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Characterisation of a novel Emaravirus identified in mosaic-diseased Eurasian aspen (*Populus tremula*)

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Abstract

Since Emaraviruses have been discovered in 2007 several new species were detected in a range of host plants. Five genome segments of a novel Emaravirus from mosaic-diseased Eurasian aspen (*Populus tremula*) have been completely determined. The monocistronic, segmented ssRNA genome of the virus shows a genome organisation typical for Emaraviruses encoding the viral RNA-dependent RNA polymerase (RdRP, 268.2 kDa) on RNA1 (7.1 kb), a glycoprotein precursor (GPP, 73.5 kDa) on RNA2 (2.3 kb), the viral nucleocapsid protein (N, 35.6 kDa) on RNA3 (1.6 kb), and a putative movement protein (MP, 41.0 kDa) on RNA4 (1.6 kb). The fifth identified genome segment (RNA5, 1.3 kb) encodes a protein of unknown function (P28, 28.1 kDa). We discovered that it is distantly related to proteins encoded by Emaraviruses, such as P4 of European mountain ash ringspot-associated virus. All proteins from this group contain a central hydrophobic region with a conserved secondary structure and a hydrophobic amino acid stretch, bordered by two highly conserved positions, thus clearly representing a new group of homologues of Emaraviruses. The virus identified in Eurasian aspen is closely associated with observed leaf symptoms, such as mottle, yellow blotching, variegation and chloroses along veins. All five viral RNAs were regularly detectable by RT-PCR in mosaic-diseased *P. tremula* in Norway, Finland and Sweden (Fennoscandia). Observed symptoms and testing of mosaic-diseased Eurasian aspen by virus-specific RT-PCR targeting RNA3 and RNA4 confirmed a wide geographic distribution of the virus in Fennoscandia. We could demonstrate that the mosaic-disease is graft-transmissible and confirmed that the virus is the causal agent by detection in symptomatic, graft-inoculated seedlings used as rootstocks as well as in the virus-infected scions used for graft-inoculation. Owing to these characteristics, the virus represents a novel species within the genus Emaravirus and was tentatively denominated aspen mosaic-associated virus.

KEYWORDS

Aspen mosaic-associated virus, genome organisation, geographic distribution, graft transmission

1 | INTRODUCTION

The objectives of this study were to (a) determine the genome of a presumably novel Emaravirus in Eurasian aspen (*Populus tremula* L.), (b) develop a reliable detection protocol, (c) provide evidence for association of the virus with the observed disease and (d) evaluate the incidence of the virus in *P. tremula* exhibiting virus-suspected symptoms in Fennoscandia.

We were observing mosaic, mottle, yellow blotching, variegation and chloroses along veins of leaves of Eurasian aspen since 1991 in Norway and since 2009 in Finland. Generally, whole branch parts or larger areas of the crown were affected and trees in the Rovaniemi area (Kivalo Research Forest) were increasingly showing signs of decline. Comparable symptoms on Eurasian aspen in Finland have first been reported by Bremer, Lehto, and Kurkela (1991). Erroneously, typical "mosaic-disease" symptoms have been considered a special genetic form of Eurasian aspen (Oskarsson & Nikkanen, 1998). This mosaic-disease of Eurasian aspen has also been documented by us since 2015 at additional sites in southern Norway and Sweden (Figure 1). Few studies have addressed plant viruses affecting poplars (*Populus* spp.), which may also contribute to observed degeneration of trees (Büttner, von Bargen, Bandte, & Mühlbach, 2013; Nienhaus & Castello, 1989). The most widespread virus in *Populus* is the poplar mosaic virus (PopMV), which has been detected in different regions of Europe, Asia and the Americas and in various species or their hybrids including Eurasian aspen. The virus is transmissible by mechanical means and is also

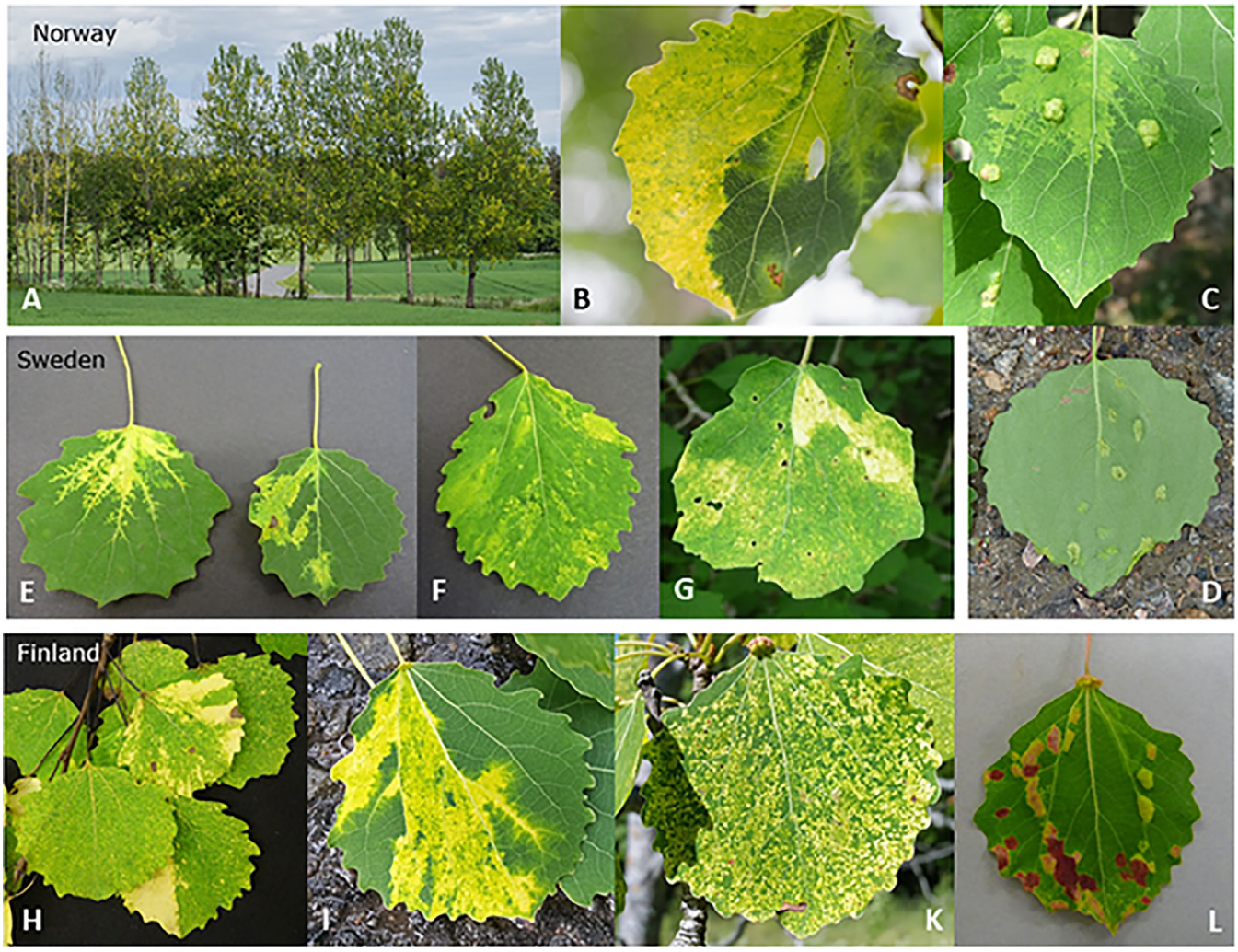

FIGURE 1 Leaf symptoms observed in Eurasian aspen (*Populus tremula*) in different locations and vegetation periods from July 2016 and July 2017. (a) Chlorotic patches in the canopy of a row of Eurasian aspen trees in Kråkstad, Norway 2016 and (b) detail of leaf with mottle, yellow blotching and chloroses along veins from Eurasian aspen in Kråkstad, Norway 2016. (c) Leaf with vein chloroses and infested by the gall mite *Phyllocoptes populi*; (d) underside of a leaf showing extensive hair production in cavities colonised by *Phyllocoptes populi*, Sellebakk, Norway 2017. (e) Leaves with vein chloroses and mottle from Eurasian aspen sampled in Säffle, Sweden in 2015, mosaic (f) variegation and mottle (g) found in a tree in Fossum, Sweden in 2016 and 2015. In Finland (h) mottle, mosaic, variegation and yellow blotches were observed in Eurasian aspen leaves in the Kivalo Research Forest, 2016, (i) mosaic and vein chloroses of a leaf sampled in Rovaniemi, 2017. (k) Leaf of Eurasian aspen with mosaic symptoms and infested with the gall mite *Eriophyes diversipunctatus* producing galls at the petiole, Vallisaari, Helsinki, 2017. (l) Leaf of Eurasian aspen infested by different gall mite species (*P. populi* and *E. diversipunctatus*) and with vein chloroses collected in the Kivalo Research Forest in 2017

