Forest Pathology WILEY-BLACKWELL

For. Path. doi: 10.1111/efp.12046

SHORT COMMUNICATION

Detection and genetic variability of *European mountain ash ringspot-associated virus* (EMARaV) in Sweden

By S. von Bargen[1,3], N. Arndt[1], J. Robel[1], R. Jalkanen[2] and C. Büttner[1]

[1]Division Phytomedicine, Department of Crop- and Animal Sciences, Humboldt-Universität zu Berlin, Lentzeallee 55/57, Berlin, 14195, Germany; [2]Finnish Forest Research Institute, Rovaniemi Research Unit, PO Box 16, Rovaniemi, FI 96301, Finland;
[3]E-mail: susanne.von.bargen@agrar.hu-berlin.de (for correspondence)

Summary

European mountain ash ringspot-associated virus (EMARaV) is a plant virus inducing characteristic ringspots and mottling in *Sorbus aucuparia* L. For the first time, EMARaV was detected in mountain ash in Sweden. All four genomic segments of the virus were detectable by RT-PCR after total RNA extraction from leaves showing chlorotic ringspots, mottling or necrotic lesions. The samples originated from southern and northern Sweden. Sequence analyses of amplified fragments revealed low genetic variability of the virus at nucleotide as well as protein level. All investigated coding regions of EMARaV were under strong purifying selection pressure.

1 Introduction

European mountain ash (syn. rowan, *Sorbus aucuparia* L.) is a common native broad-leaved tree species of the Scandinavian forests dominated by conifers up to the tree line in northern Europe.

A virus denominated *European mountain ash ringspot-associated virus* (EMARaV) induces chlorotic ringspots as well as mottling on leaves of *Sorbus aucuparia*, the only host species known to date. Affected trees are often less vigorous resulting in slow dieback. Determination of the complete genome sequence of EMARaV revealed the unique genome of the virus containing four negative-sense RNAs each encoding a single open reading frame. The characterization of the genomic structure of EMARaV led to the creation of the new genus *Emaravirus* (Mühlbach and Mielke-Ehret 2011). The ringspot disease of mountain ash is known for a long time in several parts of Europe at high incidence including the Czech Republic, Finland, Germany, Russia and the United Kingdom (Mühlbach and Mielke-Ehret 2011; Robel et al. 2013). Valkonen and Rännäli (2010) reported about ringspot symptoms in mountain ash leaves in northern Sweden for the first time, but no studies on virus occurrence were carried out.

In this study, we analysed the incidence of EMARaV in mountain ash exhibiting characteristic symptoms in different parts of Sweden by virus-specific RT-PCR, targeting all four genomic segments of the virus. Sequenced fragments were compared including available sequences from the database, to gain first insights into the population structure of EMARaV variants occurring in Sweden.

2 Material and methods

Leaf samples were taken in July 2010 from three mountain ash saplings growing as understory in the virgin coniferous forest of Norra Kvill in the province of Kalmar, southern Sweden. Additionally, eleven mountain ash growing as roadside, or park trees in the urban municipalities Luleå, Piteå, Skellefteå and Örnsköldsvik, which are all located at the east coast of the three northernmost provinces of Sweden (Norrbotten, Västerbotten and Västernorrland), were sampled in August 2010. All leaves of the sampled trees exhibited chlorotic ringspots typical for EMARaV sometimes accompanied by necrotic lesions. One tree in Norra Kvill showed chlorotic mottling of leaves instead of ringspots (Table 1). Detection of EMARaV was carried out from total RNA extracts by RT-PCR employing random hexamers for cDNA synthesis, followed by use of four primer pairs enabling amplification of specific fragments from all four genomic segments of the virus (Fig. 1), described by Mielke et al. (2008).

Fragments from all four segments of the virus genome originating from ten different trees representing the five sampled locations were sequenced using a 'BigDye Terminator v1.1 Ready Reaction Cycle Sequencing Kit' and an ABI PRISM 310 Genetic Analyzer from Applied Biosystems (USA). RNA1-, RNA2- and RNA3-specific PCR products were directly sequenced, while fragments generated with RNA4-specific primers were cloned prior to sequencing of at least two individual constructs per sampled tree. Vector-specific primers were applied in the sequencing reaction, to retrieve the full-length sequence of the 679-bp PCR product specific for RNA4 of EMARaV. Assembled and edited sequences were deposited in the EMBL database receiving accession numbers (Table 1).

