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4-2 Cherry Leaf Roll Virus in birch – an old problem or an emerging virus in Finland?

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ABSTRACT

Cherry leaf roll virus (CLRV) was first mentioned to occur in Finland in 1980 by Cooper and Edwards who described an isolate from red elderberry (*Sambucus racemosa*). CLRV affecting birch species in the country have also been confirmed sporadically from single silver birch (*Betula pendula)* trees. Since 2002 the situation changed as virus-like symptoms in birch species native to Fennoscandia started to accumulate. Leaf roll, vein banding and chlorotic patterns with subsequent necrosis of birch leaves were increasingly observed. Disease symptoms affecting downy birch (*B. pubescens* ssp. *pubescens*), curly birch (*B. pendula* var. *carelica*), mountain birch (*B. pubescens* ssp. *czerepanovii*), dwarf birch (*B. nana*), the local variety Kiilopää birch (*B. pendula* var. *appressa*) and silver birch could be associated with an infection of CLRV. Since the incidence in 2002, virus-associated symptoms are spreading in *Betula* species and are distributed all over the country at present. As causes of the sudden appearance of the disease are still unknown, possible ways of transmission and viral dissemination as well as unique features of the CLRV population occurring in Finland are discussed.

INTRODUCTION

The birches (genus *Betula*) are common trees and shrubs of the boreal and north temperate zones of the Northern hemisphere; in Finland more than one fifth of the forest is birch forest and the genus represents the most abundant group of deciduous trees providing an important raw material in the mechanical and chemical forest industry (Peltola 2006). Downy birch

(*Betula pubescens* ssp. *pubescens*) and silver birch (*B. pendula*) are used industrially for plywood, veneer (Luostarinen & Verkasalo 2000) and paper production (Viherä-Aarnio & Velling 1999). Downy and silver birch are abundant throughout the country in towns, on roadsides and most common in mixed forests. North of the Arctic Circle dwarf birch (*B. nana*), Kiilopää birch (*B. pubescens* ssp. *appressa*) and mountain birch (*B. pubescens* ssp. *czerepanovii*) are dominant and important key components of the arctic ecosystem (Walker 2000; van Wijk *et al.* 2005).

Cherry leaf roll virus, CLRV, is a plant pathogen which belongs to the genus *Nepovirus* and the family *Comoviridae*. The virus is distributed worldwide and infects various deciduous trees and shrubs (Bandte & Büttner 2001). The seed and pollen-borne virus is also transmitted by mechanical means, grafting and root connation.

Until recently CLRV has only been detected rarely in Finland and adjacent countries (Cooper & Edwards 1980; Bremer *et al.* 1991). However, since 2002 virus-related symptoms such as vein banding, leaf roll, chlorosis and subsequent necrosis on birch leaves were increasingly recorded throughout Fennoscandia. In a survey throughout Finland symptoms on birch were especially distinct during the dry summer of 2006 and it was found that several birch species were affected. Recently, CLRV was confirmed in Rovaniemi, northern Finland in several *B. pubescens* ssp. *pubescens* trees exhibiting symptoms of a viral disease (Jalkanen *et al.* 2007).

Aims of the study were to determine CLRV distribution in Finland and occurrence of the virus in different Finnish birch species. Furthermore, genetic characteristics of individual CLRV isolates obtained from different locations in Finland and *Betula* species were assessed, in order to compare the Finnish CLRV population with other known CLRV isolates.

MATERIALS AND METHODS

More than seventy trees of the genus *Betula* exhibiting characteristic symptoms of a virus infection were sampled in 2007 and 2008 all over Finland. Furthermore, selected birch trees from stands in Kittilä and Läyliäinen used for seed production were included in the study as well as four water samples collected randomly in the vicinity of symptomatic trees. Singular rowan (*Sorbus aucuparia*) trees exhibiting ringspots and mottle were also sampled as well as red elderberry (*Sambucus racemosa*) exhibiting leaf deformations (Figure 1).

