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Cronartium rust sporulation on hemiparasitic plants

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Susceptibility of potential alternate host plants to pine stem rusts belonging to *Cronartium* spp. was artificially tested in Finland during 2012–2013. Forty-three species representing 11 plant families were inoculated in the laboratory; 34 species (11 families) were inoculated in the greenhouse with aeciospores of *Cronartium flaccidum* or *C. ribicola*. Twenty-one selected species (10 families) were also exposed to natural inoculum of *C. flaccidum* in the field in two severely affected *Pinus sylvestris* stands. After 5–8 weeks' incubation, *C. flaccidum* sporulated on 17 species (nine families) in the laboratory, 17 species (eight families) in the greenhouse and seven species (five families) in the field. *Cronartium ribicola* sporulated on three species (three families) in the laboratory or greenhouse. All of the hemiparasitic plants that belong to Orobanchaceae were infected by *C. flaccidum*, and several species supported rust sporulation when exposed to natural inoculum. Susceptible species belonged to genera *Veronica*, *Euphrasia*, *Castilleja*, *Pedicularis*, *Rhinanthus*, *Saxifraga*, *Loasa*, *Ribes*, *Tropaeolum*, *Swertia*, *Physalis*, *Nicotiana*, *Hyoscyamus*, *Paeonia*, *Apocynum*, *Impatiens*, *Vincetoxicum* and *Myrica*.

Keywords: alternate hosts, forest pathology, pine stem rust, white-pine blister rust

Introduction

Cronartium ribicola causes white-pine blister rust on five-needle pines (*Pinus* section *Strobus*) in Europe (Blada, 1990; Stephan, 2004), Asia (Kim *et al.*, 2010; Zhang *et al.*, 2010) and North America (Ziller, 1974; Zampino, 2010). Since the introduction of the rust to North America, white pines have suffered severely from the rust, especially in combination with other diseases, and some species (e.g. *Pinus albicaulis*) have become endangered. In Finland, the rust was first reported in the 1800s (Liro, 1908), and destroyed most of the Finnish exotic five-needle pine plantations during the 1900s (Heikinheimo, 1956; Lähde *et al.*, 1984). Plantations of resistant *Pinus cembra* and *Pinus peuce* have survived as far north as the Arctic Circle (Kaitera *et al.*, 2013). The rust can spread via species of *Ribes* (Grossulariaceae), *Pedicularis* or *Castilleja* (Orobanchaceae) in Asia (Yokota & Uozumi, 1976; Kim *et al.*, 2010; Zhang *et al.*, 2010) and North America (McDonald *et al.*, 2006; Zampino, 2010; Mulvey & Hansen, 2011). In Finland, *C. ribicola* sporulates on wild *Ribes* (Kaitera & Nuorteva, 2006; Kaitera *et al.*, 2013), but the rust is also able to infect and sporulate on *Pedicularis* (Kaitera & Hiltunen, 2011), and species of Loasaceae and Apocynaceae under artificial conditions (Kaitera & Hiltunen, 2012; Kaitera *et al.*, 2012).

Cronartium flaccidum regularly causes severe damage to two-needle pines throughout Europe (Diamandis & De Kam, 1986; Kaitera, 2000). Recently, the rust was reported to cause significant damage to young *Pinus sylvestris*, especially in northern Fennoscandia (Samils *et al.*, 2010). *Cronartium flaccidum* is known to infect alternate hosts in the genera *Vincetoxicum*, *Melampyrum*, *Gentiana*, *Paeonia*, *Pedicularis*, *Loasa*, *Nemesia*, *Impatiens*, *Grammatocarpus*, *Schizanthus*, *Tropaeolum*, *Verbena* and *Euphrasia* (Hylander *et al.*, 1953; Gäumann, 1959; Kaitera *et al.*, 1999). In southern Europe, the rust spreads in natural pine forests via *Vincetoxicum hirundinaria* (Ragazzi, 1983), as well as *Melampyrum sylvaticum* in northern Europe (Kaitera *et al.*, 2005).

The objective of this study was to test the susceptibility of common and potential host plant species to infection by *C. flaccidum* and *C. ribicola* under laboratory, greenhouse and field conditions. In particular, hemiparasitic plants of the family Orobanchaceae were selected for susceptibility screening. The hypothesis tested was that the current list of alternate host plants for *Cronartium* is incomplete, and many other plant species are susceptible to infection and able to spread rusts from ornamental gardens to natural forests.

Materials and methods

Plant and spore material

Aeciopores of *C. ribicola* (three sources; Table 1) were collected in late May 2012 from *Pinus strobus* in an arboretum in southern Finland from sporulating aecia by cutting the aecial surface with a sterilized scalpel and gently dusting the spores into Petri dishes in the field. Aecial remnants were removed from the spore samples using forceps in a laminar flow cabinet, and spores were stored at 5°C prior to inoculation. Germination of the

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Table 1 Source data of *Cronartium* aeciospores used in inoculation trials

Source species and code	Date of collection	Host plant	Collection location
C. ribicola 1	21.5.2012	*Pinus strobus*	Viikki arboretum, southern Finland
C. ribicola 2	21.5.2012	*P. strobus*	Viikki arboretum, southern Finland
C. ribicola 3	21.5.2012	*P. strobus*	Viikki arboretum, southern Finland
C. flaccidum 2a	18.6.2012	*Pinus sylvestris*	Kolari, northern Finland
C. flaccidum 2b	18.6.2012	*P. sylvestris*	Kolari, northern Finland
C. flaccidum 2c	18.6.2012	*P. sylvestris*	Kolari, northern Finland
C. flaccidum 2d	18.6.2012	*P. sylvestris*	Kolari, northern Finland
C. flaccidum 2e	18.6.2012	*P. sylvestris*	Kolari, northern Finland
C. flaccidum 3a	18.6.2012	*P. sylvestris*	Juomukuru, northern Finland
C. flaccidum 3b	18.6.2012	*P. sylvestris*	Juomukuru, northern Finland
C. flaccidum 1	17.6.2013	*P. sylvestris*	Kolari, northern Finland
C. flaccidum 2	17.6.2013	*P. sylvestris*	Kolari, northern Finland
C. flaccidum 3	17.6.2013	*P. sylvestris*	Kolari, northern Finland
C. flaccidum 4	17.6.2013	*P. sylvestris*	Kolari, northern Finland
C. flaccidum 5	17.6.2013	*P. sylvestris*	Juomukuru, northern Finland
C. flaccidum 6	17.6.2013	*P. sylvestris*	Juomukuru, northern Finland
C. flaccidum 7	17.6.2013	*P. sylvestris*	Juomukuru, northern Finland

aeciospores varied between 49–89% among sources after 24 h incubation on 1·5% water agar. Rust aeciospores were collected from the same population that had infected *Ribes* and *Pedicularis* in a previous study (Kaitera & Hiltunen, 2011).