Comparative sequence analyses were carried out including available sequence information from the database of other EMARaV variants originating from Germany, Finland and the Czech Republic, respectively. Genetic distances were calculated of aligned nucleotide sequences omitting primer sequences of fragments amplified from all four genomic segments of EMARaV applying MEGA5 (Tamura et al. 2011).

Received: 7.2.2013; accepted: 27.3.2013; editor: V. Andrea

Table 1. Overview of investigated *Sorbus aucuparia* trees sampled in different locations in Sweden and specific detection of *European mountain ash ringspot-associated virus* (EMARaV) genome segments including databank accession numbers of sequenced fragments.

Sample ID	Location	Symptoms	Accession RNA1	Accession RNA2	Accession RNA3	Accession RNA4
Sorbus aucuparia						
E51576	Norra Kvill, Småland, Kalmar	Mottle	HE819350	HE819360	HE819379	HE819369
E51577	Norra Kvill, Småland, Kalmar	Ringspots	HE819351	–[1]	HE819380	HE819370
E51578	Norra Kvill, Småland, Kalmar	Ringspots	HE819352	HE819361	HE819381	HE819371
E51586	Piteå, Norrbotten	Ringspots	HE819353	HE819362	HE819382	HE819372
E51587	Piteå, Norrbotten	Ringspots	HE819354	HE819363	HE819383	HE819373
E51588	Piteå, Norrbotten	Ringspots	s.n.d.[2]	s.n.d.	s.n.d.	s.n.d.
E51589	Skellefteå, Västerbotten	Ringspots	HE819355	HE819364	HE819384	HE819374
E51590	Skellefteå, Västerbotten	Ringspots	HE819356	HE819365	HE819385	HE819375
E51591	Skellefteå, Västerbotten	Ringspots, necrotic lesions	s.n.d.	s.n.d.	s.n.d.	s.n.d.
E51592	Skellefteå, Västerbotten	Ringspots, necrotic lesions	s.n.d.	s.n.d.	–	–
E51593	Örnsköldsvik, Västernorrland	Ringspots	HE819357	HE819366	HE819386	HE819376
E51594	Örnsköldsvik, Västernorrland	Ringspots	HE819358	HE819367	HE819387	HE819377
E51595	Örnsköldsvik, Västernorrland	Ringspots	s.n.d.	s.n.d.	s.n.d.	s.n.d.
E51605	Luleå, Norrbotten	Ringspots, necrotic lesions	HE819359	HE819368	HE819388	HE819378
Reference sequences from EMARaV-infected *S. aucuparia* in Hamburg, Germany			AY563040	AY563041	DQ831831	DQ831828

[1]No PCR product.
[2]s.n.d., sequence not determined.

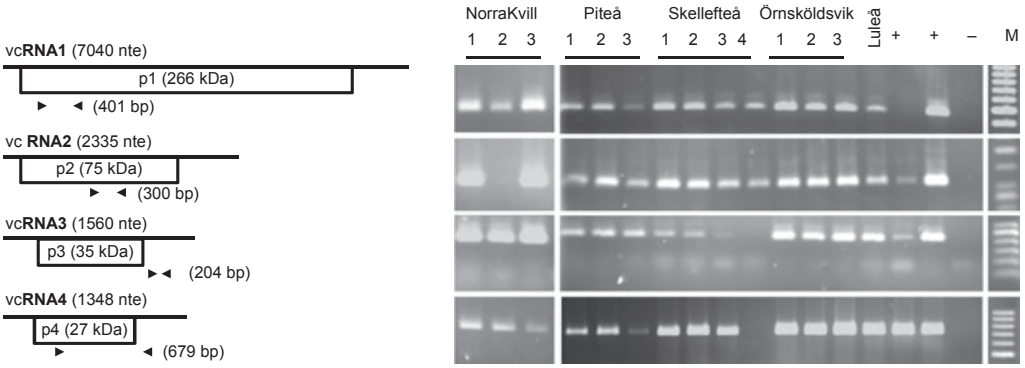

Fig 1. Schematic representation of the genome organization of *European mountain ash ringspot-associated virus* (EMARaV) (left side). Virus complementary RNAs (vcRNA) serving as mRNA are given with encoded ORFs with locations of primers (arrowheads) used for amplification of specific fragment from all four genome segments in Swedish rowan trees by RT-PCR according to Mielke et al. (2008) (right side).

