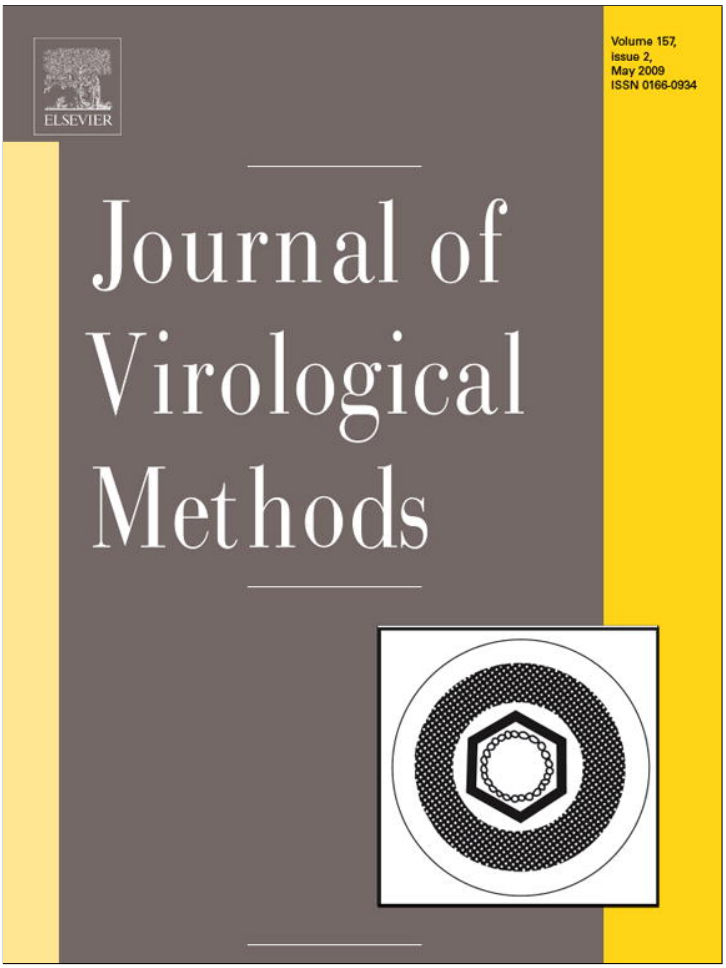

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Differentiation of *Cherry leaf roll virus* isolates from various host plants by immunocapture-reverse transcription-polymerase chain reaction-restriction fragment length polymorphism according to phylogenetic relations

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A restriction fragment length polymorphism assay (RFLP) was developed to differentiate *Cherry leaf roll virus* (CLRV) isolates according to phylogenetic clades by examining restriction patterns from partial 3′ non-coding region (NCR) genomic fragments (approx. 420 bp). The 3′ NCR fragment from 43 CLRV isolates belonging to different phylogenetic groups were compared after restriction analysis with the endonucleases Bsp143I, AluI, RsaI, EcoRI and Eco130I, and another 23 isolates were analyzed by computer assisted restriction analysis. The restriction endonucleases Bsp143I, AluI and RsaI enabled the differentiation of isolates from group B and all but two isolates belonging to group A. A major proportion of group E isolates could also be discriminated. The remainder of the group E isolates were indistinguishable from isolates belonging to phylogenetic group C or D2. Isolates belonging to group D1 could not be differentiated from two group A isolates. The method was applied successfully in an IC-RT-PCR-RFLP assay to differentiate samples from walnut, black elderberry and birch and determine their phylogenetic relationships. In future, this method will facilitate rapid phylogenetic classification of CLRV isolates detected in certain host plants by the universal immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR), and will be suitable for studying CLRV population diversity as well as genetic drift within virus populations.

1. Introduction

Cherry leaf roll virus (CLRV) is a *Nepovirus* within the *Comoviridae* family (Jones, 1985). It is distributed globally (Bandte and Büttner, 2001) and infects naturally a wide range of herbaceous and woody plants, among which are *Betula* spp., *Fagus* spp., *Fraxinus* spp., *Juglans* spp., *Ulmus* spp., *Rhamnus* spp., *Sambucus* spp., *Prunus* spp. as well as *Ligustrum vulgare* L., *Ptelea trifoliata* L. and *Cornus florida* L. (Bandte and Büttner, 2001; Obermeier et al., 2003). CLRV can lead to economic losses in walnut production by inducing walnut blackline disease, which causes necrosis at grafting unions with some English walnut and rootstock combinations (Mircetich et al., 1980). This may lead to subsequent dieback, a common disease symptom especially of woody plants, characterized by progressive death of twigs, branches, shoots, or roots, starting at the tips. Significant economic losses due to walnut blackline disease have been reported from California (Brooks and Bruening, 1995; Mircetich et al., 1980). Kegler et al. (1966) found that CLRV can cause decline and dieback in sweet cherry (*Prunus avium*), and CLRV was detected recently in several downy birch trees (*Betula pubescens* Ehrh.) in Finland (Jalkanen et al., 2007) and has to be regarded as of economic importance.

CLRV isolates from different hosts and stands may differ in their serological and molecular traits (Jones, 1976; Jones et al., 1990; Rebenstorf et al., 2006; Rowhani and Mircetich, 1988; Schmelzer, 1972; Tóbiás, 1995; Waterworth and Lawson, 1973) as well as in their host specificity and ability to induce symptoms (Jones, 1973; Rowhani and Mircetich, 1988). A strong relationship between the original host, serology and sequence based phylogeny of an approximately 375 bp fragment from the 3′ proximal non-coding region (3′ NCR) was shown by Rebenstorf et al. (2006). CLRV isolates segregate into six major groups dominated by isolates originating from birch and cherry (group A), rhubarb, ash and ground elder (group B), raspberry, sorrel and chive (group C), walnut (groups D1 and D2) and elderberry (group E) (Rebenstorf et al., 2006). However, CLRV isolates are not arranged exclusively within phylogenetic clusters according to originating host plant species, corroborating the observations, that CLRV isolates are capable of infecting not only one host, but could be transmitted between different plant species (Jones, 1973; Obermeier et al., 2003). The ecological significance of CLRV should be considered due to its wide distribution in woody plants, especially deciduous trees (Bandte and Büttner, 2001), and because its host range might not be limited to those known at present.

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Table 1
Virus isolates included in this study.