spread naturally via seeds and during vegetative clonal propagation by root runners in poplar populations. Reported leaf symptoms associated with PopMV infections vary greatly and not all varieties develop symptoms when they are infected with PopMV (Biddle & Tinsley, 1971; Navratil, 1979). Further, populations of black poplars (*Populus* × *euamericana*) in the United Kingdom have been found to contain nematode-transmissible viruses such as arabis mosaic virus (ArMV) and tobacco rattle virus (TRV). From American aspen (*P. tremuloides*) the soil-borne tobacco necrosis virus (TNV) was isolated from declining trees also showing ring blotches and chlorosis of the leaf veins (Hibben, Reese, & Borzarth, 1979). However, with the exception of PopMV, the viruses could not be confirmed as causal agents of the observed diseases of the poplars studied (Navratil, 1979). Information on the distribution and the host range of these viruses is therefore very incomplete because of the lack of systematic studies on the occurrence and importance of plant viruses affecting poplars.

Some of the Eurasian aspen trees with virus-suspected leaf symptoms surveyed in Finland, Sweden and Norway were also infested by gall mites (Acari, Eriophyidae, Figure 1), which were reported as vectors for several members of the genus *Emaravirus* (Hassan et al., 2017; Mielke-Ehret & Mühlbach, 2012). Based on these findings, an initial RT-PCR for the genus-specific detection of Emaraviruses (Elbeaino, Whitfield, Sharma, & Digiaro, 2013) was carried out using total RNA from leaf material of Eurasian aspen with mosaic, mottle and chloroses along veins collected in 2015 in Sweden in comparison with material collected from a tree without any disease symptoms. Only from diseased Eurasian aspen a specific fragment was amplified with the generic primers. This suggested that a previously unknown putative Emaravirus is associated with the observed mosaic-disease symptoms in Eurasian aspen.

The genus *Emaravirus* (family *Fimoviridae*, order *Bunyavirales*) contains enveloped plant viruses with a monocistronic, segmented RNA genome of negative polarity, which are transmitted by gall mites (Elbeaino et al., 2018). Since the first description of the type species European mountain ash ringspot-associated Emaravirus (EMARaV) in 2007 (Mielke & Mühlbach, 2007), the genus has significantly increased in numbers of acknowledged species and putative members. Recently, several previously unknown Emaraviruses were identified to be prevalent in different important fruit or desirable ornamental tree species and shrubs, respectively. For instance, they could be associated with a disease in pistachio (Buzkan et al., 2019), blue palo verde tree (Ilyas, Avelar, Schuch, & Brown, 2018), blackberry (Hassan et al., 2017), kiwi (Zheng et al., 2017), jujube tree (Yang et al., 2019) and ti plant (Olmedo-Velarde et al., 2019).

2 | MATERIALS AND METHODS

2.1 | Determination of complete viral genome segments

Different approaches were applied to determine complete genomic segments of the novel virus from mosaic-diseased Eurasian aspen trees.

Initially, Illumina RNAseq was applied to total RNA extracted from symptomatic leaf samples taken from two diseased trees originating from different sites in Sweden in 2016 (Säffle, E55056) and in 2017 Fossum (E56750) as described in Mielke and Mühlbach (2007), but omitting the phenol/chloroform extraction. The first sample from Säffle was prepared for large scale sequencing (high-throughput sequencing, HTS) including data analyses carried out by the company BaseClear (Leiden, the Netherlands), as described in von Bargen et al. (2019) using the generic PDAP213 primer (Di Bello, Ho, & Tzanetakis, 2015) for preparation of double stranded cDNA. A standard-paired end library (125 bp) was prepared by the sequencing-company BaseClear (the Netherlands) and sequenced on a HiSeq2500 machine (Illumina). For the second HTS sample E56750 RNA was extracted from fresh leaf material collected in 2017. The only other difference was that a random hexamer-primed RNAseq approach was used, in order to allow identification of additional viruses which may contribute to the observed disease. Both datasets only yielded small genome fragments of the assumed novel Emaravirus. Therefore, full-length RT-PCR for amplification, cloning and Sanger-sequencing of missing emaraviral genome segments as outlined in von Bargen et al. (2019) was carried out with total RNA leaf-extracts from the same diseased Eurasian aspen in Fossum sampled in 2016 (E55089). It was not possible to amplify the complete RNA1 of the novel virus by full-length RT-PCR from either of the samples taken in 2016 (E55089) or 2017 (E56750. As leaf material from 2016 was not available anymore, missing sequence information was generated by Sanger-sequencing of PCR amplicons generated from random hexamer primed cDNA from sample E56750 with newly developed primers (Table S1), allowing the closure of sequence gaps between scaffolds representing RNA1 of the virus. 5′ and 3′ termini of the RNA1 and the 3′ end of the RNA2 were confirmed by Sanger sequencing of at least three cloned fragments obtained by rapid amplification of cDNA ends (RACE) PCR as described in von Bargen et al. (2019).

Raw data obtained by RNAseq were quality trimmed and assembled de novo into contigs and scaffolded with the in-house standard procedure of the sequencing company BaseClear. Delivered scaffolds with a minimum length of 300 nucleotides were compared via blastX (National Center for Biotechnology Information, NCBI) against the non-redundant protein database (NRPROT). By filtering against the taxid 675,845 “Emaravirus” and taxid 10,239 “viruses” respectively, all scaffolds relating to plant viruses were identified and aligned with reference genomes in BioEdit, version 7.0.5.3 (Hall, 1999) using the integrated ClustalW option. Analysis of Sanger-sequencing generated RT-PCR products was also performed in BioEdit as well as alignments of complete genome segments of Emaraviruses. Sequence identity matrices were calculated with the according tool in BioEdit from aligned nucleotide and aa sequences.

2.2 | Analyses and comparison of viral genome segments and encoded proteins

ORF finder at NCBI (https://www.ncbi.nlm.nih.gov/orffinder/) was used to find open reading frames (ORFs) and the proteins encoded by the identified viral genome segments. All ORFs with 300 or more nucleotides were considered.

Phylogenetic analyses were conducted using MEGA version X (Kumar, Stecher, Li, Knyaz & Tamura, 2018). Trees were calculated applying the Maximum Likelihood method based on the JTT matrix-based model (Jones, Taylor, & Thornton, 1992) and boot-strapping with n = 1,000 replicates. Branches with values below 50% were collapsed. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. All positions containing gaps and missing data were eliminated.