Aeciospores of *C. flaccidum* (seven spore sources per year; Table 1) were collected from two severely affected *P. sylvestris* stands in northern Finland from fresh unopened aecia occurring on branches that were cut and transported to the laboratory in mid-June 2012 and 2013. Spores were dusted onto empty Petri dishes through a fine mesh in a laminar flow cabinet. Germination of the aeciospores ranged between 92–100% in 2012 and 46–96% in 2013 after a similar incubation to that given to the *C. ribicola* spores. Aeciospores collected from the population in Kolari were collected from trees adjacent to infected pines and are known to infect a broad range of alternate hosts (Kaitera & Hiltunen, 2011). Due to the lack of sporulating aecia in the older pine stand sampled for inoculum in a previous study (Kaitera & Hiltunen, 2011), spores from the Juomukuru population were collected from young trees located *c.* 1–2 km away from that location.

Test plant material was grown in the greenhouse of the botanical gardens at the University of Oulu from seeds sown each year in the early spring. Test plants were used in leaf inoculations in the greenhouse or transported to the field where leaves were exposed to natural inoculum. In addition, young detached leaves of the test plants were collected from perennial plants in the botanical gardens for inoculation in the laboratory. Leaves of 56 species from 13 plant families were used in the experiments (Tables S1, S2 & S3).

Inoculation of attached leaves in the greenhouse and detached leaves in the laboratory

Leaves of 34 plants (3 to 4560 per species; Table S1) were inoculated in the greenhouse as described in Kaitera & Hiltunen (2011). First, leaves of whole plants were moistened with water from a spray bottle prior to receiving an average of 127 aeciospores mm^{-2} (estimated after dusting spores on water agar and counting the number of spores using a light microscope) dusted on the lower leaf surface using an artists' pencil in mid-June 2012 (*C. flaccidum* and *C. ribicola*) and mid-June 2013 (*C. flaccidum*). Inoculated plants were then covered with a moistened plastic bag (one per plant) for 48 h to promote spore germination. After removing the bag, plants were incubated on the greenhouse table under artificial light at room temperature, and irrigated for 3 s every 30 min for 8 weeks. Individual plants of several test species were left uninoculated as controls.

Detached leaves of 43 plants (14 to 32 per species; Table S2) were floated on water in Petri dishes and inoculated by dusting spores using an artists' pencil in mid-June 2012 and mid-June 2013. Plates were incubated for 8 weeks at 18°C in artificial light in a climate chamber (Climacell). One plate of each test species was left uninoculated as a control.

Exposing plants to natural inoculum of *C. flaccidum* in the field

Plastic containers housing single or several plants of 21 species (18 to 4265 leaves per species; Table S3) were transported to *P. sylvestris* stands suffering from chronic *C. flaccidum* infection in northern Finland (i.e. Kolari and Juomukuru) during the natural sporulation period in mid-June in 2012 and 2013. Aeciospores used in artificial inoculations were also collected from these stands. Test-plant containers were filled with a mixture of peat and sand and planted adjacent to drainage ditches in order to maintain adequate moisture during the incubation period. Test plants received *C. flaccidum* spores under natural circumstances (i.e. air currents, etc.) and were placed some distance from the nearest sporulating tree. Exposed plants were incubated under field conditions and without supplementary irrigation for 5–6 weeks, after which they were transported to the laboratory (late July). In both study sites in 2012 and 2013, the prevailing climate was stable and supportive of plant growth and rust development (e.g. moist and warm) during the incubation period.

Disease assessment

Disease symptoms of Scots pine blister rust (*C. flaccidum*) and white-pine blister rust (*C. ribicola*) were assessed in terms of uredinia and telia formation on inoculated leaves (Gäumann, 1959) after 2–8 weeks of incubation. Attached leaves of whole

plants and detached leaves were examined using a stereomicroscope at 2-week intervals between late June and mid-August in 2012 and 2013. Leaves of test plants exposed to natural inoculum of *C. flaccidum* were checked for *Cronartium* uredinia and telia when terminating the experiments after 5–6 weeks. Individual samples of uredinia and telia were also checked using a light microscope. Representative leaf samples of different hosts carrying uredinia or telia were photographed using an Infinity 1 CMOS (Lumenera) stereomicroscope camera.

Molecular analysis of rust samples

A small amount of aeciospores from each source (17) was dusted into Eppendorf vials in the laboratory and stored at −20°C prior to molecular analysis. In addition, pieces of young uredinia with urediniospores or telia resulting from inoculations were cut from inoculated leaves of 15 species using a sterilized scalpel and transferred into Eppendorf vials after 2–6 weeks of incubation. A sample of mature telia was similarly collected from all species exposed to natural inoculum, depending on the number of available telia. In some cases, the analysis failed due to a small number of telia. Identification of the telia taken from six test species was conducted via PCR amplification and sequence analysis of the ITS2 region as described previously (Velmala *et al.*, 2013; Kaitera *et al.*, 2014) using GenBank as the reference database. The sequences were deposited in GenBank under accession numbers KJ959593–KJ959611, except sequences of *C. flaccidum* 2b, 2d and 3b, which were too short (<200 bp) to be accepted in the database (the sequences are available by request).

Results

Cronartium ribicola inoculation in the greenhouse and laboratory

Among the five species tested in the greenhouse, *Castilleja sulphurea* (Fig. 1a) and *Loasa tricolor* carried fruiting stages of *C. ribicola*, while *Myrica gale*, *Pedicularis lapponica* and *Impatiens glandulifera* did not (Table 2). Although leaf infection rates were <1%, spores from all three sources formed uredinia and telia on *C. sulphurea* after 2–4 weeks of incubation. Uredinia were the only fruiting stages that developed on *L. tricolor* on 1–10% of the tested leaves, and with spores from two of the three sources (Table 2) after 2 weeks of incubation.