CLRV isolate	Host plant	Accession No.	Phylogenetic group[a]	PCR product[b] [bp]
E111	*Betula pendula* Roth	AJ877122	A	416
E113	*Fagus sylvatica* L.	AJ877159	A	416
E120	*Betula pendula* Roth	AJ877118	A	416
E154	*Juglans regia* L.	–	A	416
E325	*Fraxinus excelsior* L.	AJ877158	A	416
E327	*Prunus avium* (L.) L.	AJ877127	A	415
E441	*Sambucus nigra* L.	AJ877139	A	414
E499	*Betula pendula* Roth	AJ877119	A	416
E696	*Betula pendula* Roth	AJ877121	A	416
E797	*Cornus florida* L.	AJ877161	A	416
E801	*Ulmus americana* L.	AJ877160	A	418
E803	*Prunus avium* L.	AJ877128	A	416
E805	*Rubus fruticosus* L.	AJ877163	A	415
E806	*Betula pendula* Roth	AJ877123	A	416
E836	*Betula nigra* L.	AJ877125	A	416
E896	*Betula pendula* Roth	AJ877120	A	415
E1469	*Betula pendula* Roth	AJ877124	A	416
E1472	*Prunus avium* L.	AJ877129	A	415
E1636	*Vitis vinifera* L.	AJ877164	A	417
E1771	*Rheum rhabarbarum* L.	–	A	416
Kleidocerys resedae	–	–	A	415
Polydrusus sp.	–	–	A	415
S84124RNA1	*Betula pendula* Roth	S84124	A	416
S84125RNA2	*Betula pendula* Roth	S84125	A	416
E395	*Rheum rhabarbarum* L.	AJ877165	B	412
E575	*Aegopodium podagraria* L.	AJ877157	B	412
E676	*Sambucus nigra* L.	AJ877130	B	412
E678	*Fraxinus excelsior* L.	AJ888533	B	412
E695	*Sorbus aucuparia* L.	AJ877154	B	411
E697	*Sorbus aucuparia* L.	AJ877153	B	412
E698	*Fraxinus excelsior* L.	AJ888534	B	412
S84126	*Rheum rhabarbarum* L.	S84126	B	414
E802	*Rubus idaeus* L.	AJ877162	C	412
AB168098	*Allium tuberosum* Rottl. ex Spreng.	AB168098	C	412
AB168099	*Rumex acetosella* L.	AB168099	C	412
AB168100	*Rumex acetosella* L.	AB168100	C	412
Ludmilla	*Juglans regia* L.	AJ877152	D2	417
E326	*Juglans regia* L.	AJ877146	D1	404
E648	*Juglans regia* L.	AJ877147	D1	403
E800	*Juglans regia* L.	AJ877149	D1	404
4WJUG	*Juglans regia* L.	AJ877148	D1	404
CL24694	*Juglans regia* L.	CL24694	D1	404
CTIFL	*Juglans regia* L.	AJ877151	D1	404
Gaydon	*Juglans regia* L.	AJ877126	D1	404
Z344265	*Juglans regia* L.	Z344265	D1	404
E156	*Juglans regia* L.	AJ877150	D2	421
E119	*Sambucus nigra* L.	AJ877134	E	412
E141	*Carpinus betulus* L.	AJ877156	E	412
E443	*Sambucus nigra* L.	AJ877138	E	412
E485	*Sambucus nigra* L.	AJ877131	E	412
E492	*Sambucus nigra* L.	AJ877143	E	413
E541	*Sambucus nigra* L.	AJ877137	E	412
E568	*Sambucus nigra* L.	AJ877141	E	412
E576	*Sambucus nigra* L.	AJ877142	E	412
E583	*Sambucus nigra* L.	AJ877133	E	411
E603	*Sambucus nigra* L.	AJ877132	E	412
E622	*Sambucus nigra* L.	AJ877135	E	410
E693	*Sorbus aucuparia* L.	AJ877155	E	412
E804	*Sambucus canadensis* L.	AJ877145	E	412
E839	*Sambucus nigra* L.	AJ877136	E	412
E950	*Sambucus nigra* L.	AJ877140	E	412
E1680	*Sambucus nigra* L.	–	E	412
E1682	*Sambucus nigra* L.	–	E	412
E1708	*Sambucus nigra* L.	–	E	412
E1746	*Sambucus nigra* L.	–	E	412
PV0276	*Sambucus nigra* L.	AJ877144	E	412
E2165	*Sambucus nigra* L.	AM981028	This study	
E2249	*Betula* sp.	AM981025, AM981026	This study	
W2J	*Juglans regia* L.	AM981027	This study	

[a] According to Rebenstorf (2005) and Rebenstorf et al. (2006).
[b] Obtained by primers RW1 and RW2 (Rebenstorf et al., 2006), including primer sequences.

Table 2
RFLP types determined by computer assisted and RFLP analysis of PCR products amplified with primers RW1 and RW2 from phylogenetically characterized CLRV isolates by the restriction endonucleases AluI, Bsp143I and RsaI.

RFLP type	Phylogenetic group[a]	CLRV isolates		Fragment lengths by computer assisted digestion [bp]		
		n	Members	Bsp143I	AluI	RsaI
AI	A	4	E113, E120, E154, E836	**119**[c], 297	98, 318	193, 223
AII		1	E325	**119**, 297	98, 318	Undigested
AIII		7	E111, E327, E499, E696, E803, E806, E1636	**118–120**, 297	Undigested	Undigested
AIV		2	E441, Kleidocerys resedae[b]	**118–119**, 295–297	Undigested	191–193, 222–223
AV		2	E805, E1472[b]	103, **119**, 193	Undigested	177, 238
AVI		4	E1469[b], E1771, S84124[b], S84125[b]	**119**, 297	98, 318	82, 334
AVII		1	E896[b]	**119**, 296	48, 372	192, 223
AVIII		1	Polydrusus sp.[b]	**119**, 296	48, 367	82, 110, 223
B	B	8	E395, E575, E676, E678, E695, E697, E698, S84126[b]	Undigested	**147–148, 264–266**	**82, 329–332**
C/D2/E	C	5	E802, Ludmilla[b], AB168098[b], AB168099[b], AB1680100[b]	Undigested	147–148, 264–270	Undigested
	D2	1	E156[b]	Undigested	151, 270	Undigested
	E	6	E485, E492, E603, E804, E1746, PV0276[b]	Undigested	147, 265–266	Undigested
A/D1	A	2	E797, E801	Undigested	Undigested	Undigested
	D1	8	E326, E648, E800, Gaydon [b], 4WJUG [b], CTIFL[b], Z344265[b], CL24694[b]	Undigested	Undigested	Undigested
EI	E	11	E119[b], E141, E443, E541, E576, E583, E622, E839, E1680, E1682, E1708	Undigested	**96–98, 146–147, 167**	Undigested
EII		1	E693	Undigested	80, 147, 185	Undigested
EIII		1	E568	70, 342	147, 265	Undigested
EIV		1	E950	Undigested	147, 265	59, 353

[a] According to Rebenstorf et al. (2006).
[b] Determined by computer assisted digestion only.
[c] fragments suitable for phylogenetic differentiation of CLRV isolates are printed in bold.