Two twigs of individual trees were tested by a CLRV specific IC-RT-PCR (Jalkanen *et al.* 2007) in duplicate by application of symptomatic leaves and buds, catkins or twig tips. Coat protein specific primers (CP188F and CP350R) were deduced and used alternatively in the IC-RT-PCR replacing the primer combination RW1 and RW2 established by Werner *et al.* (1997). Ten microliters per water sample were subjected to IC-RT-PCR and were also tested in duplicate. A tree/water sample was scored as CLRV positive, if a specific fragment of the expected size was amplified at least from one sample per tree/water sample. Partial fragments of the CLRV 3' non-coding region (3´ NCR) fragments were digested with AluI, Bsp143I, or RsaI respectively to determine sequence variants of CLRV isolates detected in birch samples

according to Buchhop *et al.* (in print). Selected PCR amplicons of the CLRV 3´ NCR as well as coat protein fragments obtained from CLRV contaminated samples in Finland were cloned and sequenced. PCR products were ligated into pBluescriptII SK(-)-vectors (Stratagene, USA) and transformed into chemocompetent *E. coli* using standard protocols (Sambrook *et al.* 1989). Constructs were purified from liquid bacterial cultures (InvisorbSpinPlasmid MiniII, Invitek, Germany) and inserts were sequenced from both directions by cycle sequencing and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA) by use of vector specific primers. Obtained sequences were analysed and compared to CLRV isolates characterised by Rebenstorf *et al.* (2006) applying ClustalX 1.83 (Thompson *et al.* 1997) using the incorporated neighbour-joining method for phylogenetic tree construction.

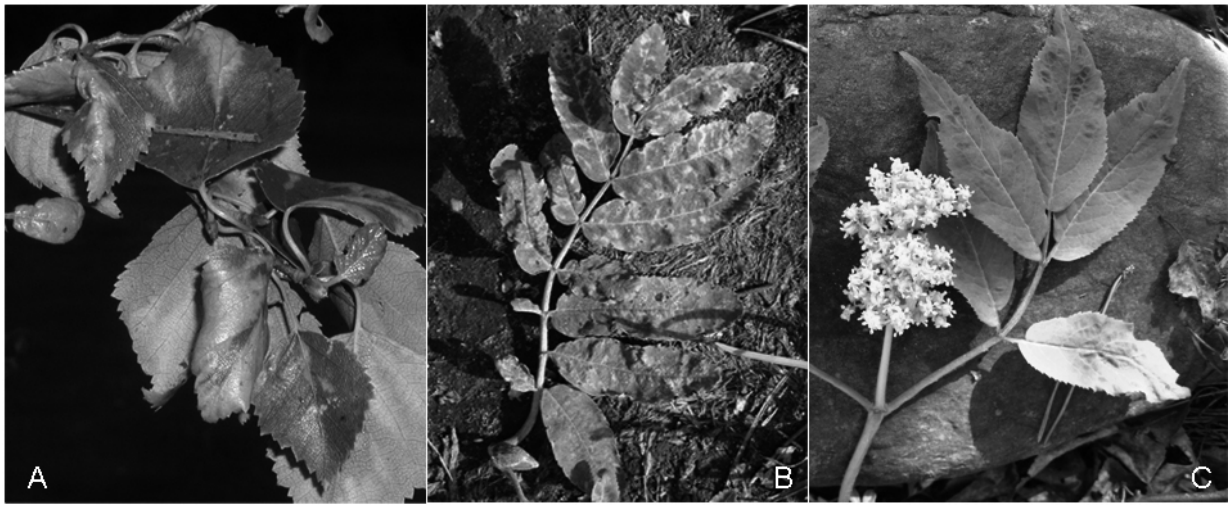

Figure 1. CLRV infected Betula sp. (A), Sorbus aucuparia (B), and Sambucus racemosa (C) exhibiting virus-like symptoms.

RESULTS

The birch trees sampled in 2007 from southern, central and northern Finland revealed numerous CLRV infections. Altogether, CLRV was proved to be in 55% of the symptomatic birch trees, thereof 18 downy birches (56%) and 12 silver birches (48%). Sampled dwarf birches (4), mountain birches (6) and Kiilopää birches (5) included in the study were limited; still, CLRV detection was successful at least in two trees per species. Additionally, the one curly birch sampled from a garden in Rovaniemi was CLRV positive (Table 1). Results confirmed that CLRV is widely distributed in different birch species throughout Finland, even north of Rovaniemi and the Arctic Circle up to northern and alpine tree line. Tree samples originated from rural areas, i.e. from alleys, parks (churchyards, schoolyard) and along roadsides in town centres but also from natural stands as for instance the samples collected in Inari. Furthermore, four symptomatic saplings — two *B. pendula* and two *B. pubescens* — originated from a 100-year-old seed-production stand in Kittilä (northern Finland) could be shown to be CLRV infected.