In the laboratory, only *Ribes nigrum* 'Mortti' was infected when inoculated with spores from two of three

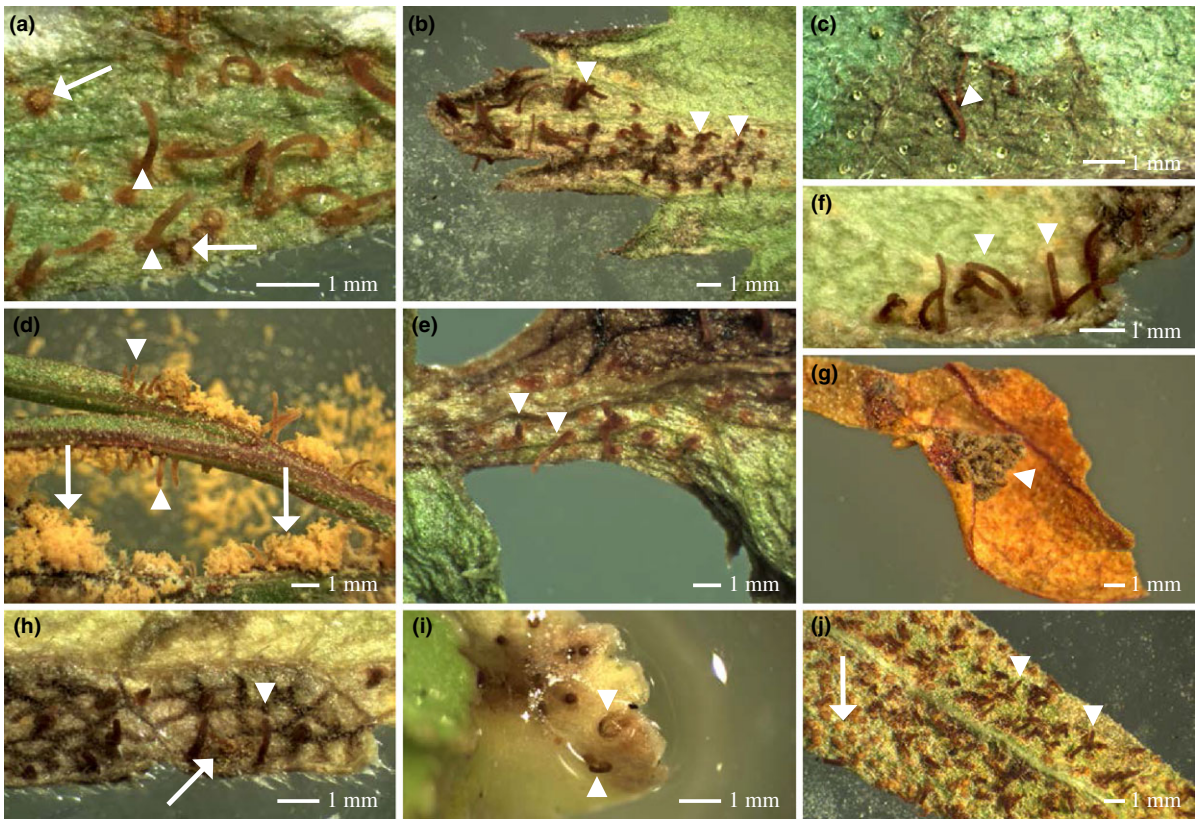

Figure 1 Uredinia (white arrows) and telia (white arrowheads) of *Cronartium ribicola* (a) and *Cronartium flaccidum* (b–j) on abaxial leaf surface of test plants inoculated in the laboratory (1) or greenhouse (2) in 2012–13; (a) uredinia and telia on *Castilleja sulphurea* (2); (b) telia on *Euphrasia brevipila* (2); (c) telia on *Myrica gale* (2); (d) uredinia and telia on *Paeonia tenuifolia* (1); (e) telia on *Pedicularis groenlandica* (2); (f) telia on *Rhinanthus aestivalis* (2); (g) telia on *Apocynum cannabinum* (1); (h) uredinia and telia on *Rhinanthus minor* (2); (i) telia on *Saxifraga hostii* (1); (j) uredinia and telia on *Castilleja miniata* (2).

Table 2 Formation of *Cronartium* uredinia and telia on inoculated leaves during the 8-week greenhouse incubation in 2012–13. Species that lacked uredinia and telia have been excluded

	Spore source																	
	C. flaccidum														*C. ribicola*			
	2012							2013							2012			
Species	2a	2b	2c	2d	2e	3a	3b	1	2	3	4	5	6	7	1	2	3	
Veronica grandis[a]	nt	nt	nt	nt	nt	nt	nt	– –	+ – 1		+ + 4	– –	nt	+ – 1	nt	nt	nt	
Veronica longifolia[a]	nt	nt	nt	nt	nt	nt	nt	– –	+ + 4	+ + 4	+ + 2	– –	nt	+ + 6	nt	nt	nt	
Veronica daurica[a]	nt	nt	nt	+ – 9[i]	nt	nt	– –	nt	nt	nt	– –	nt	nt	– –	nt	nt	nt	
Veronica krylovii[a]	nt	nt	nt	nt	nt	nt	nt	– –	+ + 8	+ + 3	– –	– –	– –	– –	nt	nt	nt	
Veronica incana[a]	nt	nt	nt	nt	nt	nt	nt	– –	+ + 20	+ + 4	+ + 4	– –	nt	– –	nt	nt	nt	
Pedicularis groenlandica[b]	nt	nt	nt	+ + 28	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	
Pedicularis sceptrum-carolinum[b]	nt	nt	nt	nt	nt	nt	nt	+ – 1	nt	nt	+ + 4	nt	nt	+ + 4	nt	nt	nt	
Euphrasia minima[b]	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	+ + 61	nt	nt	nt	nt	nt	nt	
Euphrasia brevipila[b]	nt	nt	nt	+ + 4	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	
Euphrasia officinalis[b]	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	+ + 77	nt	nt	nt	nt	nt	nt	
Rhinanthus minor[b]	nt	nt	nt	+ + 8	nt	nt	nt	nt	nt	nt	+ – 4	nt	nt	+ + 6	nt	nt	nt	
Rhinanthus aestivalis[b]	nt	nt	nt	+ + 4	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	
Castilleja sulphurea[b]	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	+ + 12	+ + 2	+ +	
Castilleja miniata[b]	nt	nt	nt	nt	nt	nt	nt	+ + 67	+ + 78	nt	+ + 100	– –	nt	+ + 60	nt	nt	nt	
Myrica gale[c]	nt	nt	nt	+ + 20	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	– –	nt	
Loasa tricolor[d]	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	+ – 1	+ – 6	– –	
Tropaeolum majus[e]	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	+ – 1	nt	nt	nt	
Vincetoxicum hirundinaria[f]	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	+ + 25	nt	nt	– –	nt	nt	nt	
Swertia fedtschenkoana[g]	nt	nt	nt	+ + 2	nt	nt	nt	nt	nt	nt	+ + 6	nt	nt	nt	nt	nt	nt	
Impatiens balsamina[h]	nt	nt	nt	nt	nt	nt	nt	+ + 8	+ + 49	+ + 40	+ + 58	– –	+ + 6	+ + 1	nt	nt	nt	

See Table 1 for spore codes.

Plant families: [a]Plantaginaceae, [b]Orobanchaceae, [c]Myricaceae, [d]Loasaceae, [e]Tropaeolaceae, [f]Apogynaceae, [g]Gentianaceae, [h]Balsaminaceae.