For protein sequence analysis, comparison and homology search of the RNA5-encoded protein of aspen mosaic-associated virus (AsMaV) ANNIE (Ooi et al., 2009) was used to predict the structural organisation of proteins. MAFFT (Katoh & Standley, 2013) was used for multiple sequence alignments. The alignment displayed using Jalview (Waterhouse, Procter, Martin, Clamp, & Barton, 2009) with the ClustalX colouring scheme. Protein secondary structure was predicted using Jpred (Cole, Barber, & Barton, 2008). Remote homology searches were run from the MPI toolkit web server (Alva, Nam, Söding, & Lupas, 2016; Zimmermann et al., 2018), using the strategy described in Kuchibhatla et al. (2014). We used the software Psi-blast (Altschul et al., 1997) and HHblits (Remmert, Biegert, Hauser, & Söding, 2012) with the standard cutoff E value of 10−3, and HHpred (Hildebrand, Remmert, Biegert, & Söding, 2009; Söding, Biegert, & Lupas, 2005) with a stringent cutoff value of 10−5. Psi-blast was run with five iterations against either the database nr50 or nr30, and HHblits was run with five iterations against Uniclust30_2018_08. HHpred was run either against the databases PDB70 or PFAM, or in pairwise mode comparison (option "Align two sequences or MSAs").

Additionally, double-stranded RNA (dsRNA) was isolated according to the protocol developed for EMARaV (Benthack, Mielke, Büttner, & Mühlbach, 2005) from 15 g of leaves from virus-infected Eurasian aspen collected in July 2016 from different locations in Norway (Kråkstad) and Finland (Kemi and Kivalo Research Forest), respectively. Double stranded-RNAs were separated by electrophoresis in a 0.8% agarose gel, in order to compare the obtained pattern and sizes with the genome segments of the novel virus determined by sequencing.

2.3 | Association of the novel virus with the observed disease

2.3.1 | RT-PCR detection

A total of 84 leaf samples were collected in 2016 and 2017 from *P. tremula* trees in Finland, Norway and Sweden and total nucleic acids were extracted as above. Random hexamer-primed reverse transcription (RT) was conducted according to the protocol established for EMARaV detection in *Sorbus* sp. as described in von Bargen et al. (2019). The integrity of the RNA and absence of PCR inhibitors were evaluated using an internal control according to Menzel, Jelkmann, and Maiss (2002). For specific detection of all genome segments of the novel virus identified in Eurasian aspen, primer pairs were developed within the coding region for each of the five RNAs, according to the scaffolds assembled from the diseased sample tree in Säffle (E55056). PCR conditions for primer pairs for each genome segment of the novel virus were optimised in order to generate specific fragments of the expected size. Amplified PCR products were bidirectionally sequenced and aligned in order to confirm that they originated from the same virus species (data not shown). The same samples were tested by RT-PCR for PopMV infection applying a primer pair established by Werner, Mühlbach, and Büttner (1997).

2.3.2 | Grafting experiment

Total of 276 Eurasian aspen scions were whip grafted or side grafted - depending on the diameter of the grafted scion—to *Populus tremula* seedlings (nursery Bunk, Germany) in March 2017 in order to confirm the graft-transmissibility of the observed disease. Twenty-eight Eurasian aspen seedlings were grafted with healthy scions from the seedling population, serving as negative controls. Grafted seedlings were cultivated for 2 months in a fogged tunnel, then transferred to the experimental garden and cultivated in 2 L pots for 2 years in peat substrate. The scions used for graft-inoculation originated from a mosaic-diseased Eurasian aspen tree growing in the Kivalo Research Forest (Finland) that tested positive for the novel virus by RT-PCR applying genus specific primers according to Elbeaino et al. (2013) and species-specific primers targeting RNA3 and RNA4 of the novel virus. For two consecutive vegetations periods the grafted seedlings were evaluated for virus-suspected symptoms such as mottle, mosaic, development of yellow blotches and/or vein yellowing. Symptomatic leaf material from 53 grafted scions and three graft-inoculated rootstocks exhibiting leaf symptoms in May and June 2018 were tested by RT-PCR targeting the RNA3—RNA5 of the novel identified virus, to confirm its presence in the scions and the rootstocks.

2.4 | Geographical distribution of the novel virus in Eurasian aspen

Primer sets targeting the RNA3 and RNA4 that produced a 317 bp long fragment of RNA 3 and a 288 bp long amplicon of the RNA4 provided the highest sensitivity when compared to the primers specific for the other genome segments. These two primer sets were therefore applied in RT-PCR based detection of the novel virus in 10 leaf samples from Eurasian aspen collected in additional locations in southern Sweden in July 2018 exhibiting mosaic, mottle and vein chloroses. Total RNA was extracted from approximately 150 mg symptomatic leaf material and cDNA-synthesis was carried out using random hexamer primers (Biolegio, the Netherlands) and the MaximaH(⁻)-Rtase (ThermoScientific, Germany) according to manufacturer's instructions. 1 µl of cDNA was applied to 24 µl PCR mix containing 0.625 u DreamTaq polymerase according to the manufacturer´s instructions (ThermoScientific, Germany). Amplification was

performed 2 min at 94°C, followed by 35 cycles of 94, 53 and 72°C each for 30 s, and final elongation for 5 min at 72°C.

The GenBank/ENA/DDBJ accession numbers for the new AsMaV.

RNA sequences are LR742461-LR742465.

http://www.ebi.ac.uk/ena/data/view/LR742461-LR742465

3 | RESULTS

3.1 | Genome determination, analyses and phylogenetic characterisation of a novel Emaravirus from mosaic-diseased Eurasian aspen

The RNAseq dataset (178 MB) received from the diseased Eurasian aspen E55056 in Säffle yielded in 485 assembled scaffolds. BlastX analyses identified nine scaffolds exhibiting significant aa identities of at least 33% to five different genome segments of several Emaraviruses. By filtering against the taxid 675,845 "Emaravirus," five scaffolds with a length between 772 and 331 nucleotides (nt) relating to the 5´ and 3´ proximal regions of RNA1 of fig mosaic virus (FMV), pigeonpea sterility mosaic virus 2 (PPSMV-2) and rose rosette virus (RRV) were found. One scaffold (2,040 nt) showed 54% aa identity and covered the complete glycoprotein-coding (GPP) region of RNA2 including the 5´terminus. One scaffold (1,060 nucleotides long) showed 61% aa identity to the complete N protein sequence of PPSMV-2, while another scaffold (547 nt) related to the MP of FMV. The ninth scaffold (835 nt) retrieved from the dataset showed closest relation to the P6 of PPSMV-2 in blastX, a protein of approximately 27 kDa with unknown function. RNAseq of the second mosaic-diseased Eurasian aspen sample E56750 from Fossum in 2017 using a random hexamer-primed dscDNA library delivered eight contigs (80–589 nt) producing similarities (cutoff E-value: 1.0e−10) to different proteins encoded by RNA1–RNA4 of Emaraviruses in blastX, but