Table 1. Detection of CLRV by IC-RT-PCR in samples from Finland

Sample	Sampled, no.	CLRV positive, no.
symptomatic birch species		
B. pubescens subsp. *Pubescens*	32	18
B. pendula	25	12
B. pubescens subsp. *Czerepanovii*	6	2
B. pubescens var. *appressa*	5	5
B. nana	4	2
B. pendula var. *carelica*	1	1
birch seed-production stands		
Kittilä (*B. pubescens* subsp. *pubescens*, *B. pendula*)	4	4
Läyliäinen (*B. pendula*)	5	2
other species and environmental samples		
Sorbus aucuparia	6	2
Sambucus racemosa	1	1
water	4	1
Total	93	50

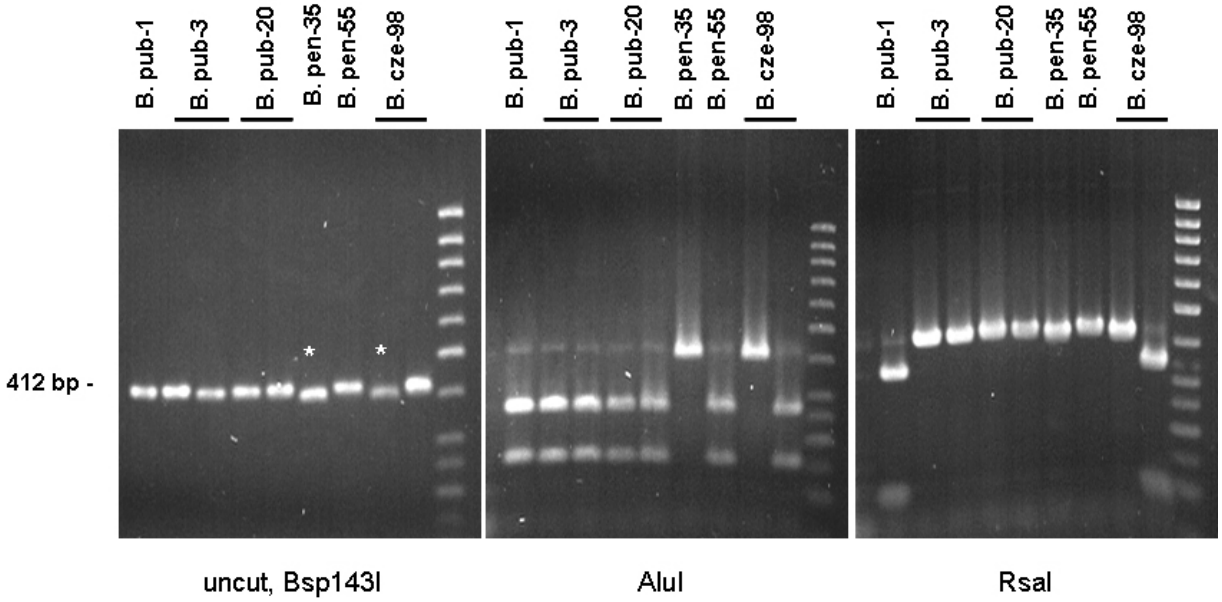

Figure 2. CLRV detection in six different birch trees by IC-RT-PCR amplification of the partial 3′ non-coding region (412 bp) with RW1 and RW2 primers developed by Werner et al. (1997) and subsequent RFLP analysis of CLRV specific fragments by use of the restriction endonucleases AluI, Bsp143I, and RsaI. Asterisks indicate shorter fragments of 404 bp. (Marker: 50 bp ladder, Fermentas)

Similar results were obtained by testing asymptomatic silver birch trees from a seed-production stand, established in 1998 in southern Finland (Läyliäinen) comprising trees from a clone collection. In two out of five randomly sampled trees CLRV was detectable, which was confirmed by sequencing of the amplified fragments of the partial coat protein-coding region. In Finland the virus is not restricted to the genus *Betula* because it was also detected in two European mountain ash trees and a singular red elderberry exhibiting virus-like symptoms. Furthermore, a CLRV positive water sample (no. 152) taken from the lake Rautavesi in the Länsi-Suomi area in May 2008 revealed the contamination of surface water with the virus.