[i]The first column represents uredinia and the second telia formation. +, present; –, absent; nt, not tested. The number in the third column refers to number of leaves with uredia or telia.

sources, while uredinia developed after 2 weeks and telia after 6 weeks of incubation (Table 3). The 14 other test species, i.e. *Impatiens*, *Pedicularis*, *Gentiana*, *Saxifraga*, *Rhinanthus*, *Euphrasia*, *Veronica*, *Paeonia* and *Vincetoxicum*, did not support uredinia or form telia after inoculation.

Cronartium flaccidum inoculation in the greenhouse and laboratory in 2012

In the greenhouse, *C. flaccidum* formed uredinia or telia in seven of nine test species after 2–4 weeks of incubation. No fruiting stages developed on *I. glandulifera* and *Saxifraga rotundifolia*. Telia or uredinia developed on 3% of leaves in *M. gale* (Fig. 1c), 7% in *Pedicularis groenlandica* (Fig. 1e), 4% in *Veronica daurica*, 3% in *Swertia fedtschenkoana*, 3% in *Rhinanthus minor* (Fig. 1h), 4% in *Rhinanthus aestivalis* (Fig. 1f) and 2% in *Euphrasia brevipila* (Fig. 1b; Table 2). In most species, fruiting stages developed only on the abaxial leaf surface, but telia occurred frequently on both abaxial and adaxial leaf surfaces in *E. brevipila* and *P. groenlandica*. This is the first time that *C. flaccidum* has been reported to fruit and sporulate on *P. groenlandica*, *R. minor*, *R. aestivalis* and *E. brevipila*.

In the laboratory, uredinia or telia developed on five of 15 test species. Detached leaves of *Impatiens nolitangere*, *Gentiana septemfida*, *R. nigrum* 'Mortti', *R. minor*, *Saxifraga paniculata*, *Euphrasia stricta*, *Veronica gentianoides*, *Gentiana asclepiadea*, *G. decumbens* and *Wulfenia carianthiaca* lacked fruiting stages, while uredinia or telia developed on leaves of *I. glandulifera*, *Pedicularis sceptrum-carolinum*, *Saxifraga hostii* (Fig. 1i), *Paeonia tenuifolia* (Fig. 1d) and *V. hirundinaria* after 2–8 weeks of incubation (Table 3). Spores from all seven sources formed abundant uredinia and six of them formed abundant telia on *P. tenuifolia*. Uredinia also developed occasionally on *P. sceptrum-carolinum* (three sources) and *I. glandulifera* (four sources). Single uredinia and telia also developed sporadically (one source) on *S. hostii* and *V. hirundinaria* (Table 3).

Table 3 Formation of *Cronartium* uredinia and telia on inoculated leaves after an 8-week laboratory incubation in 2012–13

	Spore source																	
	C. flaccidum														*C. ribicola*			
	2012							2013							2012			
Species	2a	2b	2c	2d	2e	3a	3b	1	2	3	4	5	6	7	1	2	3	
Ribes nigrum 'Mortti'[a]	– –	– –	– –	– –	– –	– –	– –	nt	nt	nt	nt	nt	nt	nt	nt	+ +	+ +	
Veronica grandis[b]	nt	nt	nt	nt	nt	nt	nt	+ +	+ +	+ +	+ +	– –	+ +	+ –	nt	nt	nt	
Veronica longifolia[b]	nt	nt	nt	nt	nt	nt	nt	+ +	+ +	+ +	+ +	– –	+ +	+ +	nt	nt	nt	
Veronica daurica[b]	nt	nt	nt	nt	nt	nt	nt	– –	– –	+ –	– –	– –	– –	– –	nt	nt	nt	
Veronica krylovii[b]	nt	nt	nt	nt	nt	nt	nt	– –	+ +	– –	+ +	– –	– –	– –	nt	nt	nt	
Physalis alkekengi[c]	nt	nt	nt	nt	nt	nt	nt	– –	– –	– –	+ –	– –	– –	– –	nt	nt	nt	
Nicotiana rustica[c]	nt	nt	nt	nt	nt	nt	nt	– –	– –	– –	+ +	– –	– –	+ –	nt	nt	nt	
Hyoscyamus niger[c]	nt	nt	nt	nt	nt	nt	nt	– –	– –	– –	– –	– –	+ –	+ –	nt	nt	nt	
Paeonia tenuifolia[d]	+ +	+ –	+ +	+ +	+ +	+ +	+ +	nt	nt	nt	nt	nt	nt	nt	nt	– –	– –	
Pedicularis sceptrum-carolinum[e]	– –	– –	– –	+ –	+ –	+ –	– –	nt	nt	nt	nt	nt	nt	nt	nt	– –	– –	
Castilleja miniata[e]	nt	nt	nt	nt	nt	nt	nt	+ +	+ +	+ +	+ +	– –	+ +	+ +	nt	nt	nt	
Saxifraga hostii[f]	– –	– –	– –	– –	– –	+ +	– –	nt	nt	nt	nt	nt	nt	nt	nt	– –	– –	
Myrica gale[g]	nt	nt	nt	nt	nt	nt	nt	– –	+ –	+ –	+ –	– –	– –	+ –	nt	nt	nt	
Vincetoxicum hirundinaria[h]	+ +	– –	– –	– –	– –	– –	– –	+ +	+ +	+ +	+ +	– –	+ +	+ +	nt	– –	– –	
Apocynum cannabinum[h]	nt	nt	nt	nt	nt	nt	nt	– –	– –	– –	– –	– –	– –	+ +	nt	nt	nt	
Swertia fedtschenkoana[i]	nt	nt	nt	nt	nt	nt	nt	– –	+ +	+ –	+ +	– –	+ +	+ +	nt	nt	nt	
Impatiens balsamina[j]	nt	nt	nt	nt	nt	nt	nt	+ +	+ +	+ +	+ +	– –	+ +	+ +	nt	nt	nt	
Impatiens glandulifera[j]	+ –	+ –	+ –	– –	– –	– –	+ +	nt	nt	nt	nt	nt	nt	nt	nt	– –	– –	

Species lacking uredinia and telia have been excluded. The first column represents uredinia and the second telia formation. +, present; –, absent; nt, not tested.

Plant families: [a]Grossulariaceae, [b]Plantaginaceae, [c]Solanaceae, [d]Paeoniaceae, [e]Orobanchaceae, [f]Saxifragaceae, [g]Myricaceae, [h]Apogynaceae, [i]Gentianaceae, [j]Balsaminaceae. See Table 1 for spore codes.