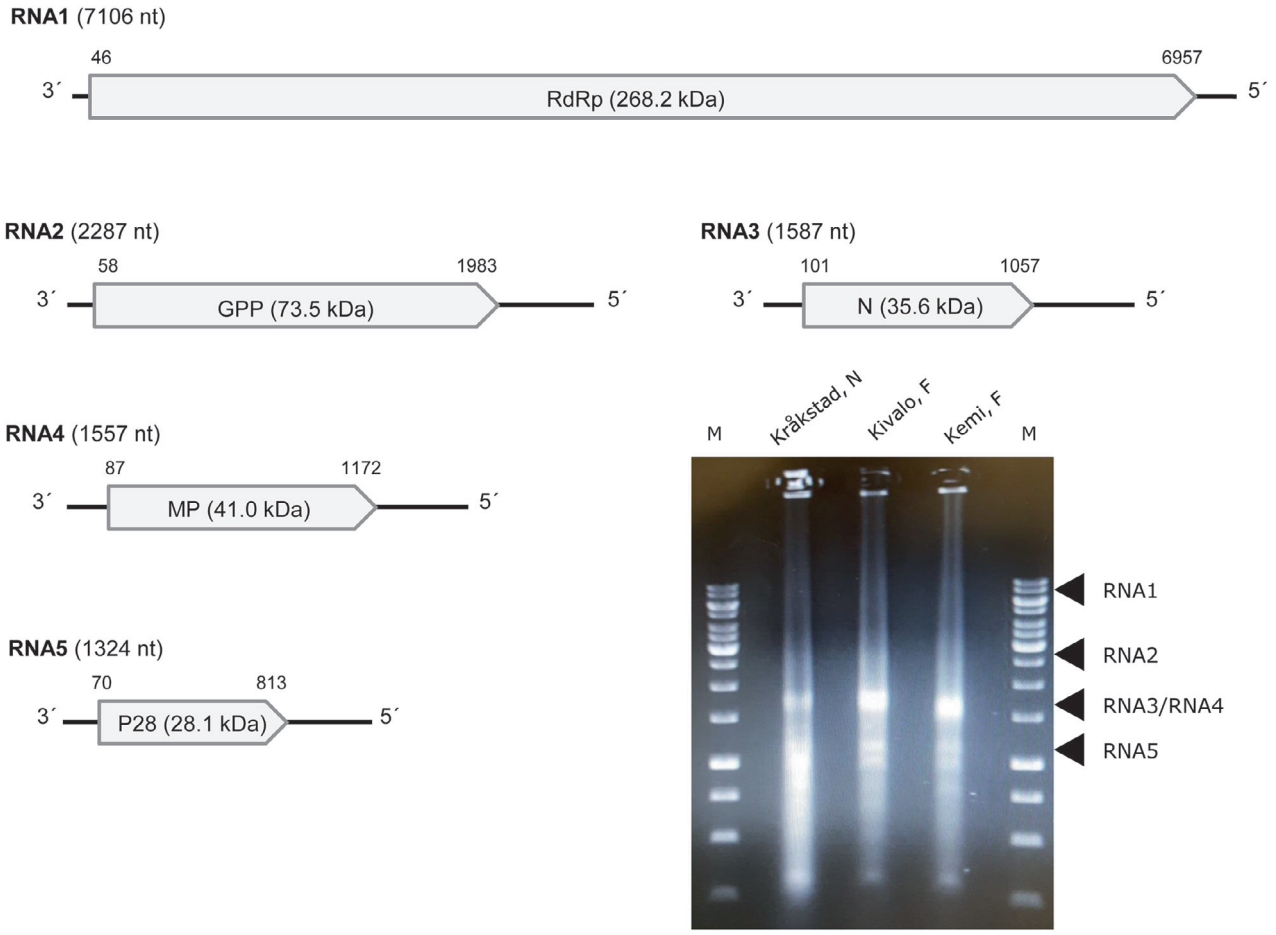

FIGURE 2 Schematic genome organisation of aspen mosaic-associated virus (AsMaV) identified in *Populus tremula*. Genome segments of the ss(−)RNA virus are displayed as mRNA with the encoded open reading frames (ORFs) as arrows indicating the first and last nucleotide above the arrow. The locations of the replicase (RdRP) on RNA1, the Glycoprotein precursor (GPP) on RNA2, the nucleocapsid protein (N) on RNA3, the movement protein (MP) on RNA4 and the protein of unknown function (P28) encoded by RNA5, are shown and are given with the deduced molecular mass. DsRNA patterns after gel electrophoresis in an 0.8% agarose gel are indicated from three different leaf samples of Eurasian aspen trees collected in Norway (N) and Finland (F), respectively, showing mosaic, mottle and vein yellowing. The corresponding RNA genome segments of the putative novel Emaravirus are indicated on the right side. (M) The 1 kb ladder (ThermoScientific) was used for comparison as size standard

no additional sequence information of the novel virus from Eurasian aspen. Furthermore, filtering the NRPROT database at NCBI with the taxid 10,239 "viruses," no additional sequences of plant viral origin could be identified by blastX in this dataset.

The sample E55089 was further analysed to determine the complete sequences of the five genome segments of the novel virus identified in mosaic-diseased Eurasian aspen (Figure 2). The complete RNA1 was assembled by Sanger sequencing of eight overlapping RT-PCR amplified fragments applying primer sets (Table S1) developed according to the available sequence information from the RNAseq data sets. Additionally, both ends of RNA1 were confirmed by RACE PCR with cDNA-synthesis-primers specific for the 5′ and the 3′ terminus respectively (Table S1). It is 7,106 nt long and encodes the viral replicase (RdRp, 2,303 aa, 268.2 kDa) as the sequence between positions 2,134–4,341 of the coding strand was recognised as the RNA dependent RNA polymerase-domain of bunyavirus' superfamily (pfam04196, *E* value 7.47e−43) in blastX. The protein sequence showed highest aa identities with the RdRps of the pistacia virus B (PiVB, 76.3%) and RRV (71.4%) (Table 1).

The complete sequences of RNA2, RNA3, RNA4 and RNA5 of the novel virus could be determined by cloning and Sanger sequencing of full-length RT-PCR products (Figure 3) excised from agarose gels. Sequences were confirmed from at least three individual clones of each segment; however, no additional genome segments could be identified by this method. The fragment with an approximate length of 2.3 kb was identified as the viral RNA2 (2,287 nt) encoding the glycoprotein precursor (GPP, 641 aa, 73.5 kDa) in blastX expressing significant homologies (*E* value 0.0) to respective proteins encoded by PiVB and PPSMV-2. The complete protein sequence showed up to 58.3% aa identities to corresponding proteins of members of the genus *Emaravirus* (Table 1). The 3′ end of the genomic RNA2 could be confirmed by RACE PCR as described above.

Cloning and sequencing of the 1.6 kb fragment amplified by full-length RT-PCR revealed that it contained two different viral genome segments. A 1,587 nt large fragment was identified as RNA3 with one open reading frame (ORF) encoding the putative N protein (318 aa, 35.6 kDa) required for encapsidation of viral RNAs. This protein showed some homologies to the N proteins of PPSMV-2 (*E* value: 2e−137) and FMV (*E* value: 1e−132) in blastX, but shared only 17.2–59.1% aa identity with the nucleocapsid proteins of other established species of the genus *Emaravirus* (Table 1).

A slightly shorter full-length RT-PCR product (1,557 nt) could be determined as RNA4 encoding the putative MP (361 aa, 41.0 kDa). The coding strand contained a P4 movement protein-domain of the Emaraviruses (pfam16505, *E* value: 8.88e−157) between nucleotides 132–1,166 identified by blastX analysis. At the aa level this protein shared highest sequence identity with the putative MP of PiVB (67.3%, Table 1).

The complete RNA5 sequence (1,324 nt) was retrieved from the cloned 1.3 kb full-length RT-PCR product encoding a protein (P28, 247 aa, 28.1 kDa) showing some identities on aa level (10.7–37.6%, Table 1) to proteins of unknown function encoded by other Emaraviruses.

