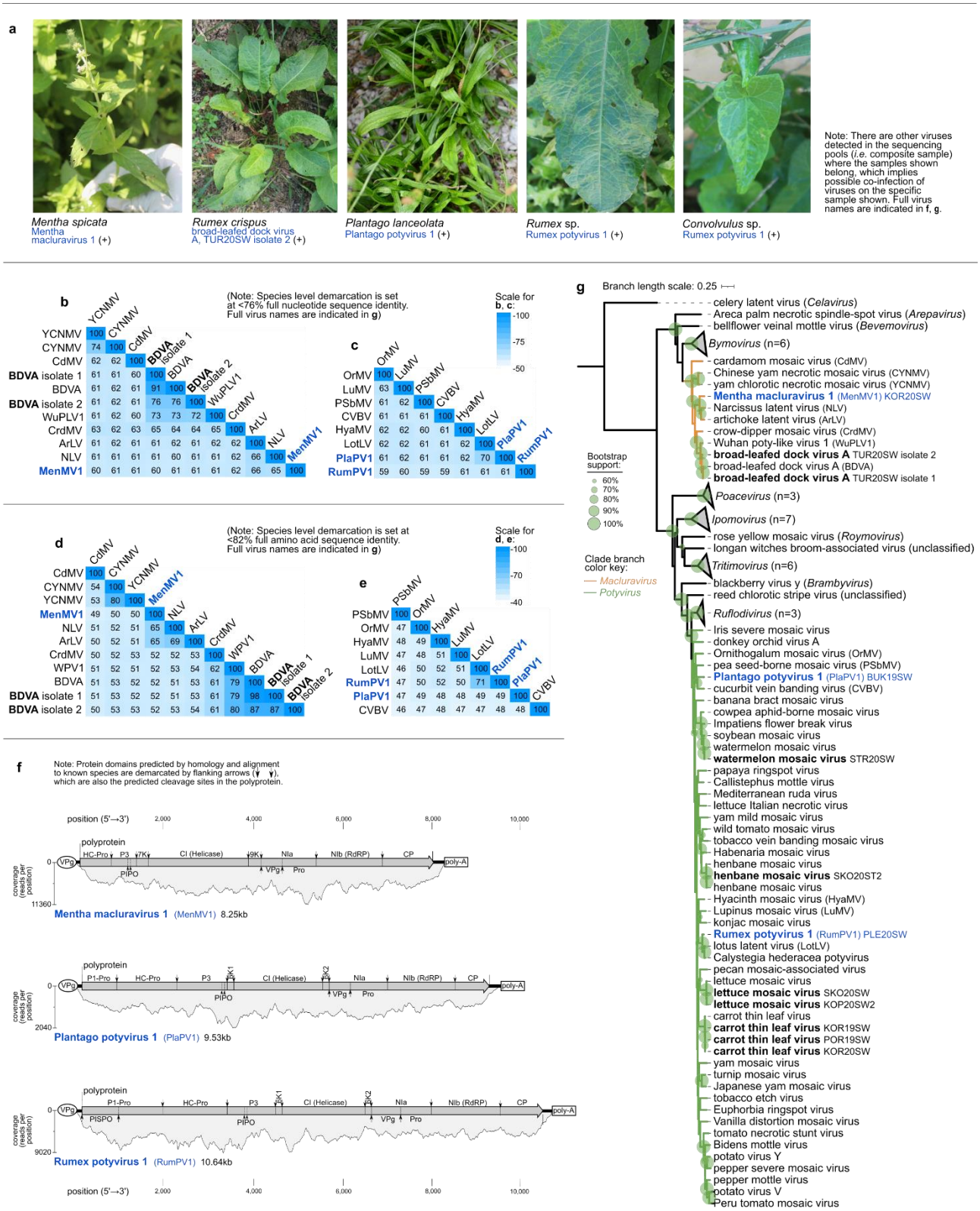

a
Mentha spicata
Mentha macluravirus 1 (+)
Rumex crispus
broad-leafed dock virus A, TUR20SW isolate 2 (+)
Plantago lanceolata
Plantago potyvirus 1 (+)
Rumex sp.
Rumex potyvirus 1 (+)
Convolvulus sp.
Rumex potyvirus 1 (+)
Note: There are other viruses detected in the sequencing pools (i.e. composite sample) where the samples shown belong, which implies possible co-infection of viruses on the specific sample shown. Full virus names are indicated in f, g.
b
(Note: Species level demarcation is set at <76% full nucleotide sequence identity. Full virus names are indicated in g)
Scale for b, c:
c
d
(Note: Species level demarcation is set at <82% full amino acid sequence identity. Full virus names are indicated in g)
Scale for d, e:
e
f
Note: Protein domains predicted by homology and alignment to known species are demarcated by flanking arrows (▼ ▼), which are also the predicted cleavage sites in the polyprotein.
position (5'→3')
polyprotein
HC-Pro
P3
CI (Helicase)
NIa
NIb (RdRP)
CP
VPg
Pro
PIPO
poly-A
coverage (reads per position)
Mentha macluravirus 1 (MenMV1) 8.25kb
P1-Pro
Plantago potyvirus 1 (PlaPV1) 9.53kb
PISPO
Rumex potyvirus 1 (RumPV1) 10.64kb
g
Branch length scale: 0.25
celery latent virus (Celavirus)
Areca palm necrotic spindle-spot virus (Arepavirus)
bellflower veinal mottle virus (Bevemovirus)
Bymovirus (n=6)
cardamom mosaic virus (CdMV)
Chinese yam necrotic mosaic virus (CYNMV)
yam chlorotic necrotic mosaic virus (YCNMV)
Mentha macluravirus 1 (MenMV1) KOR20SW
Narcissus latent virus (NLV)
artichoke latent virus (ArLV)
crow-dipper mosaic virus (CrdMV)
Wuhan poty-like virus 1 (WuPLV1)
broad-leafed dock virus A TUR20SW isolate 2