Cronartium flaccidum inoculation in the greenhouse and laboratory in 2013

Among the 25 species tested in the greenhouse, uredinia or telia of *C. flaccidum* were found on 13 of them after 2–8 weeks of incubation (Table 2). The most susceptible test species was *Castilleja miniata*, in which 72–82% of leaves had uredinia or telia (Fig. 1j) after inoculation with spores from four of five sources. Spores from source 5 did not infect any of the test leaves in the greenhouse or laboratory (Tables 2 & 3). This source was probably a homozygotic form of *C. flaccidum*, i.e. the autoecious *Peridermium pini*. Another heavily infected species was *Euphrasia officinalis*, which supported both uredinia and telia (Fig. 2b) on 79% of inoculated leaves. Three spore sources produced uredinia or telia on *P. sceptrum-carolinum* (50–80% of infected leaves among sources; Fig. 2e), *Veronica grandis* (0–9%) and *V. incana* (0–61%; Fig. 2h), and four sources (0–19%) produced uredinia or telia on *Veronica longifolia* and two sources (0–11%) on *Veronica krylovii* (Fig. 2i). Among these species, uredinia and telia were most abundant on leaves on *V. longifolia*. In addition, uredinia or telia developed variably on *V. hirundinaria* (0–10%), *S. fedtschenkoana* (11%; Fig. 2f), *Impatiens balsamina* (1–45%), *R. minor* (18–30%), *Euphrasia minima* (25%; Fig. 2a) and *Tropaeolum majus* (17%). The remaining test species (i.e. *Saxifraga rhomboidea*, *Linaria vulgaris*, *V. daurica*, *Apocynum cannabinum*, *Mitella diphylla*, *Verbascum phoenicum*, *Veronica officinalis*, *Scrophularia nodosa*, *Verbascum thapsus*, *Capsicum chinense*, *Solanum melongena* and *Physalis alkekengi*) supported neither uredinia nor telia. Observations of the development of *C. flaccidum* uredinia or telia on *C. miniata*, *E. officinalis*, *E. minima*, *V. grandis*, *V. krylovii* and *V. incana* represent the first reports of these species serving as alternative hosts.

In the laboratory, 13 of 29 inoculated test species bore uredinia or telia of *C. flaccidum* after incubation (Table 3). Uredinia or telia developed on *C. miniata*, *V. grandis* (Fig. 2g), *V. longifolia* (Fig. 2j), *V. hirundinaria* (Fig. 2k) and *I. balsamina* (Fig. 2d) when inoculated with spores from six sources, and on *S. fedtschenkoana* when inoculated with spores from five sources (Table 3). Fruiting was more sporadic, with spores from one or two sources forming single uredinia or telia, on *Veronica krylovii*, *Nicotiana rustica*, *Hyoscyamus niger* (Fig. 2c), *V. daurica*, *P. alkekengi* and *A. cannabinum* (Fig. 1g). In addition, spores from four sources formed only single uredinia on *M. gale*. Uredinia and telia did not develop on *S. rhomboidea*, *M. diphylla*, *Chrysosplenium alternifolium*, *Heuchera sanguinea*, *Astilbe* Chinensis-Group, *Astilbe* Arendsii-Group, *S. nodosa*, *Verbascum nigrum*, *V. thapsus*, *Digitalis grandiflora*, *Solanum lycopersicum*, *V. phoenicum*, *V. officinalis*, *C. chinense*, *S. melongena* and *Astilboides tabularis*.

Exposure of test plants to natural inoculum of *C. flaccidum* in the field

Three of the 13 test species that were exposed to natural *C. flaccidum* in 2012 had uredinia or telia after 5 weeks

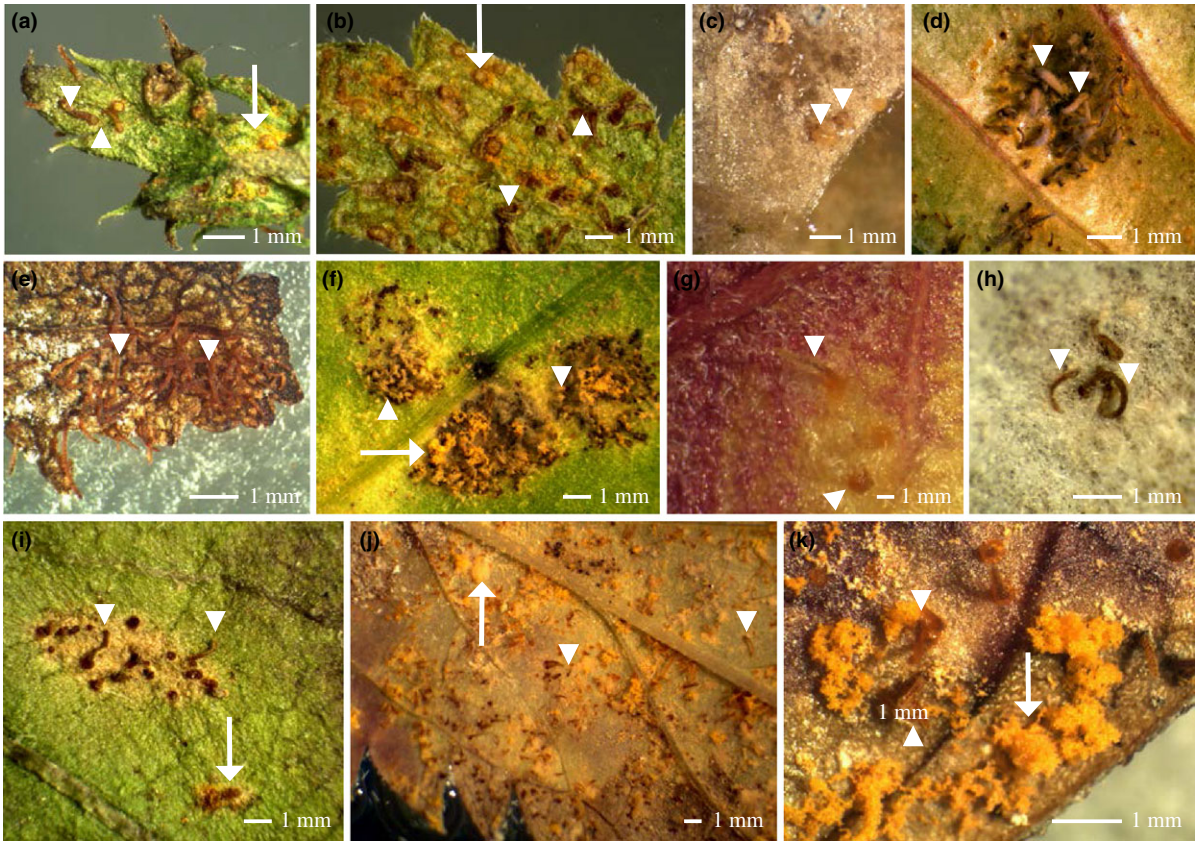

Figure 2 Uredinia (white arrows) and telia (white arrowheads) of *Cronartium flaccidum* on abaxial leaf surface of test plants inoculated in the laboratory (1) or greenhouse (2) in 2012–13; (a) uredinia and telia on *Euphrasia minima* (2); (b) uredinia and telia on *Euphrasia officinalis* (2); (c) telia on *Hyoscyamus niger* (1); (d) telia on *Impatiens balsamina* (1); (e) telia on *Pedicularis sceptrum-carolinum* (2); (f) uredinia and telia on *Swertia fedtschenkoana* (2); (g) telia on *Veronica grandis* (1); (h) telia on *Veronica incana* (2); (i) uredinia and telia on *Veronica krylovii* (2); (j) uredinia and telia on *Veronica longifolia* (1); (k) uredinia and telia on *Vincetoxicum hirundinaria* (1).