CLRV infections can be detected reliably and specifically by immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR, Werner et al., 1997). This assay is also suitable for woody hosts and has been optimized for routine testing of large sample numbers (Gentkow et al., 2007). However, studies of CLRV population structure and epidemiology will require an identification of CLRV isolates. A rapid and easy method is the analysis of characteristic fragments of the virus genome by restriction fragment length polymorphism (RFLP), which has been applied previously for differentiating among plant virus species, e.g. within the *Tobamovirus* genus (Letschert et al., 2002) as well as strains within the potyviruses, e.g. *Potato virus Y* (Blanco-Urgoiti et al., 1996) and *Soybean mosaic virus* (Kim et al., 2004).

The aim of this study was to establish an RFLP method using the amplified 3′ NCR fragment of the CLRV genome to allow an assignment of CLRV isolates to specific phylogenetic groups (Rebenstorf et al., 2006). Furthermore, the applicability of the method in an IC-RT-PCR-RFLP assay for detection and discrimination of CLRV isolates directly in different original host plants was assessed.

2. Materials and methods

2.1. Virus isolates

Sixty-six different CLRV isolates originating from six phylogenetic clusters established by Rebenstorf (2005) and Rebenstorf et al. (2006) were included in the study and are indicated by GenBank accession numbers in Table 1. Either 1 μl of purified plasmid preparation of cloned 3′ NCR fragments (amplified with primers described in Rebenstorf et al., 2006) was applied in a PCR, or IC-RT-PCR fragments were amplified directly from leaf material or approximately 2 μg virus purified from the CLRV infected propagation host *Chenopodium quinoa* L. as described by Giersiepen (1993). Additionally, leaf and fruit samples from three natural host plants walnut (*Juglans regia* L.), birch (*Betula* sp.) and black elderberry (*Sambucus nigra* L.) (Table 1), which had not been tested previously for a CLRV infection, were assayed by IC-RT-PCR-RFLP.

2.2. Amplification of CLRV genome fragments

Immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR) was carried out as described in Gentkow et al. (2007) using a concentration of 3 μg/ml of a polyclonal CLRV antiserum produced against a European ash isolate of CLRV for immunocapture. This antibody was used previously by Rebenstorf et al. (2006) and is suitable for detecting phylogenetically and serologically diverse CLRV isolates from a wide range of natural woody hosts. Coated reaction tubes were either used directly after coating or were stored up to 14 days at 4 °C until further use. Either 10 μl of plant material homogenized in 10 volumes of sample buffer or purified virus were applied to coated tubes and incubated overnight at 4 °C. First-strand cDNA synthesis was done directly

Table 3
Characterization of new CLRV isolates from woody host plants by determination of the RFLP types received by computer assisted and RFLP analysis of PCR products amplified with primers RW1 and RW2 and sequence analysis.

CLRV isolate	RFLP type	Fragment lengths after digestion [bp]			Sequence analysis	
		Bsp143I	AluI	RsaI	Phylogenetic group[a]	Fragment length [bp]
E2249, clone 24	AIII	120–123, 295–311	Undigested	Undigested	A	416
E2249, clone 21	AIV	120–123, 295–311	Undigested	189–193, 225	A	416
W2J	C/D2/E	Undigested	153–154, 268–273	Undigested	D2	416
E2165	EI	Undigested	104, 154, 175	Undigested	E	412

[a] According to Rebenstorf et al. (2006).

in the immunocapture tubes in a total reaction volume of 10 μl using 100 units Moloney murine leukemia virus reverse transcriptase (M-MuLV-Rtase), 1 mM of each dNTP, 5 μM antisense primer (RW1: 5-GTCGGAAAGATTACGTAAAAGG-3) in M-MuLV-Rtase reaction buffer supplied by Fermentas (St. Leon-Rot, Germany). PCR amplification was undertaken in a total volume of 50 μl using 10 μl of the reverse transcription product, 2.0 mM $MgCl_2$, 5 units Taq DNA polymerase (Promega, Madison, USA), 0.5 μM antisense primer, and 0.5 μM sense primer (RW2: 5-TGGCGACCGTGTAACGGCA-3) in PCR buffer supplied by Promega. The PCR was performed in a Robocycler (Stratagene, La Jolla, USA); the cycling parameters were 5 min of denaturation at 94 °C followed by 30 cycles consisting of 94 °C denaturation for 1 min, 55 °C annealing for 45 s, 72 °C extension for 1 min, and a final extension for 5 min at 72 °C. The PCR products were separated on 1% TBE-agarose gels and visualized after staining with ethidium bromide under UV-light.

PCR amplification using cloned CLRV fragments was carried out in a total volume of 50 μl PCR reaction mix with 2 U of Taq polymerase as described above.

2.3. Restriction fragment length polymorphism (RFLP) analysis

Computer assisted restriction analysis of selected sequences of CLRV isolates (accession nos. AJ877118, AJ877158, AJ877127, AJ877139, AJ877165, AJ877162, AJ877146, AJ877150, AJ877132, AJ877155, S84124, S84125) representing different phylogenetic groups was done with NEBcutter V2.02 (Vincze et al., 2003) in order to identify suitable restriction endonucleases. The enzymes Bsp143I, EcoRI, AluI, Eco130I and RsaI were selected to digest the PCR amplified 3′ NCR fragments of the CLRV isolates.

Restriction assays were carried out in a total volume of 15 μl using 7 μl of PCR product and 0.3–0.4 units/μl of restriction enzyme (Fermentas, Germany and Promega, USA). Restriction fragments were separated in 2–4% high resolution agarose gels. Fragment lengths were determined with the software BioDocAnalyze (Biometra, Göttingen, Germany) and were compared to the expected fragment lengths based on computational restriction analysis.

If the expected and actual fragments were not similar in size, the restriction analysis was repeated with a plasmid from another clone or an RW1/RW2 primed IC-RT-PCR product directly amplified from a *C. quinoa* plant infected with the corresponding CLRV isolate.