CLRV infection of three downy, two silver birches and one mountain birch was also confirmed by restriction analysis and sequencing of the amplified 3´ NCR fragment. The samples originated from various locations in Finland, i.e. Rovaniemi and Inari (North), Lieksa (East) and Vaasa (West) and display size differences between 404 and 412 bp as well as sequence variability after restriction analysis (Figure 2). RFLP-types of CLRV strains from Finnish birch samples differed from virus variants characterised from other geographical origins. This was supported by sequence comparison of 3´ NCR fragments with CLRV strains characterised previously by Rebenstorf *et al.* (2006). Analysis revealed that fragments of CLRV strains obtained from Finnish birches shared highest sequence identities to CLRV isolates belonging to phylogenetic group B, D or E (data not shown). Sequencing of individual clones of the partial coat protein-coding region (161 bp) of selected *B. pendula* trees (no. 137, 140 and 256) and the positive water sample followed by phylogenetic analysis showed close relationships of samples from Finland (98.2–100% sequence identities); these samples are clearly distinguished from other CLRV strains characterised previously by Rebenstorf *et al.* (2006) (Figure 3). Partial coat protein sequences of CLRV strains obtained from birches in Germany and the United Kingdom enclosed in phylogenetic group A (I2, E441, E120) are most distantly related to virus strains found in Finnish birch species sharing only between 75.0–80.3% sequence identity at the nucleotide level of the analysed 112 bp.

DISCUSSION

CLRV has been confirmed in birch trees from several places in Finland by molecular means, revealing that the virus is widely distributed in the country and also affects at least six birch species or varieties native to Fennoscandia. The main route of CLRV dispersal in birch in natural habitats is assumed to be pollen and seed transmission, which has been studied in detail before (Cooper 1976, 1979; Cooper *et al.* 1984). Cross pollination resulting in *Betula* hybrids is commonly reported from the genus (Anamthawat-Jónsson & Thórsson 2003; Atkinson 1992) and may be a reason why the virus had been spread between different *Betula* species. In our investigations we found CLRV infected seedlings in a seed production stand in Kittilä in northern Finland as well as asymptomatic *B. pendula* trees harvested for seeds in the southern part of the country. Thus, contaminated seed could be a possible route of CLRV dispersal into planted birch populations. However, most trees with CLRV symptoms especially in the coun-