of incubation in the field (Table 4). Among these, 5% of leaves in *V. hirundinaria* carried uredinia or telia at both sites; 1–6% for *V. daurica* and 0–1% for *V. longifolia*. Leaves of *I. glandulifera*, *S. fedtschenkoana*, *T. majus*, *S. rotundifolia*, *P. sceptrum-carolinum*, *L. vulgaris*, *Pedicularis palustris* subsp. *palustris*, *V. phoenicum*, *L. tricolor* and *M. gale* lacked uredinia or telia after incubation in the field.

Of the test species exposed in 2013, seven of 15 bore telia of *C. flaccidum* (Table 4). *Castilleja miniata* was the most heavily infected species, where 14% of leaves had telia after 6 weeks of incubation (Fig. 3a). Of the other test species infected in both locations, 2–5% of leaves in *V. hirundinaria* (Fig. 3g), 1% of *I. balsamina* (Fig. 3c), 0·4–1% of *E. stricta* (Fig. 3b) and 0·3–0·5% of *V. longifolia* (Fig. 3f) developed uredinia or telia. Two percent of leaves of *V. daurica* (Fig. 3e) were infected at Kolari, and 0·2% of *M. gale* (Fig. 3d) in Juomukuru. *Saxifraga rhomboidea*, *L. vulgaris*, *R. minor*, *S. fedtschenkoana*, *V. phoenicum*, *M. diphylla*, *M. gale*, *V. thapsus* and *V. officinalis* remained unaffected when exposed to natural inoculum.

Molecular identification of rusts

Molecular identification of the aeciospores showed that all 14 spore sources of *C. flaccidum* were 99–100% similar to strains of *C. flaccidum* and *P. pini* in GenBank. Likewise, spores from three sources of *C. ribicola* were 100% identical to strains of *C. ribicola* in GenBank.

Urediniospores and telia developed on test plants after inoculation with *C. flaccidum* in the greenhouse or in the laboratory in 2012 and were 100% identical to strains of *C. flaccidum* in GenBank on *R. aestivalis* and *M. gale*. Identification was confirmed for seven uredinial samples on *C. miniata* (inoculation with spore sources nos. 1–4 and 7), four uredinial and telial samples on *V. hirundinaria* (nos. 1, 2, 4 and 7), three uredinial and telial samples on *I. balsamina* (nos. 2, 3 and 7), three uredinial and telial samples on *V. longifolia* (nos. 2, 3 and 7), three uredinial and telial samples on *S. fedtschenkoana* (nos. 2 and 6), and one uredinial sample on *E. officinalis* and *E. minima* (spore source no. 4) after 2–4 weeks of incubation in 2013. In addition, telial samples from *V. grandis*, *V. krylovii*, *V. incana*, *A. cannabinum* and

Table 4 Formation of uredinia and telia of *Cronartium flaccidum* on inoculated leaves exposed to natural inoculum for 5–6 weeks in 2012–13. Species without fruitbodies have been excluded

	Location			
	Kolari		Juomukuru	
Species	2012	2013	2012	2013
Veronica longifolia[a]	+ – 3[f]	+ + 2	– –	– + 4
Veronica daurica[a]	+ + 8	+ + 2	+ + 2	– –
Euphrasia stricta[b]	nt	+ + 4	nt	– + 2
Castilleja miniata[b]	nt	+ + 181	nt	nt
Myrica gale[c]	– –	– –	– –	– + 1
Vincetoxicum hirundinaria[d]	+ + 56	+ + 26	+ + 23	+ + 13
Impatiens balsamina[e]	nt	+ + 6	nt	– –

Plant families: [a]Plantaginaceae, [b]Orobanchaceae, [c]Myricaceae, [d]Apocynaceae, [e]Balsaminaceae.
[f]The first column represents uredinia and the second telia formation. +, present; –, absent; nt, not tested. The number in the third column refers to the number of leaves with uredinia or telia.

P. sceptrum-carolinum were identified after 8 weeks of incubation.

Telia that developed on test plants after exposure to natural inoculum in the field were 100% identical to *C. flaccidum* in GenBank on *V. hirundinaria* in 2012 and on *C. miniata*, *V. longifolia*, *I. balsamina*, *E. stricta* and *V. daurica* in 2013.

Discussion

In this study, the hemiparasite *C. sulphurea* (Orobanchaceae) was the most susceptible species to *C. ribicola*, although the infection rate was low. *Castilleja* are known alternate hosts for *C. ribicola* in North America (McDonald *et al.*, 2006; Mulvey & Hansen, 2011). Results presented here show that European strains of *C. ribicola* are pathogenic on *Castilleja*. *Cronartium ribicola* also formed uredinia on *L. tricolor* (Loasaceae) on live plants in the greenhouse. In earlier studies, species of *Loasa* were shown to support sporulation of *C. ribicola* (Kaitera & Hiltunen, 2012; Kaitera *et al.*, 2012). Although *Loasa* are evidently more resistant to *C. ribicola* than *Ribes* (Grossulariaceae) – the main wild alternate hosts for *C. ribicola* and most susceptible species (Zampino, 2010; this study) – individual species of Loasaceae, e.g. *Mentzelia lindleyi*, are susceptible to the rust (Kaitera & Hiltunen, 2012). On the other hand, tested species of *Impatiens*, *Pedicularis*, *Gentiana*, *Saxifraga*, *Rhinanthus*, *Euphrasia*, *Veronica*, *Paeonia*, *Myrica* and *Vincetoxicum* were fully resistant to *C. ribicola* indicating a relatively narrow alternate host range that may be specialized to members of Grossulariaceae. This has been noted in earlier inoculation studies with *C. ribicola* (Kaitera & Hiltunen, 2011, 2012; Kaitera *et al.*, 2012). While Kaitera & Hiltunen (2011) found *P. palustris* subsp. *palustris* to be susceptible, the present study inoculated two species of *Pedicularis* with *C. ribicola* and found no signs of infection at the end of the incubation period, suggesting resistance/susceptibility is variable in this genus. In North America, species of *Pedicularis* are known to be alternate hosts for *C. ribicola* (McDonald *et al.*, 2006; Mulvey & Hansen, 2011) in natural settings.