2.4. Cloning and sequence analysis

CLRV specific IC-RT-PCR amplification products from natural host plants were ligated into a pBluescriptII SK(-) based T-vector and transformed into chemocompetent *E. coli* using standard protocols (Sambrook et al., 1989). Plasmids from two clones of each vector construct were purified and sequenced from both directions using a BigDye® Terminator v1.1 Ready Reaction Cycle Sequencing Kit using standard protocols and an ABI PRISM® 310 Genetic Analyzer from Applied Biosystems (Foster City, USA). Sequences were assembled in BioEdit 7.4 (Hall, 1999) and submitted to the EMBL database.

For nucleotide sequence based classification of 3′ NCR fragments obtained from new CLRV isolates, phylogenetic trees were calculated using the maximum parsimony, maximum likelihood and neighbor joining methods. The phylogenetic trees were compared in order to confirm the biological relevance of the calculations. Neighbor joining was done using the bootstrap option of ClustalX 1.8 (Thompson et al., 1997) with 1000 bootstrap trials. Gap positions were excluded and multiple substitutions were corrected. The maximum likelihood algorithm was applied using the program Dnaml of the PHYLIP package (Felsenstein, 2005). Global rearrangements of possible subtrees were done; the other software options were kept at the default settings. The maximum parsimony algorithm was applied by use of the program Dnapars of the PHYLIP package, the input order of sequences was randomized, and other options were left in the default settings. Calculated trees were plotted using NJPlot (Perriere and Gouy, 1996) and major phylogenetic groups were adapted from phylogenetic studies of Rebenstorf (2005) and Rebenstorf et al. (2006).

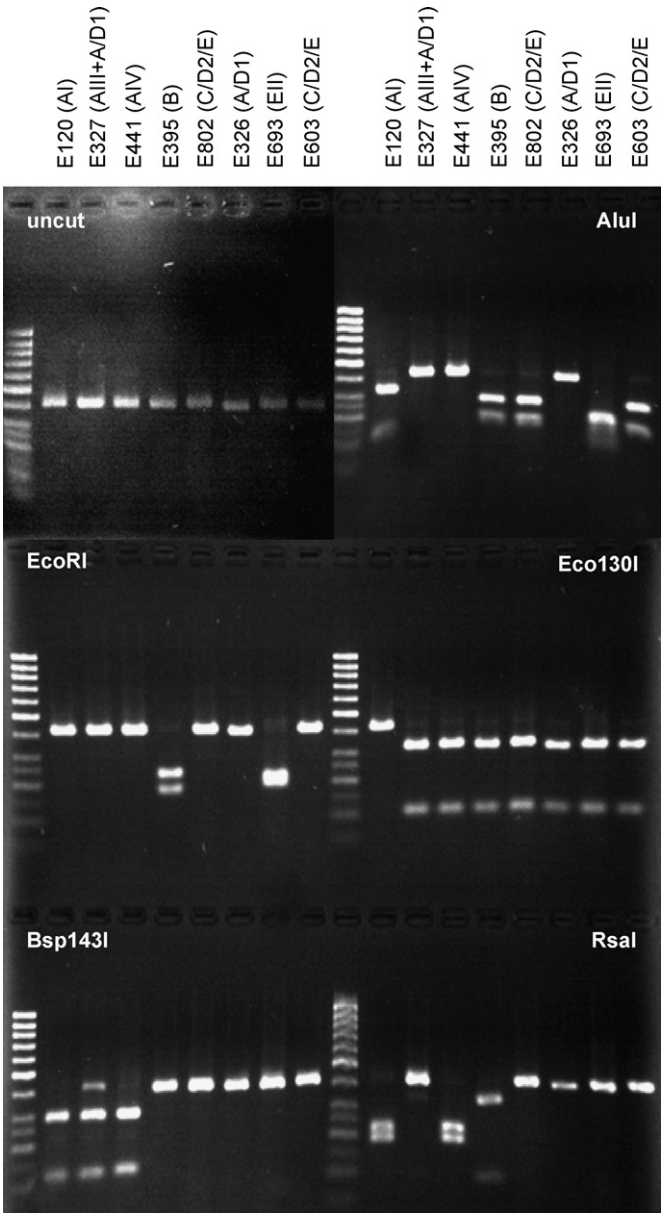

Fig. 1. Detection and discrimination of phylogenetically different CLRV isolates by IC-RT-PCR-RFLP generated from virus purifications. Corresponding RFLP types are indicated in parentheses. Sizes of the marker bands (bottom to top): 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1 000 bp.

3. Results

3.1. Differentiation of CLRV isolates by restriction fragment length polymorphism (RFLP)

PCR fragments amplified by RW1-RW2 from cloned fragments of 43 different CLRV isolates were digested with AluI, Bsp143I, EcoRI, Eco130I and RsaI. Corresponding sequences from the 3′ NCR fragments obtained from the database were also analyzed by

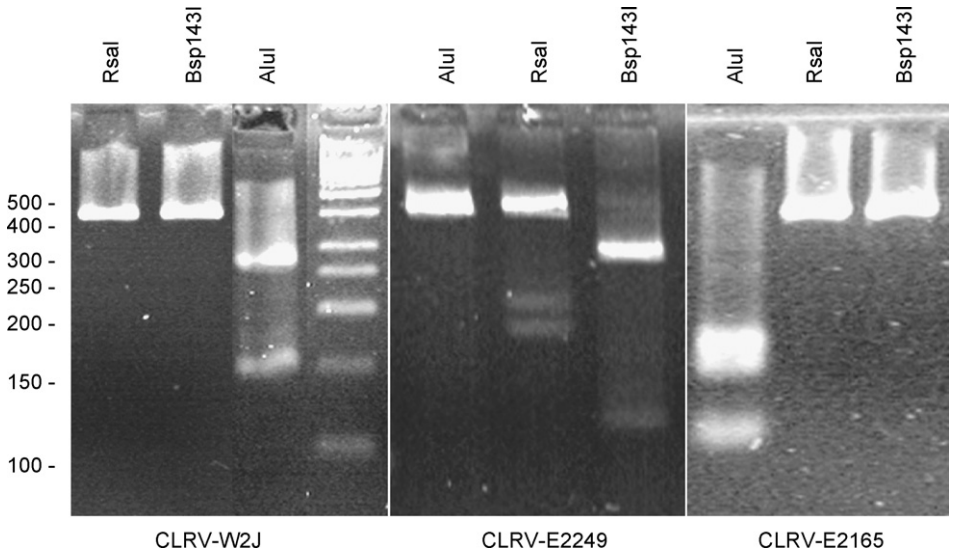

Fig. 2. Differentiation of CLRV isolates from woody host plants by IC-RT-PCR-RFLP. Some sizes of marker fragments (50 bp Ladder, Fermentas) are indicated on the left.

computational digestion. Twenty-three additional CLRV-sequence fragments from the database were analyzed by digestion with the computer program NEBcutter. Virus isolates were categorized in 15 different RFLP types by restriction analysis of the RW1-RW2 amplified fragments of the CLRV 3′ NCR with AluI, Bsp143I and RsaI (Table 2).