broad-leafed dock virus A (BDVA)
broad-leafed dock virus A TUR20SW isolate 1
Poacevirus (n=3)
Ipomovirus (n=7)
rose yellow mosaic virus (Roymovirus)
longan witches broom-associated virus (unclassified)
Tritimovirus (n=6)
blackberry virus y (Brambyvirus)
reed chlorotic stripe virus (unclassified)
Ruflodivirus (n=3)
Iris severe mosaic virus
donkey orchid virus A
Ornithogalum mosaic virus (OrMV)
pea seed-borne mosaic virus (PSbMV)
Plantago potyvirus 1 (PlaPV1) BUK19SW
cucurbit vein banding virus (CVBV)
banana bract mosaic virus
cowpea aphid-borne mosaic virus
Impatiens flower break virus
soybean mosaic virus
watermelon mosaic virus
watermelon mosaic virus STR20SW
papaya ringspot virus
Callistephus mottle virus
Mediterranean ruda virus
lettuce Italian necrotic virus
yam mild mosaic virus
wild tomato mosaic virus
tobacco vein banding mosaic virus
Habenaria mosaic virus
henbane mosaic virus
henbane mosaic virus SKO20ST2
henbane mosaic virus
Hyacinth mosaic virus (HyaMV)
Lupinus mosaic virus (LuMV)
konjac mosaic virus
Rumex potyvirus 1 (RumPV1) PLE20SW
lotus latent virus (LotLV)
Calystegia hederacea potyvirus
pecan mosaic-associated virus
lettuce mosaic virus
lettuce mosaic virus SKO20SW
lettuce mosaic virus KOP20SW2
carrot thin leaf virus
carrot thin leaf virus KOR19SW
carrot thin leaf virus POR19SW
carrot thin leaf virus KOR20SW
yam mosaic virus
turnip mosaic virus
Japanese yam mosaic virus
tobacco etch virus
Euphorbia ringspot virus
Vanilla distortion mosaic virus
tomato necrotic stunt virus
Bidens mottle virus
potato virus Y
pepper severe mosaic virus
pepper mottle virus
potato virus V
Peru tomato mosaic virus
Bootstrap support:
60%
70%
80%
90%
100%
Clade branch color key:
Macluravirus
Potyvirus