Although a wide range of species from different plant families was infected, *C. flaccidum* sporulated most frequently on hemiparasitic live plants (Orobanchaceae).

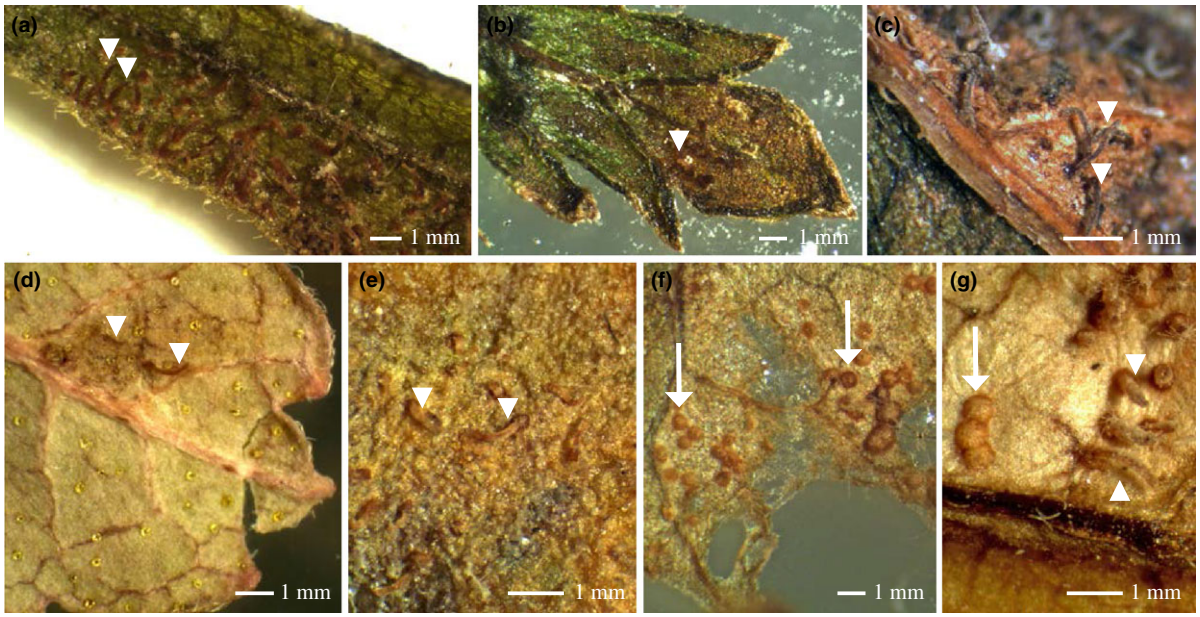

Figure 3 Uredinia (white arrows) and telia (white arrowheads) of *Cronartium flaccidum* on abaxial leaf surface of test plants exposed to natural inoculum of *C. flaccidum* in 2012–13; (a) telia on *Castilleja miniata*; (b) telia on *Euphrasia stricta*; (c) telia on *Impatiens balsamina*; (d) telia on *Myrica gale*; (e) telia on *Veronica daurica*; (f) uredinia on *Veronica longifolia*; (g) uredinia and telia on *Vincetoxicum hirundinaria*.

Hemiparasitic plants in this family have roots that grow inside the roots of host plants, from which they draw resources (Kalela, 1963), although they are capable of photosynthesis. The hemiparasites of *Pedicularis*, *Rhinanthus*, *Euphrasia*, *Bartsia* and *Melampyrum* show little host specificity and can parasitize various species of Poaceae, Cyperaceae and even other hemiparasites (Jalas, 1980). All of the *Pedicularis*, *Rhinanthus* and *Euphrasia* species tested were successfully infected in all greenhouse experiments. Sporulation was generally lower on detached leaves of hemiparasites probably due to slow loss of vitality, e.g. *Rhinanthus* and *Euphrasia*. Among the genera of Orobanchaceae, several species of *Pedicularis* (Klebahn, 1914; Kaitera & Hiltunen, 2011, 2012) and *Euphrasia* (Gäumann, 1959; Kaitera & Hiltunen, 2012) have been reported as alternate hosts for *C. flaccidum* elsewhere. In this study, all species of *Euphrasia* were highly susceptible to *C. flaccidum*, as was *E. stricta* (also reported by Kaitera & Hiltunen, 2012; Kaitera *et al.*, 2012). Different varieties of *E. stricta* grow commonly throughout Finland in gardens and alongside roads and rivers (Hämet-Ahti *et al.*, 1998), and thus *E. stricta* has a clear potential to spread *C. flaccidum*. *Pedicularis* spp. were also moderately susceptible with some variation among species. *Pedicularis sceptrum-carolinum* was moderately infected in this study, while it was resistant or only slightly susceptible in earlier studies (Kaitera & Hiltunen, 2011, 2012; Kaitera *et al.*, 2012). *Pedicularis* spp. have become rare and occur sporadically on river banks, lake shores and coastal locations (Hämet-Ahti *et al.*, 1998). Attached leaves of live *R. minor* proved to be slightly susceptible, while detached leaves were fully resistant to *C. flaccidum*, which agrees with results of earlier inoculation trials (Kaitera *et al.*, 2012). This species grows commonly throughout Finland along roadsides and field edges and on dry grassland (Hämet-Ahti *et al.*, 1998). Surprisingly, both attached and detached leaves of *C. miniata*, also a member of Orobanchaceae, proved to be highly susceptible to *C. flaccidum*, where almost all the inoculated leaves were totally covered with uredinia and telia after 2–4 weeks of incubation. Susceptibility of *Castilleja* to *C. flaccidum* had never been tested before, although species of this genus are reported as alternate hosts for *C. ribicola* in North America (Hiratsuka & Maryama, 1976; McDonald *et al.*, 2006; Mulvey & Hansen, 2011). Besides *C. flaccidum* (in this study) and *C. ribicola*, *C. miniata* is also an alternate host for *Cronartium coleosporioides*, which also sporulates on hemiparasites such as *Rhinanthus* and *Pedicularis* (Ziller, 1974). The authors know of no reports concerning the artificial or natural sporulation of *C. flaccidum* on the hemiparasites *R. minor*, *R. aestivalis*, *P. groenlandica*, *E. brevipila*, *E. minima*, *E. officinalis* and *C. miniata*.