Generally, restriction with AluI, Bsp143I and RsaI of amplified PCR fragments of the CLRV 3′ NCR corresponded with theoretical computer analyses. However, out of the 43 isolates that were analyzed with the three restriction endonucleases, three (E499, E698 and E801) gave repeatedly different results than expected from computer assisted analysis. Amplicons originating from virus isolates E499 and E689, obtained from cloned fragments as well as from infected bioassay plants, were not digested by AluI or EcoRI, respectively, as predicted by computer analyses, which is obviously due to sequencing artifacts. Furthermore, CLRV-E801 restriction patterns exhibited an additional fragment around 140 bp, which did not correspond with the expected bands around 99 and 319 bp produced by Eco130I.

It was also found that restriction patterns obtained by Bsp143I, AluI and RsaI, were able to identify virus isolates according to their phylogenetic grouping. Bsp143I digestion provided a restriction pattern that allowed us to assign these isolates to phylogenetic group A. Digestion with Bsp143I produced a 118–120 bp fragment characteristic for all isolates of phylogenetic group A with the exception of the isolates E797 and E801. The latter were not cut by Bsp143I, AluI or RsaI and clustered together with D1 isolates. Isolates exhibiting a restriction fragment of 118–120 bp length after digestion with Bsp143I could be identified as members of phylogenetic group A.

All fragments obtained from eight CLRV isolates belonging to phylogenetic group B produced bands around 147 bp and 265 bp after AluI digestion. They could only be differentiated from isolates belonging to group C, D2 or E by an RsaI restriction site, which is absent in isolates belonging to the other phylogenetic groups. Therefore, comparing the restriction pattern after digestion with AluI and RsaI allowed differentiation among isolates from distinct phylogenetic groups.

For the 20 CLRV isolates belonging to phylogenetic group E, neither a group specific restriction enzyme nor specific fragment sizes were found. However, 11 out of 20 tested isolates contained two AluI restriction sites resulting in 96–98 bp, 146–147 bp and 167 bp fragments, which are characteristic of half the isolates belonging to this phylogenetic cluster.

On the contrary, EcoRI and Eco130I could not be used to assign CLRV isolates to different phylogenetic groups. For instance, 14 of 24 CLRV isolates belonging to group A were cut by Eco130I producing bands around 99 and 317 bp. However, all group B isolates as well as eight out of 20 isolates belonging to group E and the five isolates of the phylogenetic cluster C produced identical patterns. Similar results were observed for EcoRI, as a single recognition sequence around position 190 was present not only in all B isolates, but also in some isolates belonging to groups A and E. Therefore, EcoRI was not useful in the discrimination of phylogenetically distinct isolates (examples are given in Fig. 1).

Digestion with AluI, RsaI and Bsp143I was suitable for differentiating virus isolates from several phylogenetic groups. Group A isolates could be distinguished from isolates belonging to groups B, C, D2 or E. Group B isolates were distinguishable from isolates belonging to all the other groups. A subset of isolates belonging to group E differed from all the other groups; however, some group E isolates were indistinguishable from group C and group D2 isolates. CLRV isolates belonging to phylogenetic group D1 were indistinguishable from two isolates grouped in cluster A.

3.2. Validation of IC-RT-PCR-RFLP method

After establishing restriction patterns produced from cloned 3′ NCR fragments originating from different CLRV isolates, amplification of CLRV specific 3′ NCR by IC-RT-PCR was coupled with restriction analysis (RFLP) and the method was validated using virus preparations. Expected patterns after restriction of PCR fragments with AluI, Bsp143I, EcoRI, Eco130I and RsaI as well as predicted by computational digestion were reproducible by IC-RT-PCR-RFLP received from purified virus isolates CLRV-E120, CLRV-E327, CLRV-E441 (phylogenetic group A), CLRV-E395 (group B), CLRV-E802 (group C) CLRV-326 (group D), CLRV-693 and CLRV-E603 (group E). However, digestion of the CLRV-E327 amplicon with Bsp143I was incomplete, producing bands of 415 bp as well as the digestion products with a size of 118 and 297 bp. IC-RT-PCR products, as well as restriction patterns after treatment with AluI, Bsp143, EcoRI, Eco130I and RsaI, are shown in Fig. 1.

The IC-RT-PCR-RFLP method was applied to samples collected from CLRV infected black elderberry (CLRV-E2165), walnut (CLRV-W2J) and birch (CLRV-E2249). The resulting CLRV specific PCR fragments from the three isolates were digested with the restriction endonucleases AluI, Bsp143I and RsaI (Fig. 2). The banding pattern of the IC-RT-PCR-RFLP product from the black elderberry isolate