3 Results and discussion

European mountain ash ringspot-associated virus was detected in all fourteen sample trees; however, only in twelve of them, specific fragments from all four genomic RNAs could be amplified by RT-PCR (Fig. 1). In sample E51577 from Norra Kvill, the RNA2 of EMARaV was not detectable, while in another sample E51592 from Skellefteå neither the RNA3- nor the RNA4-specific PCR product was amplified. Schlatermund (2008) demonstrated by qRT-PCR that viral RNAs were only present in low concentrations in European mountain ash leaves during the summer months, and content of vRNA differed considerably between trees, which was attributed to different age and vitality of EMARaV-affected rowans. However, relative abundance remained quite constant between different genome segments of the virus, with RNA2 always showing highest concentrations in diseased plant tissues. It seems unlikely that this virus segment was not detected in sample tree E51577, due to low concentration of the virus in the tissue, because fragments of vRNA1, vRNA3 and vRNA4 were readily amplified.

Fragments originating from all four genomic segments of EMARaV exhibited low variability at nucleotide level (Table 2). Mean nucleotide distances after pairwise comparison ranged between 0.031 (vRNA3) and 0.014 (vRNA4). These values are in the range of the lowest genetic diversities of populations reported for other RNA plant virus species (Garcia-Arenal et al. 2001). In general, the coding regions including partial sequences of the p1 (361 bp of vRNA1) representing the viral RNA-dependent RNA polymerase (RdRp), the p2 (261 bp of vRNA2) and the p4 (471 bp of vRNA4) exhibited lower nucleotide diversity with a maximum of 0.049 in the vRNA2 fragment encoding p2 than the partial 3′ untranslated region (3′ UTR)

Table 2. Genetic distances of nucleotide and amino acid sequences received from all four genomic RNAs of *European mountain ash ringspot-associated virus* (EMARaV) detected in Sweden after pairwise comparison with corresponding sequences available in the NCBI database including calculated evolutionary constraints of partial coding regions (cds) of vRNA1, vRNA2 and vRNA4.

		Pairwise nucleotide distance[2]				Pairwise amino acid distance[3]				Selective constraint[4]		
	n[1]	[bp][5]	Mean (SE)	Min	Max	[aa]	Mean (SE)	Min	Max	dS (SE)	dN (SE)	dN/dS
RNA1												
Partial cds	11	361	0.015 (0.006)	0.000	0.026	120	0.006 (0.003)	0.000	0.017	0.064 (0.018)	0.002 (0.001)	0.031
RNA2												
Partial cds	10	261	0.016 (0.005)	0.000	0.049	87	0.005 (0.003)	0.000	0.023	0.062 (0.019)	0.003 (0.002)	0.048
RNA3												
Partial 3′ UTR	33	156	0.031 (0.007)	0.000	0.071	–[6]	–	–	–	–	–	–
Partial 3′ UTR	11[7]	158	0.027 (0.008)	0.000	0.046	–	–	–	–	–	–	–
RNA4												
Partial cds +3′ UTR	11	632	0.014 (0.003)	0.000	0.031	–	–	–	–	–	–	–
Partial cds	11	472	0.007 (0.003)	0.000	0.015	156	0.004 (0.002)	0.000	0.013	0.024 (0.009)	0.002 (0.001)	0.083
Partial 3′ UTR	11	160	0.037 (0.011)	0.000	0.091	–	–	–	–	–	-	–

[1]Number of sequences included in the pairwise comparison.
[2]Calculated by application of the maximum composite likelihood model implemented in MEGA5.
[3]Calculated by the equal input model implemented in MEGA5.
[4]Codon-based evolutionary divergence between sequences by estimation of number of synonymous differences per synonymous site (dS), number of non-synonymous differences per non-synonymous site (dN) from between sequences and their ratio as indicators for selection pressure.
[5]Compared fragment length without primer sequences.
[6]Not applicable.
[7]Only reference sequence and EMARaV variants originating from Swedish locations.

determined for vRNA3 (max. 0.071) and vRNA4 (max. of 0.091), respectively. The increased size of the analysed dataset (33) available for the partial 3′ UTR of vRNA3 sequences originating from several geographical locations contributes to the observed higher variability of this genome region. However, mean nucleotide distance was still considerably higher (0.027) than mean values (0.007–0.016) calculated for the coding regions comparing only the samples originating from Swedish locations and the reference sequence.