Figure 8

Figure 8 | Characteristics of (+)ssRNA viruses from family *Potyviridae* (order *Patatavirales*) from the present study. a Selected photographs of samples that tested positive in RT-PCR assays for the viruses presented herewith. **b-e** Heatmaps of the pairwise identities of *Macluravirus* and *Potyvirus* species based on alignment and comparison of full genome nucleotide sequences (**b-c**) and based on full polyprotein amino acid sequence (**d-e**). **f** Genome organization with predicted cistrons and read coverage for novel potyviruses from this study. **g** Maximum likelihood phylogenetic tree based on the alignment of conserved RdRp amino acid sequence of RefSeq species from the *Potyviridae* family (*Macluravirus* and *Potyvirus* genera). Branch length scale represents amino acid substitution per site. Virus names and acronyms in blue bold font are the novel viruses, while those in black bold font are known viruses from the present study. Indicated after the acronym are the isolate IDs of the viruses. Full virus names of those abbreviated in the pairwise identity matrices (**b-e**) can be found in the genome organization and phylogenetic trees (**f-g**).

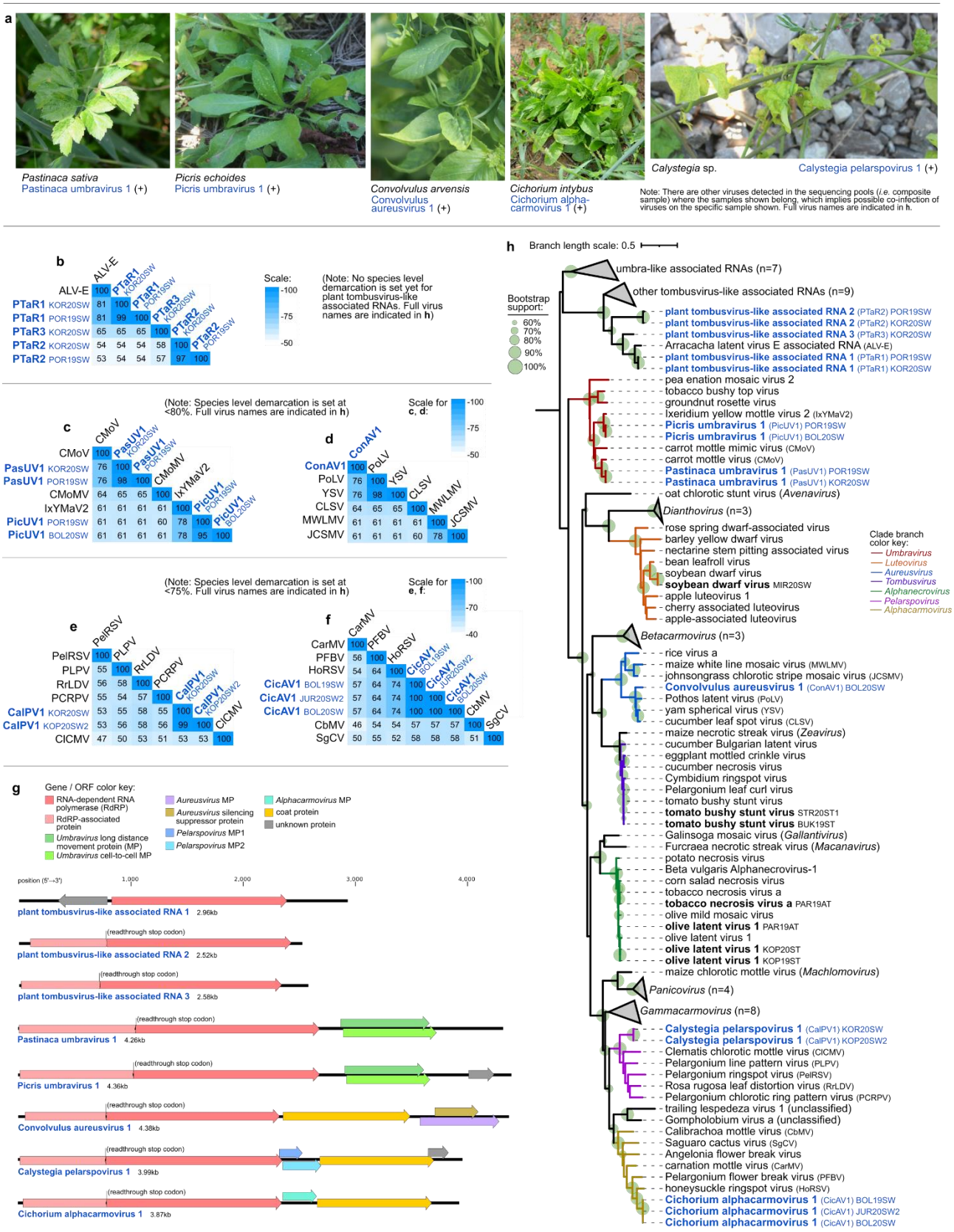

a
Pastinaca sativa
Pastinaca umbravirus 1 (+)
Picris echoides
Picris umbravirus 1 (+)
Convolvulus arvensis
Convolvulus aureusvirus 1 (+)
Cichorium intybus
Cichorium alpha-carmovirus 1 (+)
Calystegia sp.
Calystegia pelarspovirus 1 (+)
Note: There are other viruses detected in the sequencing pools (i.e. composite sample) where the samples shown belong, which implies possible co-infection of viruses on the specific sample shown. Full virus names are indicated in h.
b
(Note: No species level demarcation is set yet for plant tombusvirus-like associated RNAs. Full virus names are indicated in h)
Scale:
c
d
(Note: Species level demarcation is set at <80%. Full virus names are indicated in h)
Scale for c, d:
e
f
(Note: Species level demarcation is set at <75%. Full virus names are indicated in h)
Scale for e, f:
g
Gene / ORF color key:
RNA-dependent RNA polymerase (RdRP)
RdRP-associated protein