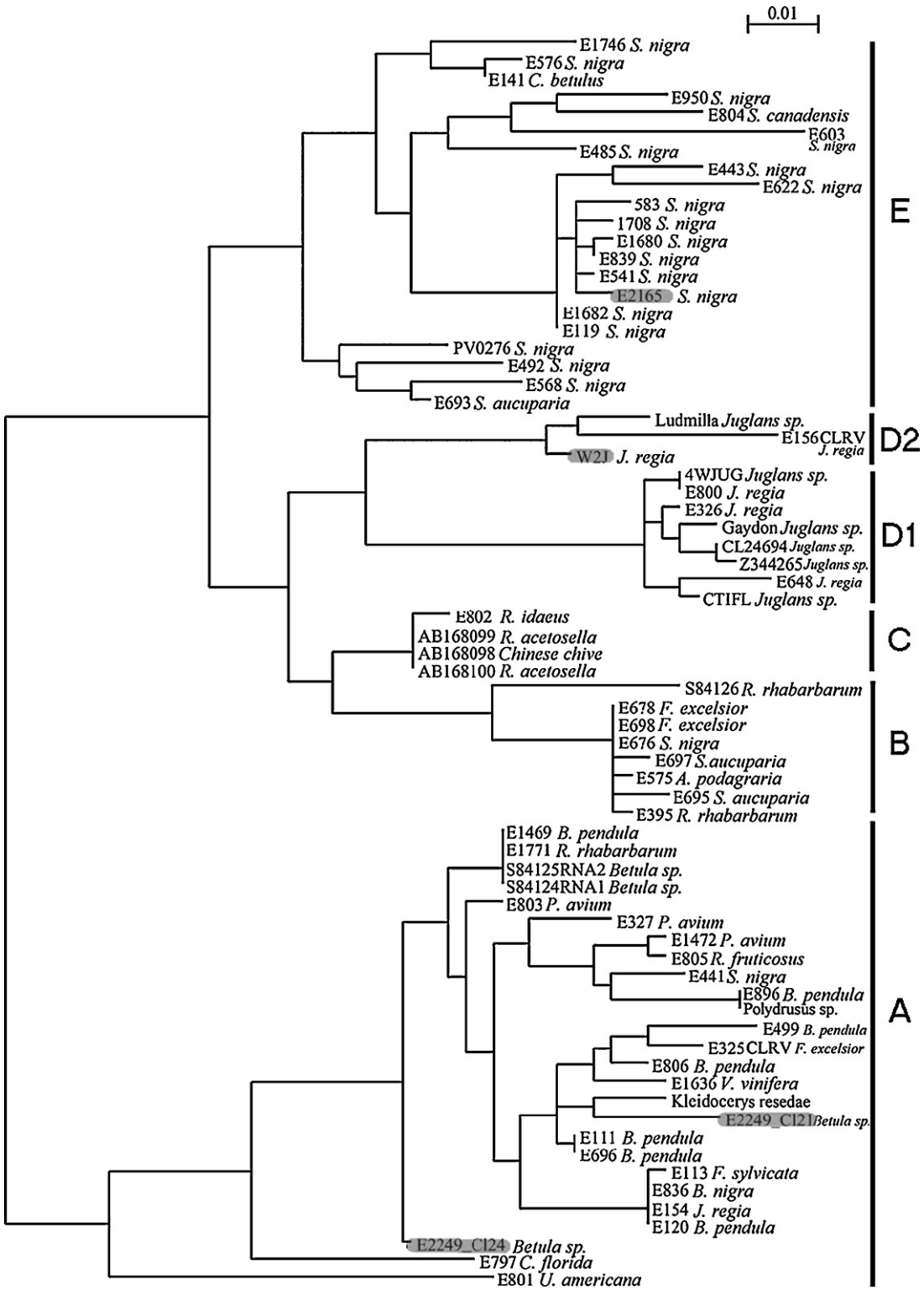

Fig. 3. Phylogenetic tree calculated with Dnapars using 3′ NCR sequence fragments of 70 CLRV isolates. Phylogenetic groups A–E according to Rebenstorf et al. (2006) are indicated on the right side. The new CLRV isolates E2165, E2249 and W2J are marked.

corresponded to the RFLP type EI (Table 3), which was typical of most isolates from phylogenetic group E. IC-RT-PCR using primers RW1 and RW2 with leaf and infructescence tissues from isolate W2J resulted in a band of the expected size (~420 bp) that exhibited the restriction pattern of RFLP type E/C/D2 (Table 3). Amplification by IC-RT-PCR was repeated for the birch isolate E2249 with four independent leaf samples and one sample from an infructescence. CLRV was detectable in three out of the four leaf samples as well as in the infructescence sample. Two PCR products were sufficiently concentrated for an RFLP assay. The 420 bp fragment was only partially digested by RsaI into two fragments of approximately 190 and 230 bp. The isolate was assigned to the phylogenetic group A, because the obtained restriction patterns corresponded to RFLP types AIII and AIV (Table 3).

In order to confirm the phylogenetic classification assumed by RFLP patterns, the CLRV specific IC-RT-PCR amplification products for the isolates E2165, E2249 and W2J were cloned and two individual clones per isolate were sequenced. Both cloned sequences from E2165 were identical; the two W2J clones showed a single nucleotide exchange whereas the sequence identities from the E2249 fragments were 96.8%. The CLRV sequences obtained from the birch sample were analyzed with the computer program NEBcutter. Comparison of the cloned 3′ NCR fragments of isolate E2249 revealed that in one clone a restriction site for RsaI was present, but absent in the other one. This finding was consistent with the restriction of the direct amplification product which was only partially digested by RsaI. Computer assisted restriction of the isolates E2165 and W2J corresponded with the results of the digestion with restriction endonucleases. When the three new isolates were compared to the 66 phylogenetically characterized CLRV sequences, the phylogenetic trees generated by different algorithms resulted in similar arrangements of the CLRV isolates. The arrangements corresponded largely to the analyses of Rebenstorf (2005) and Rebenstorf et al. (2006).

The tree calculated using the maximum parsimony algorithm is shown in Fig. 3, while trees calculated with maximum likelihood and neighbor joining methods are not shown. The sequence based phylogenetic analysis confirmed the RFLP classifications; isolate E2165 clustered within group E, isolate W2J clustered within group D2, and both sequences obtained from isolate E2249 clustered within group A.

4. Discussion

The partial 3′ NCR of the CLRV genome is variable among virus isolates that are diverse serologically and which are phylogenetically separate (Rebenstorf et al., 2006). Restriction analysis of the partial 3′ NCR of CLRV can replace sequencing if an isolate is grouped by its RFLP type to phylogenetic cluster A, B or E. However, this method cannot distinguish all the phylogenetic groups established by Rebenstorf et al. (2006). For example, the method did not differentiate group D1 from the two group A isolates E797 and E801. The observed RFLP pattern of these CLRV isolates supports previous findings by Rebenstorf (2005) based on the comparison of 280 bp of the 3′ NCR. Phylogenetic analysis using this shorter sequence fragment placed the isolates E797 and E801 not in phylogenetic group A, which harbors mainly cherry and birch isolates, but into separate phylogenetic groups, F and G, respectively. This indicates a more distant relationship of these two CLRV isolates to other group A isolates and is consistent with their origin from different host plants. Therefore, the lack of the Bsp143I restriction site in isolates E797 and E801, which is conserved in all other examined group A strains, reflects these initial sequence based phylogenetic allocations. CLRV isolates currently within group D1 originated exclusively from walnut trees and this group forms a single phylogenetic clade defined by the host plant species. Isolates exhibiting the RFLP type A/D1 from a host plant other than walnut should be examined by sequencing to determine conclusively their phylogenetic classification. If a new CLRV isolate exhibits RFLP type E/C/D2, an analysis of the sequence is imperative to resolve the phylogenetic relationship. There is no pattern that differentiates group E isolates with the RFLP type E/C/D2 from other phylogenetic group E isolates or that hints towards clusters with isolates from group C and D2. The group E isolates with RFLP type E/C/D2 are scattered within different subclusters of the major phylogenetic group E and do not form a distinct subgroup within group E. Also, the original hosts (black and golden elderberry) are common hosts of many group E isolates.