Pairwise comparison of peptide sequences performed from fragments including partial coding regions of vRNA1, vRNA2 and vRNA4, respectively, confirmed high conservation of the partial p1, p2 and p4 of EMARaV exhibiting similar mean distances between 0.004 and 0.006. Also, analyses of seventeen EMARaV variants originating from Finland and Russia by Kallinen et al. (2009) revealed strong conservation of the RNA3 region encoding the nucleocapsid (max. 3% diversity, 944 bp), whereas a 442-bp fragment of the 3′ UTR of this genome segment was more variable exhibiting up to 6% diversity at the nucleotide level. Interestingly, the partial protein-coding regions compared in the present study did not include any conserved domains characteristic for an RdRp (p1) or predicted structural elements such as signal peptides, transmembrane helices or N-glycosylation sites (p2), which may permit higher genetic variability of the encoded peptide. We could not find a correlation between geographical distribution and genetic variability of the Swedish EMARaV variants including fragments of all four genomic segments of the virus confirming the investigation of Kallinen et al. (2009) who analysed the RNA3 region of EMARaV (data not shown).

The amount of non-synonymous and synonymous substitutions per site (dN and dS, respectively) and their ratio (dN/dS) are important indicators of selection pressure at the protein level. They are therefore useful for studying the mechanisms of DNA sequence evolution. dN/dS ratios of <1 indicate purifying or stabilizing selection, 1 neutral mutations, and >1 imply diversifying or positive selection. To examine selective constraint on the partial coding regions of vRNA1, vRNA2 and vRNA4, dN and dS values and their ratios dN/dS were calculated as the overall average of the codon sites in each gene fragment using the Nei–Gojobori method implemented in MEGA5. Amino acid change as dN/dS ratios were limited between 0.031 (120 aa of p1) and 0.082 (156 aa of p4) indicating a strong purifying selection acting on all three gene loci of EMARaV analysed in this study. Values were low as compared with estimations derived from other virus genera ranging between 0.013 and 0.308 including structural proteins as well as viral proteins involved in replication (Garcia-Arenal et al. 2001).

Sequence stabilization of the coding regions of the genome of a plant virus will be maintained by necessary interactions with the host plant as well as the natural vector during multiplication and transmission (Garcia-Arenal and Fraile 2008). Genetic stability of all analysed regions covering the four genome segments of EMARaV is high and can be explained by the fact that this virus seems to be highly specialized, because it has been found only in mountain ash to date, and it is most likely transmitted in a circulative manner by the pear blister mite *Phytoptus pyri* under natural conditions (Mielke-Ehret et al. 2010). Implying a close co-evolution of this virus with its only host plant and natural dissemination by a single vector

species serving as population bottlenecks, these features may cause the strong purifying selective pressure on all EMARaV-encoded proteins. Another reason for the observed low genetic diversity can be a genetic bottleneck originating from recent introduction of EMARaV into Sweden, known as a founder effect (Garcia-Arenal et al. 2001). However, the observed low genetic diversity within the analysed region of vRNA3 comparing Swedish EMARaV variants with accessions from other geographical regions, that is, Finland, Russia, the Czech Republic and Germany, did not corroborate this hypothesis. Further, ringspot symptoms on mountain ash have been recognized for decades (Robel et al. 2013).

Levels of genetic heterogeneity observed for regions encoding structural proteins of the related *Tomato spotted wilt virus* (TSWV), a virus that exhibits a very wide host range, were found to be higher than values determined for sequences encoding orthologous EMARaV proteins. Tsompana et al. (2005) reported average pairwise nucleotide differences between 0.024 and 0.031 of the TSWV sequence encoding the nucleocapsid protein (N) and the glycoprotein precursor (G1–G2), respectively. In another study investigating the population structure of TSWV infecting peanuts in the USA, Kaye et al. (2011) showed that the dN/dS ratios estimated for the N and RdRp gene of the virus were similar (0.045 and 0.039, respectively) to the value determined for the p1 fragment of EMARaV analysed in this study. On the other hand, purifying selection pressure on the non-structural movement protein of TSWV (NSm) was lower (0.099) and in the same range as determined for the partial p4 of EMARaV.