TABLE 1 Comparison of sequence identities on amino acid level of deduced proteins (RdRp, GPP, N, MP, P28) encoded by RNA1–RNA5 of the novel Emaravirus from Eurasian aspen (*P. tremula*) with corresponding proteins from established and putative members of the genus *Emaravirus*

	Emaravirus from *Populus tremula* (sequence identities in percent)				
	RNA1	RNA2	RNA3	RNA4	RNA5
Emaraviruses	RdRp	GPP	N	MP	P28
AcCRaV	47.2	37.3	33.9	29.6	12.4 (P5, 27 kDa)
BLMaV*	68.6	53.3	54.4	53.9	29.4 (P5, 26 kDa)
Bpvbv*	31.1	21.4	17.1	16.5	–
EMARaV	48.6	36.1	35.8	28.9	10.7 (P4, 27 kDa)
FMV	71.1	51.6	57.8	65.3	30.7 (P6, 26 kDa)
HPWMoV	29.6	20.0	17.2	15.8	–
JYMaV*	31.6	32.6	20.7	14.3	–
PiVB*	**76.3**	**58.3**	56.1	**67.3**	**37.6 (P6, 28 kDa)**
PPSMV-1	53.7	42.5	39.4	37.1	30.7 (P6, 27 kDa)
PPSMV-2	70.2	53.6	**59.1**	63.4	31.1 (P6, 27 kDa)
RLBV	31.2	22.1	20.4	14.2	–
RRV	71.4	53.9	52.3	26.4	36.0 (P6b, 27 kDa)
RYRSaV	47.0	37.6	37.9	30.8	12.0 (P5, 26 kDa)
TiRSaV*	31.7	20.0	19.5	11.6	–

Notes: Highest percentage of amino acid identities are highlighted in bold. The corresponding proteins of Emaraviruses and putative members of the genus (*) identified as homologues of P28 of AsMaV are indicated in brackets (name, size) in the last column. Viruses are given with their approved acronyms. BLMaV, blackberry leaf mottle-associated virus; PiVB, pistacia virus B; BPVBV, blue palo verde broom virus; TiRSaV, Ti ringspot-associated virus; JYMaV, jujube yellow mottle-associated virus.

Psiblast detected some of these proteins as homologues of the P28 protein encoded by the novel virus identified in Eurasian aspen; i.e., the P6 of PiVB (GenBank accession no. QAR18008), P6 of pigeon pea sterility mosaic virus 1 (PPSMV-1, ANQ90719) and PPSMV-2 (CDN67483), the P6b of RRV (AJW66845), the P6 of FMV (BAM13854) and P5 of blackberry leaf mottle-associated virus (BLMaV, AQX45477).

However, more distant homologues can frequently be identified by either of two approaches: (a) looking for marginal hits (i.e., for hits having an E-value above the significance cutoff) that belong to related taxa; or (b) by carrying out homology searches on proteins from related taxa in the hope of identifying a reciprocal match (Kuchibhatla et al., 2014). We thus looked for more distant homologues by systematically running searches on all other known proteins of Emaraviruses, using Psiblast and the more recent, powerful software HHblits. By so doing, we identified the P4 protein of EMARaV as a distant homologue of P28 with the following workflow.

Psiblast on EMARaV P4 (YP_003104766) detected homologues only in redbud yellow ringspot-associated virus (RYRSaV, P5, ANJ21384), actinidia chlorotic ringspot-associated virus (AcCRaV, P5, ALX00131 and EMARaV P27, VFU05380). We aligned these homologues and used the resulting alignment as a query in Psiblast and HHblits. HHblits detected a similarity with the P6 of FMV (itself a close homologue of the RNA5 encoded P28 identified above), with a marginal *E* value of 0.03, slightly below the significance cutoff of 10–3. This *E* value, although not significant, strongly suggests that EMARaV P4 and FMV P6 (and thus P28 of the aspen virus) might be homologous, because they belong to related taxa.

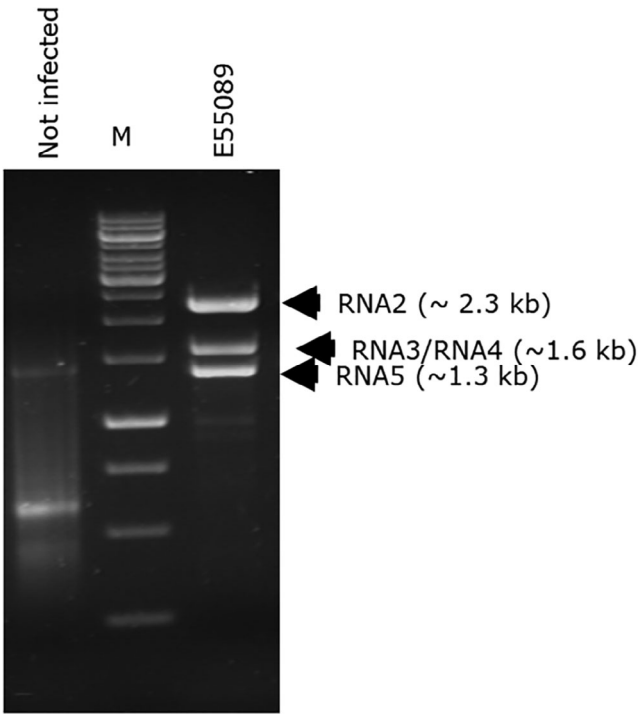

FIGURE 3 Gel electrophoresis of amplification products after full-length PCR applying the PDAP213-primer targeting the conserved terminal sequence regions of Emaraviruses according to Di Bello et al. (2015). Positions of emaraviral genome segments amplified from PDAP213-primed cDNA of the AsMaV-infected sample E55089 from Fossum, Sweden as confirmed by Sanger-sequencing of at least three individual clones are indicated at the right side of the gel. At the left side of the gel, unspecific amplification products from an Eurasian aspen sample without leaf symptoms are displayed. M = 1 kb ladder (ThermoScientific)

To verify this, we compared the alignment of P28 and its close homologues identified above with the alignment of the close homologues of EMARaV P4 identified above, by using HHpred in pairwise comparison mode. HHpred reported that their central region is similar, with an *E* value of $3.6 \times 10–8$, below the stringent significance cutoff of 10–5, thereby confirming homology. In contrast, we could not detect homologues of P28 in High Plains wheat mosaic virus (HPWMoV), raspberry leaf blotch virus (RLBV) or in the three recently reported Emaraviruses blue palo verde broom virus (BPVBV, Ilyas et al., 2018), jujube yellow mottle-associated virus (Yang et al., 2019), or Ti ringspot-associated virus (Olmedo-Velarde et al., 2019).

Figure 4 presents a sequence alignment of the central region of P28 of the novel virus from Eurasian aspen and of its homologues. All homologues of the P28 contain a hydrophobic region, bordered by two highly conserved positions R/K and G, indicated above Figure 4. Notably, the homologues of P28 bear a variety of names (P4, P5, P6, P6b), reflecting the size and order in which the RNAs that encode them were discovered; thus, harmonising their names, and perhaps choosing a unique name, will probably be necessary in the future.

Additional fragments amplified by full-length RT-PCR of 1 kb and 0.85 kb (Figure 3) were identified as partial genome segments representing the 3´terminal region of the RNA4 and RNA5, respectively, of the novel virus identified in Eurasian aspen.

The length of RNA1–RNA5 of the novel virus discovered by the different amplification strategies and sequencing approaches is in accordance with dsRNA patterns observed after gel electrophoresis of extracts of three mosaic-diseased *P. tremula* trees originating from Kråkstad (Norway), Kemi and the Kivalo Research Forest (Finland) (Figure 2). Additional small dsRNAs with a size of approximately 1 kb, 0.85 kb and 0.3 kb were visible. As the upper two dsRNAs correspond in length to the 1 kb and 0.85 kb products amplified by the full-length RT-PCR, it is assumed that they also represent truncated genome segments of the virus.

Phylogenetic trees inferred from the aa alignments of the RdRp encoded by RNA1 and the putative N protein (RNA3) of the novel virus found in Eurasian aspen and members of the genus *Emaravirus* (Figure 5) produced trees exhibiting different subgroups. One group was always composed of RLBV, HPWMoV and blue palo verde broom virus (BPVBV); the second contained EMARaV, AcCRaV and RYRSaV. The novel virus from Eurasian aspen clustered consistently with PiVB, RRV and PPSMV-2 supporting a closer relation with these viruses. Furthermore, high bootstrap values clearly demonstrate that AsMaV represents a separate novel species within the genus.

As a result of the observed symptoms in Eurasian aspen, the determined genome structure and phylogenetic relations of the novel virus, we propose the name aspen mosaic-associated virus (AsMaV).