IC-RT-PCR-RFLP enabled the correct classification of the new CLRV isolates E2165 and E2249 as confirmed by phylogenetic sequence analysis. This information can be used on epidemiological studies; for example isolates of elderberry that occur in phylogenetic groups B and E can be distinguished rapidly. As isolates in phylogenetic group B are derived from a range of different host plants, the question arises whether isolates of this type are able to infect a broader range of host plants and whether there might be a risk given that economically or ecologically important plants might be or become hosts. Furthermore, the ability of CLRV to infect woody hosts like elderberry and cherry has been demonstrated by Jones (1973) for instance for one rhubarb isolate of the B group (S84126) as well as for the golden elderberry strain (CLRV-E804, group E). Thus the IC-RT-PCR-RFLP method developed above is a useful tool for studying the epidemiology of CLRV as well as the host range of isolates, because as Rebenstorf et al. (2006) outlined, CLRV isolates from groups A and B differ considerably in their original host plant species (group A harbors CLRV isolates from 12 species and group B comprises isolates from five plant species).

RFLP analysis of 3′ NCR fragments generated directly after IC-RT-PCR using virus purification, or natural host plant material, revealed partial digestions in two cases. Virus preparation of the group A isolate E327 was only digested partially with Bsp143I, resulting in a pattern similar to RFLP types AIII and A/D1 respectively, suggesting the presence of two sequence variants. Also, the amplification of two distinct 420 bp sequence variants of the 3′ NCR determined for the birch isolate E2249 is uncommon for CLRV. This was outlined by Rebenstorf et al. (2006), who found the nucleotide sequence of this part of the CLRV genome to be strictly conserved in individual isolates, even when propagated for more than 13 years in *C. quinoa*. The partial restriction by RsaI for isolate E2249 was found in two independent IC-RT-PCR-RFLPs; one using leaf and one using infructescence tissue as sample, and probably is not due to PCR artifacts. As the nucleotide differences between the two individual clones are diverse and probably are the result of several mutational events. It is, therefore, unlikely that one of the sequence variants had evolved from the other one but represents CLRV variants within a single host plant. This was confirmed by the phylogenetic analysis which placed the two isolates in distinct subclades within phylogenetic group A. Such genetic variability within sequences obtained from one virus isolate also has been detected by RFLP assays in another nepovirus, e.g. *Grapevine fanleaf virus* (Fattouch et al., 2005; Vigne et al., 2004) as well as in a potexvirus *Pepino mosaic virus* (Martínez-Culebras et al., 2002). Therefore, the IC-RT-PCR-RFLP described above has the potential to contribute to the identification of different CLRV sequence variants within a single isolate. Furthermore, it allows us to estimate the ratios of each virus isolate present in one host by comparing band intensities as reported for instance by Willment et al. (2001). It would be interesting to know whether one of the two E2249 sequence variants exhibits a higher fitness than the other causing RFLP fragments of one sequence variant to fade or to be lost over time. Such an alteration in the RFLP pattern of a GFLV isolate was found by Naraghi-Arani et al. (2001).

With IC-RT-PCR-RFLP analysis, it is critical to amplify the specific PCR product in sufficient amounts to produce visible bands after restriction. This can be difficult particularly when applied to woody host plants. CLRV specific 3′ NCR fragments were amplified easily from black elderberry and from walnut, however, this was more difficult when testing samples from birch. Werner et al. (1997) detected successfully CLRV not only in birch leaves but also in male inflorescences, leaf buds, single seeds and cortical tissues of young twigs using IC-RT-PCR. Grüntzig et al. (1996) described that the virus concentration in leaf tissue was lower than in buds, inflorescences, infructescences and young shoots. In this study, amplicons of sufficient yield were obtained from younger leaves and from a birch infructescence. Differing concentrations of PCR products might have been due to secondary plant compounds present in *Betula* spp. and especially in leaves (Riipi et al., 2002), because

polysaccharides and phenolic compounds have been reported to inhibit PCR (Demeke and Adams, 1992), but the results also may have been due to an irregular distribution of CLRV.

Several approaches might be suitable to increase the efficiency of the IC-RT-PCR. Werner et al. (1997) found a two-tube reaction to be more sensitive than a one-tube reaction. In addition, buffer additives in reaction mixes could lead to increased amplification efficiency in PCR despite the presence of inhibitory compounds (Demeke and Adams, 1992). Selection of suitable sampling material (e.g. inflorescences) might also contribute to higher yield of PCR products.

In conclusion, the restriction analysis of 420 bp fragments from the CLRV 3′ NCR with the enzymes AluI, RsaI and Bsp143I succeeded in differentiating isolates belonging to different phylogenetic groups infecting various host plant species. Therefore, this method can be useful for studying the phylogeny and epidemiology of CLRV as well as processes of virus evolution in woody hosts. The virus may be amplified universally from original hosts by IC-RT-PCR and subsequent RFLP analysis is used to discriminate among certain phylogenetic clusters and potentially sequence variants. Thus this method could provide a tool for monitoring genetic drift of CLRV strains over time in infected trees during adaptation processes of the virus to the host plant as was postulated by Rebenstorf et al. (2006). Furthermore, it may be used to investigate coevolution processes of CLRV within the plant-virus interactions. Overall, the established IC-RT-PCR-RFLP protocol is suitable for the rapid phylogenetic classification of CLRV isolates derived from original host plants and for the study of population structure of the virus.

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References

Bandte, M., Büttner, C., 2001. A review of an important virus of deciduous trees—cherry leafroll virus: occurrence, transmission and diagnosis. Pflanzenschutzberichte 59, 1–19.

Blanco-Urgoiti, B., Sánchez, F., Dopazo, J., Ponz, F., 1996. A strain-type clustering of potato virus Y based on the genetic distance between isolates calculated by RFLP analysis of the amplified coat protein gene. Archives of Virology 141, 2425–2442.

Brooks, M., Bruening, G., 1995. Transient gene expression of antisense RNA and coat protein-encoding sequences reduced accumulation of cherry leafroll virus in tobacco protoplasts. Virology 208, 132–141.

Demeke, T., Adams, R.P., 1992. The effects of plant polysaccharides and buffer additives on PCR. BioTechniques 12, 332–334.

Fattouch, S., Acheche, H., M'hirsi, S., Mellouli, L., Bejar, S., Marrakchi, M., Marzouki, N., 2005. RT-PCR-RFLP for genetic diversity analysis of Tunisian Grapevine fanleaf virus isolates in their natural host plants. Journal of Virological Methods 127, 126–132.