Umbravirus long distance movement protein (MP)
Umbravirus cell-to-cell MP
Aureusvirus MP
Aureusvirus silencing suppressor protein
Pelarspovirus MP1
Pelarspovirus MP2
Alphacarmovirus MP
coat protein
unknown protein
position (5'→3')
plant tombusvirus-like associated RNA 1 2.96kb
plant tombusvirus-like associated RNA 2 2.52kb
plant tombusvirus-like associated RNA 3 2.58kb
Pastinaca umbravirus 1 4.26kb
Picris umbravirus 1 4.36kb
Convolvulus aureusvirus 1 4.38kb
Calystegia pelarspovirus 1 3.99kb
Cichorium alphacarmovirus 1 3.87kb
(readthrough stop codon)
h
Branch length scale: 0.5
Bootstrap support:
umbra-like associated RNAs (n=7)
other tombusvirus-like associated RNAs (n=9)
Dianthovirus (n=3)
Betacarmovirus (n=3)
Panicovirus (n=4)
Gammacarmovirus (n=8)
Clade branch color key:
Umbravirus
Luteovirus
Aureusvirus
Tombusvirus
Alphanecrovirus
Pelarspovirus
Alphacarmovirus

Figure 9

Figure 9 | Characteristics of (+)ssRNA viruses in family *Tombusviridae* (order *Tolivirales*) from the present study. a Selected photographs of samples that tested positive in RT-PCR assays for the viruses presented herewith. **b-f** Heatmaps of pairwise identities of plant tombusvirus-like associated RNAs based on alignment and comparison of RdRp amino acid sequences (**b**), *Umbravirus* species based on full genome nucleotide sequences (**c**), *Aureusvirus* species (**d**), *Pelarspovirus* species (**e**), and *Alphacarmovirus* species (**f**) based on RdRp amino acid sequences. **g** Genome organization of novel *Tombusviridae* species and tombusvirus-like associated RNAs from this study. Open reading frames (ORFs) and the proteins they code for are color coded accordingly. **h** Maximum likelihood phylogenetic tree based on the conserved amino acid sequence of RdRp. Virus names and acronyms in blue bold font are the novel viruses, while those in black bold font are known viruses from the present study. Indicated after the acronym are the isolate IDs of the viruses. Branch length scale represents amino acid substitution per site. Full virus names of those abbreviated in the pairwise matrices in (**b-f**) are in the phylogenetic tree (**h**).