Cronartium flaccidum also sporulated on several species in other plant families. The only tested *Paeonia*, *P. tenuifolia*, was highly susceptible to *C. flaccidum* as expected, whereas other species of this genus are variably resistant to *C. flaccidum* (Klebahn, 1901; Gäumann, 1959; Roll-Hansen, 1973; Kaitera *et al.*, 1999, 2012). The only species of Tropaeolaceae tested, *T. majus*, was also infected. Several species of *Tropaeolum* are common garden plants and have previously been reported as alternate hosts for *C. flaccidum* (Hylander *et al.*, 1953; Gäumann, 1959; Kaitera & Hiltunen, 2012; Kaitera *et al.*, 2012). The only tested member of Myricaceae, *M. gale*, was also infected but poor development and low frequency of fruiting stages suggest this species to be only slightly susceptible to *C. flaccidum*. A similar response was observed by Kaitera *et al.* (2012). *Myrica gale* is also known as an alternate host for *Cronartium comptoniae* in North America (Ziller, 1974). This plant species occurs in southern and central Finland mainly in coastal and lake-shore habitats, and rarely on peatlands (Hämet-Ahti *et al.*, 1998), where it may spread *C. flaccidum*.

Among the species of Plantaginaceae tested, only those in *Veronica* were infected by *C. flaccidum*. *Veronica longifolia* was the only moderately susceptible species among *Veronica*, while *V. krylovii*, *V. daurica*, *V. grandis* and *V. incana* were only slightly susceptible. In addition, *V. officinalis* and *V. gentianoides* were completely resistant, emphasizing the variation in rust resistance among species within this genus. Among species of *Veronica*, *V. officinalis* is distributed widely in southern and central Finland and *V. longifolia* occurs throughout Finland (Hämet-Ahti *et al.*, 1998). *Veronica officinalis* grows in upland forests with dry grass–herb vegetation, grazing grounds, open-cut areas, dry fields and rocks, whereas *V. longifolia* grows especially well in coastal habitats, lake shores and river banks. Earlier, *V. daurica* (Kaitera *et al.*, 2012) and *V. longifolia* have been reported as alternate hosts for *C. flaccidum*, and thus, *V. krylovii*, *V. grandis* and *V. incana* are reported here as new alternate hosts for the rust.

In line with earlier results (Kaitera & Hiltunen, 2012; Kaitera *et al.*, 2012), each of the species of *Gentiana* (Gentianaceae) tested here were fully resistant to *C. flaccidum*. However, according to Gäumann (1959), a form of *C. flaccidum* specific to *Gentiana* occurs in central Europe. Although northern spores of *C. flaccidum* infect a wide diversity of species in several plant families, *Gentiana* spp. are evidently exceptions to that. However, a species of *Swertia* (Gentianaceae) was also infected. *Swertia* have previously been reported as alternate hosts for *C. flaccidum* (Gäumann, 1959; Kaitera *et al.*, 2012).

Most of the Solanaceae species tested proved to be resistant to *C. flaccidum*, but three species of *Physalis*, *Nicotiana* and *Hyoscyamus* supported weak sporulation. Therefore, Solanaceae also contains alternate hosts for the rust, and Gäumann (1959) listed *Schizanthus* as an alternate host for *C. flaccidum*. Similarly, the majority of the species of Saxifragaceae tested were either fully resistant or very slightly susceptible to *C. flaccidum*. Within *Saxifraga*, only a few leaves of *S. hostii* supported uredinia and telia formation, while three others were resistant. In Finland, species of *Saxifraga* are rather rare and grow in nutrient-rich peatlands and open fields of the north. As such, it is unlikely members of this genus are a signifi-

cant vector for the spread of *C. flaccidum*. Kaitera *et al.* (2012) have previously reported the slight susceptibility of *S. hostii* and two other *Saxifraga* to *C. flaccidum*.

Impatiens spp. were reported as alternate hosts for *C. flaccidum* in the early 1900s (Klebahn, 1905). Within the genus *Impatiens* (Balsaminaceae), *I. balsamina* was highly susceptible and *I. glandulifera* highly resistant. This supports the results of recent inoculation trials with these two species (Kaitera & Hiltunen, 2012; Kaitera *et al.*, 2012). Although ornamental varieties of *I. glandulifera* have escaped gardens and can be found in natural settings, it is unlikely that they provide an effective route through which *C. flaccidum* might spread to natural forests. On the other hand, *I. balsamina* is clearly capable of spreading *C. flaccidum* from garden to garden, where the rust might access new alternate hosts.

Similar to earlier results (Kaitera & Hiltunen, 2011, 2012; Kaitera *et al.*, 2012), the known alternate host of Apocynaceae, *V. hirundinaria*, supported sporulation in all experiments here. Another species of this family, *A. cannabinum*, was also weakly susceptible showing rust sporulation on single leaves, and represents a new host record for *C. flaccidum*. Although Apocynaceae contains several species of varying susceptibility, there is no evidence yet reported that species other than *V. hirundinaria* might spread the rust efficiently. The only species of Scrophulariaceae tested, *S. nodosa*, was not infected in these trials, although this species was listed as an alternate host for *C. flaccidum* by Gäumann (1959). Previously, *Nemesia* spp. (Scrophulariaceae) have been reported as alternate hosts for *C. flaccidum* (Hylander *et al.*, 1953). In recent inoculation trials, *Nemesia* spp. were weakly susceptible to *C. flaccidum* (Kaitera & Hiltunen, 2012). Therefore, no species in this family are known to be highly susceptible to *C. flaccidum*.

In the field, several species that are widely distributed in Finland were infected by natural inoculum of *C. flaccidum* with a relatively low percentage of infected leaves. This was confirmed by genetic identification. Of the known alternate hosts of *C. flaccidum*, *V. hirundinaria* was moderately infected. Of those species infected in the greenhouse, *E. stricta*, *V. longifolia*, *V. daurica*, *M. gale* and *I. balsamina* were only slightly infected. Nevertheless, these species were shown to support sporulation in natural forests and may spread *C. flaccidum* from their specific biotopes to surrounding forests. Finally, *C. miniata* was severely infected in artificial inoculations as well as under a natural spore load of *C. flaccidum*. Therefore, *C. miniata* can act as a vector and spread *C. flaccidum* to two-needle pines, if this rust were ever to be introduced to North America.

In the future, the ability of *C. ribicola* to infect pines with inocula from the new alternate hosts should be studied. In particular, species in the genus *Mentzelia* (Loasaceae) should be studied in detail. Another essential target of future study for both *C. flaccidum* and *C. ribicola* is their frequency of sporulation on the new alternate hosts in their natural habitats.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. Number of plant leaves inoculated in the greenhouse in 2012–13.

Table S2. Number of plant leaves inoculated in the laboratory 2012–13. See Table 1 for spore and Table S1 for plant family codes.

Table S3. Number of plant leaves exposed to natural inoculum of *C. flaccidum* in two locations in northern Finland in 2012–13. See Table S1 for plant family codes.