Analyses of the partial EMARaV-p4 revealed that this non-structural protein of unknown function exhibited highest sequence conservation but also showed that purifying selection pressure of this genome region was lower than for the p1 necessary for replication, the p3 and p2, which are both components of the viral capsid or envelope, respectively.

These studies confirm the presence of EMARaV in parts of southern and northern Sweden. Further evaluation of its geographical distribution in the country has to be carried out. Simultaneously, it will be interesting to determine genetic variability and evolutionary constraints of the complete p4 in comparison with p2 and p3 as major structural components of the virus particle within the EMARaV population found in Sweden and to include EMARaV variants from other countries.

Acknowledgements

S. von Bargen is supported by a grant of the German Research Foundation (DFG, BU890/14-1). We thank Renate Junge for skilled technical assistance.

References

Garcia-Arenal, F.; Fraile, A., 2008: Questions and concepts in plant virus evolution: a historical perspective. In: Plant Virus Evolution. Ed. by Roosinck, M. J. Berlin: Springer, pp. 1–14.

Garcia-Arenal, F.; Fraile, A.; Malpica, J. M., 2001: Variability and genetic structure of plant virus populations. Ann. Rev. Phytopathol. **39**, 157–186.

Kallinen, A. K.; Lindberg, I. L.; Tugume, A. K.; Valkonen, J. P., 2009: Detection, distribution, and genetic variability of *European mountain ash ringspot-associated virus*. Phytopathology **99**, 344–352.

Kaye, A. C.; Moyer, J. W.; Parks, E. J.; Carbone, I.; Cubeta, M. A., 2011: Population genetic analysis of *Tomato spotted wilt virus* on peanut in North Carolina and Virginia. Phytopathology **101**, 147–153.

Mielke, N.; Weber, M.; Khan, S.; Mühlbach, H.-P., 2008: Detection of European mountain ash ringspot-associated virus (EMARAV) in *Sorbus aucuparia* L. by a specific antiserum and reverse transcription-PCR. Forest Pathol. **38**, 371–380.

Mielke-Ehret, N.; Thoma, J.; Schlatermund, N.; Mühlbach, H.-P., 2010: Detection of European mountain ash ringspot-associated virus-specific RNA and protein P3 in the pear leaf blister mite *Phytoptus pyri* (Eriophyidae). Arch. Virol. **157**, 987–991.

Mühlbach, H.-P.; Mielke-Ehret, N., 2011: Emaravirus. In: Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. Ed. by King, A.; Lefkowitz, E.; Adams, M. J.; Carstens, E. B. San Diego: Elsevier Academic Press, pp. 767–770.

Robel, J.; Bandte, M.; Mühlbach, H.-P.; von Bargen, S.; Büttner, C., 2013: Ein neuartiges Virus in *Sorbus aucuparia* L.: Nachweis und Verbreitung des *European mountain ash ringspot-associated virus* (EMARaV). In: Jahrbuch der Baumpflege 2013. Ed. by Dujesiefken, D., Braunschweig: Haymarket Media, pp. 47–53.

Schlatermund, N., 2008: Lokalisation und Quantifizierung der RNAs des *European mountain ash ringspot-associated virus* (EMARAV) in der Eberesche (*Sorbus aucuparia* L.). PhD thesis. Universität Hamburg, 114 pp.

Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Masatoshi Nei, M.; Kumar, S., 2011: MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. **28**, 2731–2739.

Tsompana, M.; Abad, J.; Purugganan, M.; Moyer, W., 2005: The molecular population of the *Tomato spotted wilt virus* (TSWV) genome. Mol. Ecol. **14**, 53–66.

Valkonen, J. P. T.; Rännäli, M., 2010: First report of *European mountain ash ringspot-associated virus* (EMARaV) in eastern Karelia in Russia. Plant Dis. **94**, 921.