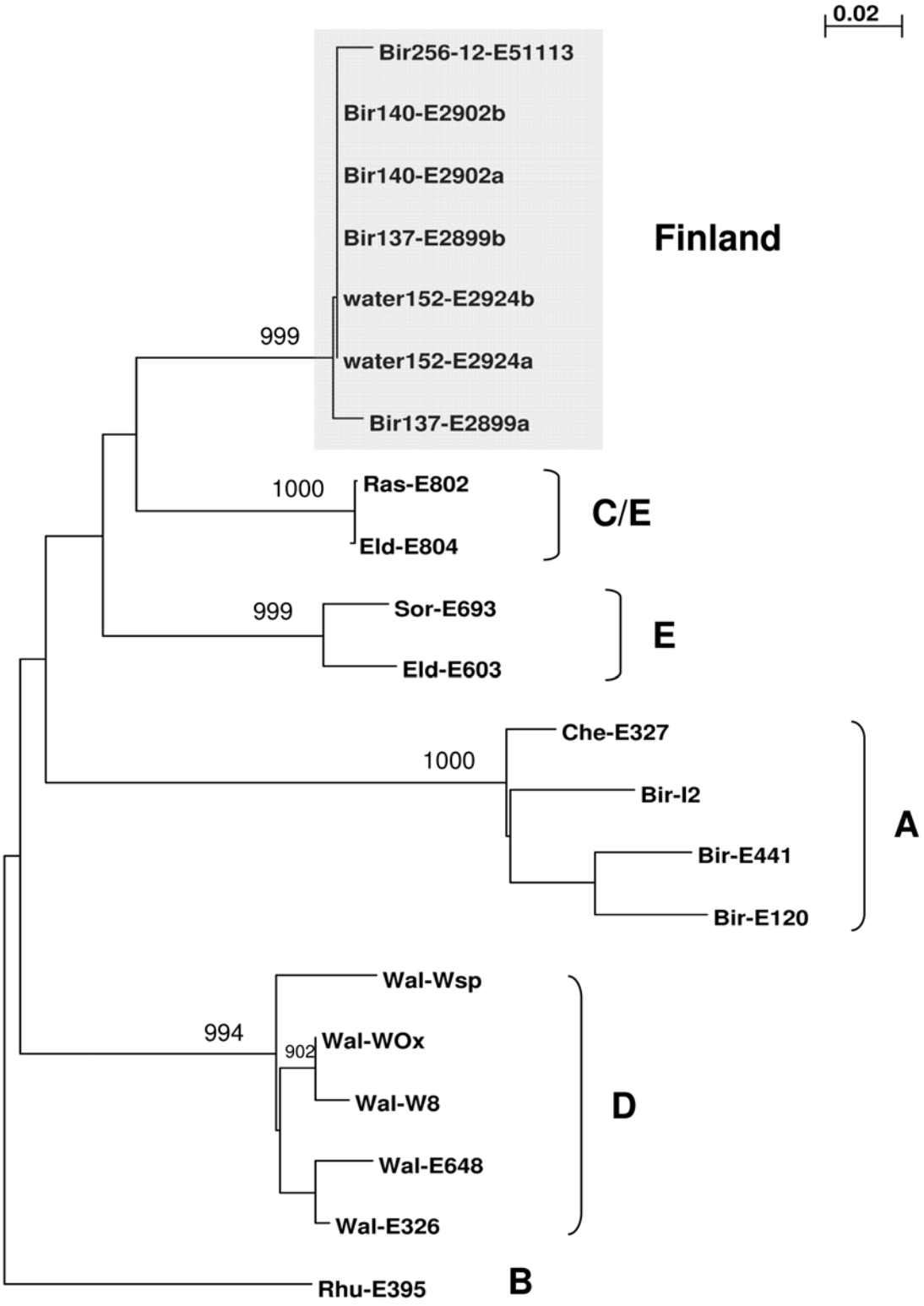

Figure 3. Neighbour-joining phylogenetic tree calculated with ClustalX 1.83 from 112 nucleotides of the partial CLRV coat protein-coding region amplified with primers CP188F/CP350R. Bootstrap analysis was performed with 1000 replicates; values above 900 are indicated on branches. The bar length represents substitutions per nucleotide. Samples from Finland are boxed. Major groups (A–E) defined by Rebenstorf et al. (2006) are indicated by the respective character on the right side.

tryside are naturally born. Further, most sampled birches from alleys and town birches are rather old, 40 to 80 years, suggesting that contaminated seed is not involved in the rapid spread of CLRV unless the trees were infected latently without symptom expression until recently. The role of insects as virus vectors has not been studied satisfactory, but the birch catkin bug *Kleidocerys resedae* as well as weevils (*Polydrusus* sp.) has been shown to carry the virus (Werner *et al.* 1997; Rebenstorf 2005). Potential insect vectors have never been under investigation in Finland; however, the contamination of surface water with CLRV may indicate towards an additional route of virus dissemination in the environment as was postulated already by Bandte *et al.* (2007).

Sequence comparisons of the partial 3´ NCR revealed unusual phylogenetic relationships of Finnish CLRV isolates. Until now, phylogenetically characterised CLRV isolates of birch trees from the United Kingdom and Germany exclusively clustered within clade A (Rebenstorf *et al.* 2006). They concluded that co-evolution of CLRV and host plant is a major factor that led to quick adaptation of virus populations within one host species, which could be genetically differentiated according to infected plant species. The majority of CLRV isolates from Finnish birch trees were found to relate to other phylogenetic clades which was supported by analysis of the partial coat-protein-coding region. CLRV isolates from Finland clustered with characterised strains originating from a wider range of host plant species including ash (*Fraxinus excelsior*), rowan and *Sambucus* sp. (Rebenstorf *et al.* 2006). These species are also native to southern Finnish ecosystems (Mikk & Mander 1995; Simola 2006) and may have been the source of CLRV strains now affecting birch species in Finland. This speculation is substantiated by our findings that mountain ash trees as well as a red elderberry sampled in 2008 were found to be CLRV infected, and our analyses of sequence data which indicates towards a different virus population to be present in Finnish birches. Birches may have recently acquired CLRV from other host plants and the virus population is not adapted to specific host plant species yet. Lack of adaption of the virus to the host species may have induced severe symptom development in birches and the presence of a mixed virus population may be responsible for the aggressive spreading of the virus disease in Finland in a very short time. However, this may also be due to the new introduction of CLRV into birch species of this north European region and cannot be secured because of the few individuals tested and virus isolates characterised so far.

It is of particular importance to monitor the dissemination of CLRV in the Finnish environment and the development of virus populations found in Finnish plant species, because they differ considerably from previous findings. The virus may even represent a threat to the Finnish forest industry relying on birch logs as source for pulpwood and therefore, the epidemiology of the virus has to be elucidated, and the impact of the pathogen for the *Betula* genus has to be investigated in future studies.

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