3.2 | Identification of AsMaV as causal agent of the mosaic-disease affecting *P. tremula*

All five RNAs could be confirmed in leaf material from the mosaic-diseased *P. tremula* sampled in consecutive years (2016, 2017 and 2018) in Säffle and Fossum by RT-PCR using specific primer pairs targeting each genome segment. Primers were derived from the RNAseq-generated sequences of the virus variant in the Eurasian aspen tree E55056 from Säffle (Table S1).

RNA3–RNA5 were also consistently detected by this RT-PCR in 59 leaf samples of 62 mosaic-diseased Eurasian aspen trees collected from different locations in Norway, Sweden and Finland in the years 2016 and 2017 (Table 2). However, RNA1 and RNA2 specific fragments could only be amplified in 53 leaf samples showing mosaic, mottle, vein chloroses and/or chlorotic ringspots with the respective primer pairs, but RNA1 was also detectable with the generic primer set (Elbeaino et al., 2013) in these samples. Sequencing of selected PCR products confirmed that the mosaic-diseased trees were infected by AsMaV (data not shown). In none of the 22 Eurasian aspen trees with no typical virus-suspected leaf symptoms sampled adjacently to the diseased *P. tremula* could AsMaV be detected by RT-PCR using RNA1-RNA5 specific primers. These results demonstrate a clear

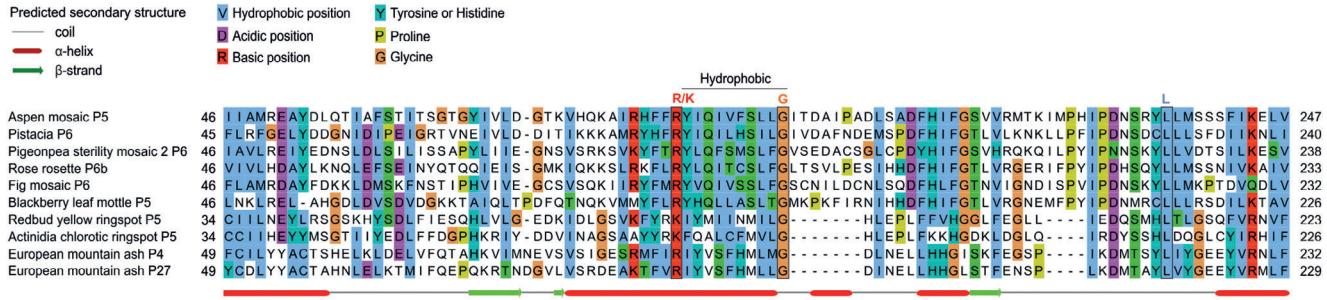

FIGURE 4 Sequence alignment of the homologues of the P28 protein encoded by RNA5 of AsMaV. Strictly conserved or semi-conserved positions are boxed, and the corresponding amino acid is indicated above the alignment

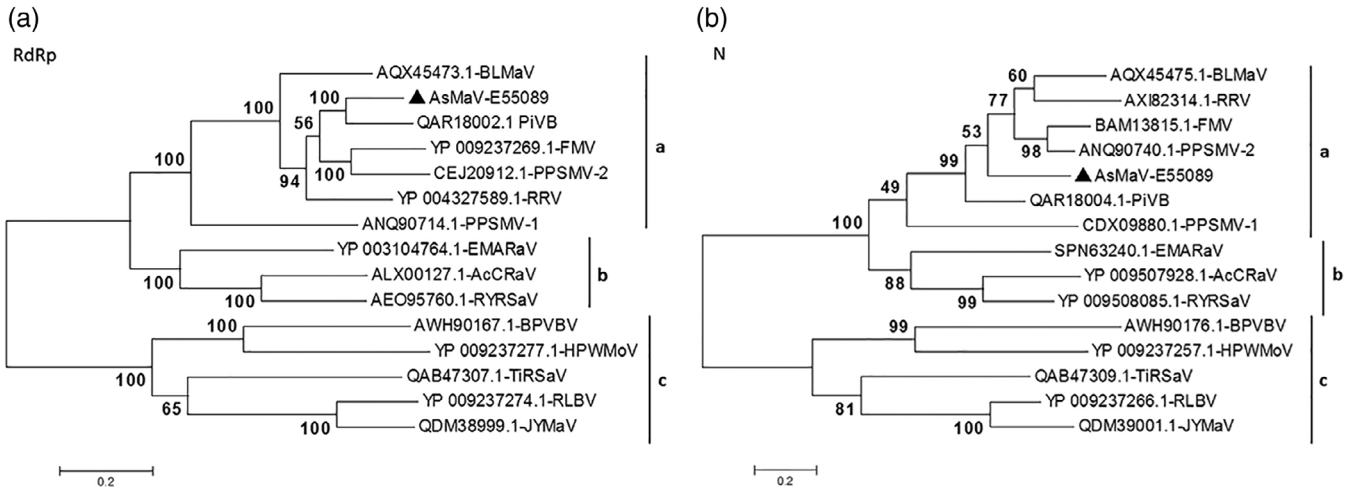

FIGURE 5 Molecular phylogenetic analysis by Maximum Likelihood method of RdRp protein encoded by RNA1 (a) and N protein encoded by RNA3 (b) of aspen mosaic-associated virus (AsMaV). Groups a to c of Emaraviruses described in Elbeaino et al. (2018) are indicated at the right side of the trees. The evolutionary analyses were conducted in MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018) and were inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. All positions containing gaps and missing data were eliminated. For analysis of the RdRp amino acid sequence there were a total of 2,141 positions in the final dataset and the tree with the highest log likelihood (−50,723.06) is shown. The tree inferred from the N aa sequence contained a total of 253 analysed positions in the final dataset and the tree with the highest log likelihood (−6,840.36) is shown. The analysis involved 15 amino acid sequences of established and putative members of the genus *Emaravirus*, which are indicated by their approved acronyms and GenBank accession numbers. Acronyms of putative members are BLMaV, blackberry leaf mottle-associated virus; PiVB, pistacia virus B; BPVBV, blue palo verde broom virus; TiRSaV, Ti ringspot-associated virus; JYMV, jujube yellow mottle-associated virus. Sequences determined from aspen mosaic-associated virus (AsMaV) are indicated by a black triangle

association of AsMaV with observed symptoms of the mosaic-disease of Eurasian aspen, especially as an infection of PopMV of the 84 investigated trees was excluded by RT-PCR (Table 2) applying a virus-specific primer set (Table S1). On the other hand, when testing nine poplar trees (*Populus* sp.) from three different locations in Germany (Berlin, Mecklenburg-Pomerania) showing leaf symptoms such as

TABLE 2 Detection of viruses by RT-PCR and association with leaf symptoms observed in Eurasian aspen (*P. tremula*) in Finland and Scandinavia sampled in 2016 and 2017

Location	Leaf symptoms	No. of sampled trees	AsMaV-infected	PopMV-infected
Finland	None	12	0	0
	Mosaic, mottle, vein chloroses, chlorotic ringspots	37	37	0
Norway	None	7	0	0
	Mosaic, mottle, vein chloroses, chlorotic ringspots	19	16	0
Sweden	None	3	0	0
	Mosaic, mottle, vein chloroses	6	6	0
Total	None	22	0	0
	With symptoms	62	59 (95.2%)	0

Notes: Applied primer pairs targeting RNA1 to RNA5 of AsMaV and the carlavirus PopMV are given in Table S1.