Data availability

Raw sequencing reads were submitted in the NCBI Sequence Read Archive (SRA), with the BioProject accession number PRJNA772045. All sample and sequencing metadata, and SRA accession numbers are in Supplementary Tables 1-3. All data related to virus genome assembly and identification, and their NCBI GenBank accession numbers are listed in Supplementary Tables 4-6. All other data, expanded or detailed figures, and raw results (*i.e.*, data source) of all analyses are provided in the 'Supplementary information' section of this paper.

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Contributions

M.R., D.K., and I.G.A. acquired funding. D.K., M.R., and M.P.S.R. formulated and designed the study. D.K. and M.R. supervised the study. M.P.S.R. did the laboratory and greenhouse work, the data analyses, and wrote the first draft of the manuscript. K.B. did the electron microscopy, and A.P. did a part of the nanopore sequencing of inoculated plants and data analyses. The rest of the authors significantly helped in sampling and sample processing. All authors contributed to editing the final manuscript.

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Ethics declarations

Competing interests

The authors declare no competing interests.

Additional information

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Supplementary information

Supplementary File 01: Supplementary Table 1. Individual plant samples, their species names, corresponding plant families and sampling locations. **Supplementary Table 2.** Composite samples used in high-throughput sequencing, their composition, year of collection, farm type and other associated information. **Supplementary Table 3.** Sequence read archive (SRA) metadata of the sequencing experiments, read counts, and other associated information.

Supplementary File 02: Supplementary Table 4. List summarizing all viruses detected in the composite samples. **Supplementary Table 5.** List of analyses done in the reconstruction and identification of individual virus genomes. **Supplementary Table 6.** List of circular RNAs assembled using a custom pipeline based on SLS-PFOR2, with filtering using BLASTn and BLASTx searches.

Supplementary File 03: Supplementary Figure 1. Genome organization of novel viruses, first full genomes of known viruses showing known and putative open reading frames and the protein they code for and predicted secondary structures of selected viroid-like circular RNAs detected in this study.

Supplementary File 04: Supplementary Table 7. RT-PCR primers and PCR conditions used in confirmation of associated plant hosts of selected viruses and putative viroids. **Supplementary Table 8.** RT-PCR thermocycling conditions used in the detection of selected viruses and putative viroids in associated plant hosts. **Supplementary Figure 2**. Orientation and annealing sites of the primers designed for the amplification of the circular genome of Taraxacum viroid-like circular RNA 1, and results of RT-PCR confirmation of the circular genome. **Supplementary Table 9.** List of confirmed associated plant hosts of selected viruses and putative viroids.

Supplementary File 05: Supplementary Figure 3. Representative field photos of confirmed associated plant hosts of selected viruses and putative viroids.

Supplementary File 06: Supplementary Table 10. Model selection and other parameters used for phylogenetic analyses. **Supplementary Figure 4.** Phylogenetic trees up to the virus family or order level showing relationships of identified known and novel viruses in this study.

Supplementary File 07 (Data Source): Supplementary Table 11-22. Summary of all results from the DnaSP v. 6 analyses.

Supplementary File 08 (Data Source): Supplementary Table 23-42. Summary of all pairwise identity values from the SDT v. 1.2 analyses.

Supplementary File 09 (Data Source): Supplementary Table 43-47. Summary of all results from the protein domain prediction using InterPro scans, and homology alignments with known species of potyviruses to identify start and cleavage sites of proteins.

Supplementary File 10: Supplementary Figure 5. RT-PCR, TEM and nanopore sequencing results on the confirmation of infectivity of Plantago tobamovirus 1 in solanaceous hosts.

All supplementary files were deposited in FigShare, and can be accessed and cited through this digital object identifier (doi): https://doi.org/10.6084/m9.figshare.20200769.v2