Felsenstein, J., 2005. PHYLIP (Phylogeny Inference Package) Version 3.6. Department of Genome Sciences, University of Washington, Seattle [distributed by the author].

Gentkow, J., von Bargen, S., Petrik, K., Büttner, C., 2007. Detection of *Cherry leaf roll virus* (CLRV) in woody plants by serological and molecular methods. In: Dujesiefken, D., Kockerbeck, P. (Eds.), Yearbook of Arboriculture. Haymarket Media, Braunschweig, pp. 279–284.

Giersiepen, R., 1993. Untersuchungen zur Charakterisierung des Kirschenblattrollvirus (CLRV) als Ursache einer Eschenvirose. PhD Thesis. Universität Bonn.

Grüntzig, M., Fuchs, E., Hentsch, T., 1996. Occurrence and serological detection of cherry leaf roll nepovirus (CLRV) and apple mosaic ilarvirus (ApMV) in Betula spp. Journal of Plant Disease and Protection 103, 571–581.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41, 95–98.

Jalkanen, R., Büttner, C., von Bargen, S., 2007. *Cherry leaf roll virus*, CLRV, Abundant on *Betula pubescens* in Finland. Silva Fennica 41, 755–762.

Jones, A.T., 1973. A comparison of some properties of four strains of *Cherry leaf roll virus*. Annals of Applied Biology 74, 211–217.

Jones, A.T., 1976. Serological specificity of isolates of *Cherry leaf roll virus* from different natural hosts. Agriculturae Conspectus Scientificus 39, 527–532.

Jones, A.T., 1985. *Cherry leaf roll virus*. Descriptions of Plant Viruses No. 306, CMI/ABB.

Jones, A.T., Koenig, R., Lesemann, D.E., Hamacher, J., Nienhaus, F., Winter, S., 1990. Serological comparison of isolates of *Cherry leaf roll virus* from diseased beech and birch trees in a forest decline area in Germany with other isolates of the virus. Journal of Phytopathology 129, 339–344.

Kegler, H., Richter, J., Schmidt, H.B., 1966. Untersuchungen zur Identifizierung und Differenzierung des Blattrollvirus der Kirsche (*Cherry leaf roll virus*). Phytopathologische Zeitschrift 56, 313–330.

Kim, Y.H., Kim, O.S., Roth, J.H., Moon, J.K., Sohn, S.I., Lee, S.C., Lee, J.Y., 2004. Identification of soybean mosaic virus strains by RT-PCR/RFLP analysis of cylindrical inclusion coding region. Plant Disease 88, 641–644.

Letschert, B., Adam, G., Lesemann, D.E., Willingmann, P., Heinze, C., 2002. Detection and differentiation of serologically cross-reacting tobamoviruses of economical importance by RT-PCR and RT-PCR-RFLP. Journal of Virological Methods 106, 1–10.

Martínez-Culebras, P.V., Lázaro, A., Abad Campos, P., Jordá, C., 2002. A RT-PCR assay combined with RFLP analysis for detection and differentiation of isolates of Pepino mosaic virus (PepMV) from tomato. European Journal of Plant Pathology 108, 887–892.

Mircetich, S.M., Sanborn, R.R., Ramos, D.E., 1980. Natural spread, graft-transmission, and possible etiology of walnut blackline disease. Phytopathology 70, 962–968.

Naraghi-Arani, P., Daubert, S., Rowhani, A., 2001. Quasispecies nature of the genome of Grapevine fanleaf virus. Journal of General Virology 82, 1791–1795.

Obermeier, C., Rebenstorf, K., Bandte, M., Büttner, C., 2003. Impact and distribution of *Cherry leaf roll virus* (CLRV) in forest trees and horticultural plants. In: Dujesiefken, D., Kockerbeck, P. (Eds.), Yearbook of Arboriculture. Thalacker Medien, Braunschweig, pp. 219–225.

Perriere, G., Gouy, M., 1996. WWW-Query: an on-line retrieval system for biological sequence banks. Biochimie 78, 364–369.

Rebenstorf K., 2005. Untersuchungen zur Epidemiologie des *Cherry leaf roll virus* (CLRV): genetische und serologische Diversität in Abhängigkeit von der Wirtspflanze und der geographischen Herkunft. PhD Thesis. Humboldt-Universität zu Berlin, Berlin.

Rebenstorf, K., Candresse, T., Dulucq, M.J., Büttner, C., Obermeier, C., 2006. Host species-dependent population structure of a pollen-borne plant virus, *Cherry leaf roll virus*. Journal of Virology 80, 2453–2462.

Riipi, M., Ossipov, V., Lempa, K., Haukioja, E., Koricheva, J., Ossipova, S., Pihlaja, K., 2002. Seasonal changes in birch leaf chemistry: are there trade-offs between leaf growth and accumulation of phenolics? Oecologia 130, 380–390.

Rowhani, A., Mircetich, S.M., 1988. Pathogenicity on walnut and serological comparisons of cherry leafroll virus strains. Phytopathology 78, 817–820.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning—A Laboratory Manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Schmelzer, K., 1972. Nachweis der Verwandtschaft zwischen Herkünften des Kirschenblattroll-Virus (*Cherry leaf roll virus*) und dem Ulmenmosiak-Virus (elm mosaic virus). Zentralblatt für Bakteriologie Abteilung II 127, 140–144.

Tóbiás, I., 1995. Characterisation of different *Cherry leaf roll virus* (CLRV) isolates. Horticultural Science 27, 69–73.

Thompson, J.D., Gipson, T.J., Plewaniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24, 4876–4882.

Vigne, E., Bergdoll, M., Guyader, S., Fuchs, M., 2004. Population structure and genetic variability within isolates of Grapevine fanleaf virus from a naturally infected vineyard in France: evidence for mixed infection and recombination. Journal of General Virology 85, 2435–2445.

Vincze, T., Posfai, J., Roberts, J.R., 2003. NEBcutter: a program to cleave DNA with restriction enzymes. Nucleic Acids Research 31, 3688–3691.

Waterworth, H.E., Lawson, R.H., 1973. Purification, electron microscopy, and serology of the dogwood ringspot strain of cherry leafroll virus. Phytopathology 63, 141–146.

Werner, R., Mühlbach, H.P., Büttner, C., 1997. Detection of cherry leaf roll nepovirus (CLRV) in birch, beech and petunia by immunocapture RT-PCR using a conserved primer pair. European Journal of Forest Pathology 27, 309–318.

Willment, J.A., Martin, D.P., Rybicki, E.P., 2001. Analysis of the diversity of African streak mastreviruses using PCR-generated RFLPs and partial sequence data. Journal of Virological Methods 93, 75–87.