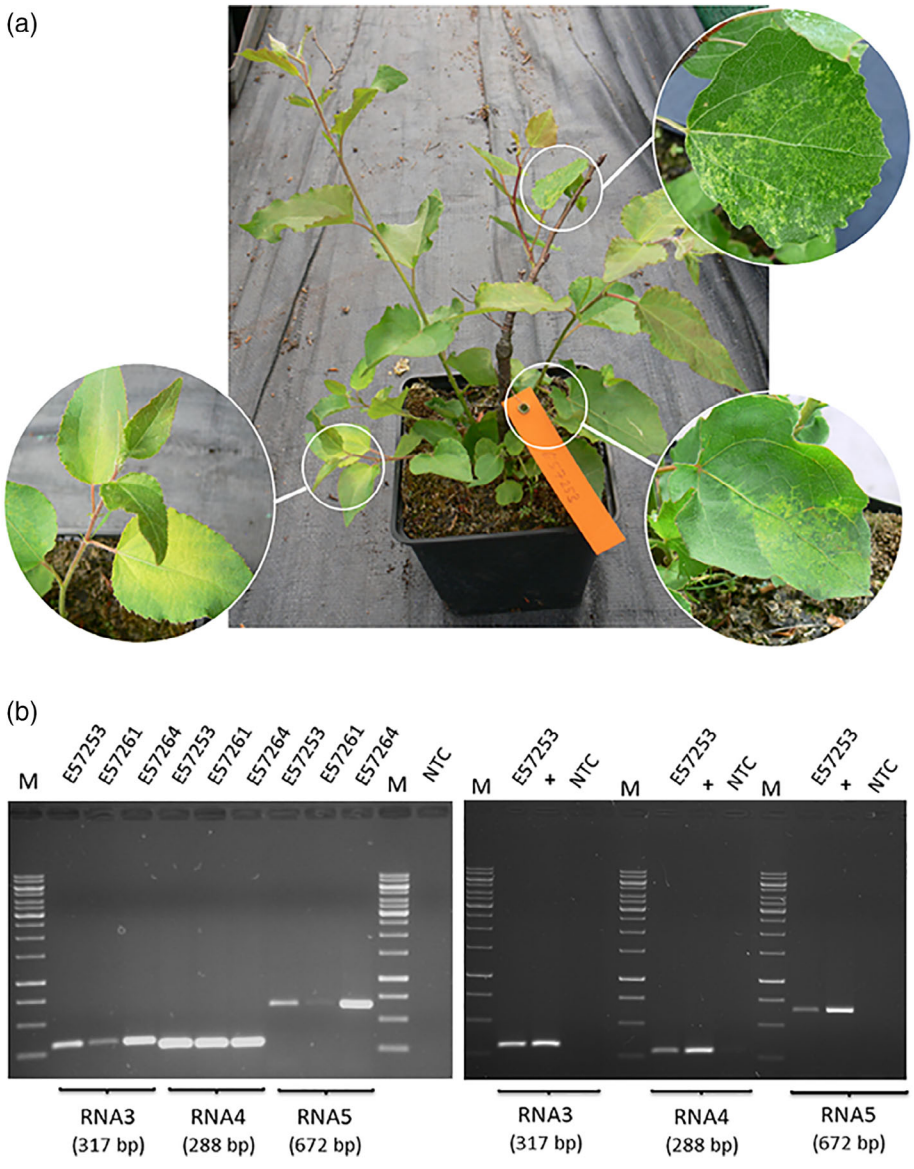

FIGURE 6 Transmissibility of mosaic-disease by grafting of AsMaV-infected scion to healthy Eurasian aspen seedling. (a) Mosaic and mottle observed in leaves of grafted scion (detail picture at the top) and leaves showing yellow blotches, mottle and vein yellows in the shoots grown from the rootstock (detail pictures at the bottom) of Eurasian aspen seedling E57253 15 months after grafting. (b) Detection of RNA3, RNA4 and RNA5 of AsMaV by RT-PCR in rootstocks with virus-suspected symptoms of grafted seedlings E57253, E57261 and E57264 (left) and in grafted scion E57253 (right). +, Positive control; NTC, nontemplate control; M, 1 kb ladder (ThermoScientific)

chlorotic ringspots, line pattern, mosaic and mottle, PopMV was detectable by RT-PCR in these samples, while AsMaV could not be confirmed applying species-specific primers targeting RNA1-RNA5 (data not shown).

In scions received from an AsMaV-infected *P. tremula* from the Kivalo Research Forest, Finland, grafted in 2017 onto healthy *P. tremula* rootstocks mottle and mosaic symptoms first appeared 2 months after grafting at the end of May in leaves of 17 seedlings. In July of the first vegetation period 70 scions showed mosaic-disease symptoms and AsMaV was confirmed by RT-PCR in 10 symptomatic scions applying RNA3 and RNA5 specific primers (Table S1).

However, in the first vegetation period no symptoms could be observed in grafted rootstocks. Sixty scions died during this period and 30 of these seedlings were discarded. From remaining 246 grafted seedlings 53 scions produced symptoms of the mosaic-disease in the second vegetation period. Furthermore, also in shoots of rootstocks of three grafted seedlings (E57253, E57261, E57264) yellow blotching, mosaic and vein chloroses were observed in June and they tested positive for AsMaV with RNA3-RNA5 specific RT-PCR (Figure 6).

Sequencing of PCR products confirmed the infection with AsMaV in the investigated rootstocks. In the case of sample E57253, which was the only symptomatic rootstock with a surviving scion, PCR products amplified from the infected grafted scion and corresponding rootstock produced identical sequences. The 28 control seedlings grafted with healthy scions never exhibited any virus-suspected leaf symptoms in 2017 and 2018. Furthermore, the virus was not detectable in six of these seedlings tested by RT-PCR targeting RNA3–RNA5 of AsMaV of pooled leaf samples collected from grafted scion and rootstock of every seedling. Our results confirm the graft transmissibility of the mosaic-disease observed in Eurasian aspen and suggest AsMaV as the causal agent of the disease.

3.3 | Distribution of the novel Emaravirus in diseased Eurasian aspen in Fennoscandia

Distribution of AsMaV and occurrence of mosaic-diseased Eurasian aspen was evaluated in parts of Norway, southern Sweden and Finland each July between 2016 and 2018 (Figure 7). AsMaV could be detected by species-specific RT-PCR in a total of 69 Eurasian aspen showing mosaic, mottle, variegation, yellow blotching of leaves and chloroses along veins. In the first 2 years of the survey, RT-PCRs targeting the RNA3 and RNA4 proved to be the best for reliable detection of the virus in symptomatic leaves collected at two locations in south western Sweden (Fossum, Säffle), three different sites in southern Norway (Sellebakk, Kråkstad, Ørje), in the area of Helsinki, Finland and from different locations in Finnish Lapland (Rovaniemi, Vaatunki, Lustila, Kemi, Maula, and Sipera and Hyypiökivalo in the Kivalo Research Forest). Both primer sets were therefore applied for successful detection of AsMaV by RT-PCR in additional 10 symptomatic leaf samples collected at three additional sites of southern Sweden (Baldernäs Herrgård, Fengersfors, Stora Djulö) in 2018.

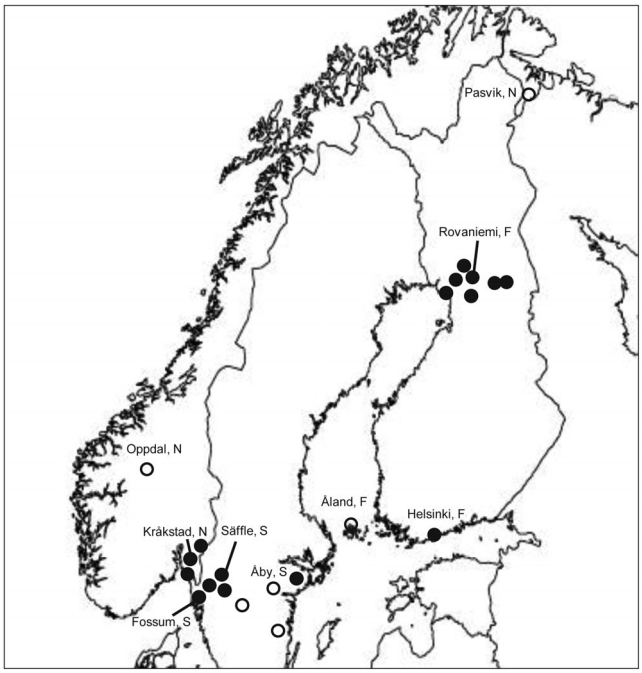

FIGURE 7 Distribution of symptoms of the mosaic-disease and confirmation of aspen mosaic-associated virus (AsMaV) in Eurasian aspen (*Populus tremula*) in Fennoscandia. Black dots indicate locations with AsMaV-infected *P. tremula* confirmed by RT-PCR in 2015–2018. Black circles indicate locations with mosaic-diseased Eurasian aspen trees observed in 2015–2019

It could be demonstrated that the virus is constantly detectable in mosaic-diseased Eurasian aspen in southern Sweden and Norway and also occurs in affected *P. tremula* obtained from Finnish Lapland. That the disease is far more widespread in Fennoscandia is also supported by this study (Figure 7). Mosaic, mottle, yellow blotching and veinal chloroses in Eurasian aspen have been observed between 2015 and 2019 in other parts of southern Sweden including Åby, in Norway (Oppdal, Pasvik), and even at one location on the Åland islands (Finland, Figure 7).

4 | DISCUSSION

The overall genome structure of the novel virus identified in mosaic-diseased *P. tremula* is in accordance with the genus *Emaravirus* (Elbeaino et al., 2018). (a) RNA1–RNA4 encode the viral replicase, structural proteins (GPP and N) of the virus particle and the MP essential for cell-to-cell transport of nucleoprotein complexes. RNA5 encodes a small protein (P28), which is distantly related to other emaraviral proteins (P4–P6, Table 1). Thus, we have discovered a new group of homologous Emaravirus proteins of similar size (26-28 kDa), in addition to the four groups known before (RdRP, N, GPP, MP). The function of proteins in this group is unknown, to our knowledge. Interestingly, not all members of the Emaraviruses seem to encode a homologue protein. For RLBV, HPWMoV, TiRSaV, JYMV and BPVBV showing the highest evolutionary distance to AsMaV in phylogenetic

analyses of RdRps and N proteins, such a homologous small protein has not been found to date. (b) The five identified genome segments are monocistronic RNAs with conserved terminal sequence motifs (13 nt), allowing their full-length amplification by RT-PCR with the terminal generic PDAP213 primer (Di Bello et al., 2015). (c) These conserved termini exhibit high complementarity necessary for circularisation of the ssRNA genome segments by formation of a panhandle structure (Kormelink, Garcia, Goodin, Sasaya, & Haenni, 2011). Our findings clearly demonstrate that the virus identified in Eurasian aspen is a member of the genus.

Amino acid sequence diversity among the RNA1 encoded RdRp of AsMaV and PiVB were slightly lower than the cutoff of 25% diversity on protein level established for the genus *Emaravirus* (Elbeaino et al., 2018, https://talk.ictvonline.org/ictv-reports/ictv_online_report/negative-sense-rna-viruses/bunyavirales/w/fimoviridae). However, the much higher diversity found among RNA2 encoded GPPs, the N proteins encoded by RNA3, and the putative movement proteins (RNA4) of all established species of the Emaraviruses are evidence that AsMaV has to be considered as a novel species of the genus.

Whether RNA1–RNA5 represent the complete genome of AsMaV has to be further investigated. In full-length RT-PCR as well as in dsRNA preparations more fragments could be generated from AsMaV-infected mosaic-diseased Eurasian aspen leaves with estimated lengths of 1.0, 0.85 and in the case of the dsRNA patterns 0.35 kb. Other Emaraviruses contain up to eight different genome segments (Elbeaino et al., 2018) emphasising the apparent flexibility of the genome structure within this genus. For instance, in RLBV 3 RNAs with a length of 1,089 nt (RNA7), 1,095 nt (RNA6) and 1,273 nt (RNA8a) have been identified (Lu et al., 2015) all encoding related proteins, which may play a role in RLBV pathogenicity. The presence of additional genome segments can therefore not be excluded for AsMaV.

Epidemiological issues regarding the novel Emaravirus identified in Eurasian aspen needs to be addressed in future studies in order to further characterise AsMaV and to estimate its economic and ecological impact. As an Emaravirus, eriophyid mites are most likely vectors of the virus (Elbeaino et al., 2018). At least two different gall types induced by different mite species (*Phyllocoptes populi* and *Eriophyes diversipunctatus*) were regularly found in AsMaV-affected Eurasian aspen trees in Fennoscandia (Figure 1). Preparation and identification of mite species inducing different galls in *P. tremula*, as well as transmission experiments needs to be established to identify the putative vector species of the virus.

We could successfully transmit the mosaic-disease symptoms by grafting scions of AsMaV-infected plants to healthy rootstocks. Further, the virus could be detected by RT-PCR in affected scions as well as in diseased rootstocks, thus confirming AsMaV as causal agent of the mosaic-disease of Eurasian aspen. However, the disease could be induced only in three rootstocks of 276 grafted seedlings (1.1% transmission rate). This very low transmission rate indicates that graft transmission of AsMaV is in general possible, but seems not very effective. This is in accordance with a report from Bremer et al. (1991) stating that their trials to transmit the mosaic and veinbanding symptoms in Eurasian aspen by grafting were not successful at all. However, as natural propagation of Eurasian aspen most often occurs vegetatively via suckers grown from root runners (Caudullo & de Rigo, 2016; MacKenzie, 2010), this mechanism has to be considered an important mode of AsMaV transmission. Effective virus dispersal via vegetative propagation may also explain our observations that many Eurasian aspen trees growing as clonal populations next to each other in different investigated locations and sampled sites (Figure 1) show the characteristic mosaic-disease symptoms and tested positive for the virus. In parallel our investigation of the geographic distribution of AsMaV in Eurasian aspen trees needs to be continued in the future and extended to other regions of the wide natural range of the host plant in Europe and Asia.

Our results also change the knowledge of the assumed prevalent distribution and impact of PopMV in poplar species (Biddle & Tinsley, 1971; Navratil, 1979). We could demonstrate that symptoms such as mosaic, mottle, variegation, yellow leaf blotches and veinal chloroses in leaves of Eurasian aspen are rather caused by an infection with the novel Emaravirus AsMaV and are not associated with PopMV. We could show that the mosaic-disease is widely distributed in Eurasian aspen in Scandinavia and Finland. AsMaV could be detected in many of the affected trees, but not in other investigated diseased poplar species from different European locations. Leaf symptoms especially vein chloroses and yellow blotches, which are similar to the observed mosaic-disease in Eurasian aspen that could be associated with AsMaV in this study, have also been described to occur in North American aspen (*P. tremuloides*) and the hybrid *P. x euamericana* (Navratil, 1979). However, no virus could be identified as the causal agent to date. European aspen readily hybridises with *P. tremuloides* (MacKenzie, 2010) and other aspen species (Caudullo & de Rigo, 2016). As a result of this fact and to the resemblance of observed symptoms in leaves in different species, most probably the virus affects other aspen and poplar species. The investigation of susceptibility of other *Populus* species to AsMaV should therefore be studied in more detail in the future including European-wide monitoring. Decline of Eurasian aspen trees affected by this novel Emaravirus over the last 10 years have been observed by us, for instance in the Kivalo Research Forest, Finland. Whether the virus contributes to this decline and how it interacts with additional biotic and abiotic factors has to be investigated. It is of major importance to estimate these impacts of AsMaV to tree populations of managed forest stands as well as of natural or disturbed sites, as aspen is a keystone pioneer species, because of its fundamental ecological importance for other species such as herbivores, birds, saprophytic invertebrates, fungi and lichens.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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