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Tracking the Footsteps of an Invasive Plant-Pathogen: Intercontinental Phylogeographic Structure of the White-pine-blister-rust Fungus, *Cronartium ribicola*

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Abstract – Presently, little is known about the worldwide genetic structure, diversity, or evolutionary relationships of the white-pine-blister-rust fungus, *Cronartium ribicola*. A collaborative international effort is underway to determine the phylogeographic relationships among Asian, European, and North American sources of *C. ribicola* and closely related taxa. Here, we present preliminary information on phylogenetic relationships among selected Eurasian and North American populations of *C. ribicola* using DNA sequences from four nuclear loci totaling over 2,100 bp. Geographic regions included eastern and western North America, northern Germany, Korea, Japan, and northeastern China. Phylogenetic and network analyses suggest *C. ribicola* comprises at least three distinct clades. Isolates from Korea and China formed one clade, and Japanese isolates formed a second clade that was intermediate the third clade, which comprised isolates from USA and Germany. Identifying the evolutionary relationships and potential origin(s) of *C. ribicola* that spread through Eurasia and North America, and determining the phylogenetic relationships of its hosts are critical toward understanding this pathogen's native ecology. Such information will help evaluate risks of cryptic introductions, contribute to the development of biological controls, identify sources of host resistance and develop appropriate regulatory practices.

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Introduction

Invasive plant pathogens pose a worldwide threat to natural and agricultural ecosystems, yet little is known about their origins or how they interact with other biotic and abiotic components of their native ecosystems. Phylogenetic and population genetic research is a critical first step in mitigating impacts of invasive plant pathogens through better understanding of their biology, epidemiology and ecology. This research could elucidate potential sources of invasive plant pathogens, which can improve measures to prevent introductions, identify potential biological control agents, and determine sources of host resistance.

Cronartium ribicola, the white-pine-blister-rust pathogen, was first reported in eastern Europe during the 1850s before spreading to western Europe and subsequently to North America in the late 1800's to early 1900's (Spaulding 1922, McDonald and Hoff 2001). Before these records, the occurrence and movement of blister rust is unknown, but origins of eastern Asia have been speculated (Leppik 1970). Today outside of North America, incidence of *C. ribicola* has been mainly found in plantations of introduced eastern white pine (*Pinus strobus*) in northern Europe. In Asia, white pine blister rust has been reported on Korean white pine (*P. koraiensis*) in the Korean peninsula and northeastern China, Japanese stone pine (*P. pumila*) in Japan, eastern Siberia and Kamchatka, Siberian pine (*P. sibirica*) in northcentral Asia and Siberia, and Himalayan blue pine (*P. wallichiana*) and Chinese white pine

(*P. armandii*) in the Himalayas and central China (reviewed in McDonald *et al.* 2005). Varying alternate hosts have been reported among these regions, including 1) predominately *Pedicularis* spp. in South Korea, hereafter referred to as Korea (La and Yi 1995, Stephan and Hyun 1983); 2) *Pedicularis*, *Castilleja* and *Ribes* spp. in Siberia (Azbukina 1995, Kakishima *et al.* 1995); and 3) *Ribes* spp. in northern India (Bagshee 1950). Across much of Asia, blister rust disease incidence is usually minor, suggesting this pathosystem is highly coevolved in these regions.

In this study, we used four nuclear loci, over 2,100 bp of DNA sequence data, to elucidate the genetic relationships among *C. ribicola* that occurs in different continents and regions. We examined isolates collected from *Pedicularis* in Japan, *P. koraiensis* in Korea and China, *Ribes* from Germany, *P. strobus* in the eastern USA, and *P. albicaulis* in the western USA. The objectives of this study are to assess the phylogenetic relationships among *C. ribicola* from diverse geographic regions, and evaluate the potential origin(s) of populations that are extant in North America and western Europe.

Material and Methods

Rust isolates were collected either from the *Ribes* / *Pedicularis* (telial) or five-needled, white pine (aecial) hosts, placed in desiccant for shipment following the permit guidelines of the USDA Animal and Plant Health Inspection Service. DNA was extracted from aeciospores following the protocol of Zambino (2002). For telial host infections, three to four telial columns were placed directly into 50 μL of Lyse-N-Go™ reagent (Thermo Scientific) and processed according to the manufacturer's thermocycling protocol. PCR reagents were prepared with the following concentrations: 0.2 mM dNTPs, 4 mM $MgCl_2$, 0.5 μM of DNA primers, 10X PCR buffer, 1 U DNA polymerase, and 1 μL of the DNA template (i.e., Lyse-N-Go mixture™ diluted 10X with dH_2O) in a total volume of 30 μL. PCR was performed using fungal specific primers 5.8SR and LR7R for the large subunit (LSU, 28S) of the ribosomal DNA (Moncalvo *et al.* 2000) with the following thermocycling parameters: 95°C for 1 min and 35 cycles of 94°C for 30 sec, 49°C for 45 sec and 72°C for 1.5 min. A nested primer (5'-TTAAAAAGCAAAGGAGTG) was then used for the 5' sequencing reaction. Previously developed primers and thermocycling reactions were used for the other three loci: Dcon03, Dcon10, Dcon57 that showed highest homology to elongation factor 1-α, glutamine synthetase, and cytochrome P-450 monooxygenase, respectively (Joly *et al.* 2005). DNA sequencing was performed at the University of Wisconsin Biotechnology Center with an Applied Biosystems 3700 automated sequencer using both forward and reverse primers.

Sequence alignments were compiled with SEQUENCHER ver. 4.5. A partition-homogeneity test was performed with PAUP* 4.0b10 (Sinauer Publishing, Sunderland, MA) using 1,000 heuristic searches and TBR branch swapping to evaluate the concordance the four loci. Statistical parsimony

Table 1. Geographic location and host information of *Cronartium ribicola* isolates in this study

Sample name	Location	Host	Longitude	Latitude
Ch1	Jiaohe, China	*Pinus koraiensis*	127°28	42°10
Ch12	Jiaohe, China	*P. koraiensis*	127°28	42°10
Ch3	Jiaohe, China	*P. koraiensis*	127°28	42°10
Ger2	Walsieverdorf, Germany	*Ribes nigrum*	14°03	52°32
Ger6	Walsieverdorf, Germany	*R. nigrum*	14°03	52°32
JJ127_56	Jinbu, Korea	*P. koraiensis*	128°31	37°29
JJ127_6	Jinbu, Korea	*P. koraiensis*	128°31	37°29
LJ13	Jinbu, Korea	*P. koraiensis*	128°06	37°29
LJ14	Jinbu, Korea	*P. koraiensis*	128°06	37°29
Mt_K_B	Mt. Kisokomagatake, Japan	*Pedicularis* spp.	137°48	35°46
Mt_K_C2	Mt. Kisokomagatake, Japan	*Pedicularis* spp.	137°48	35°46
Mt_N_A	Mt. Norikura, Japan	*Pedicularis* spp.	137°48	35°46
Mt_N_D	Mt. Norikura, Japan	*Pedicularis* spp.	137°48	35°46
MN2-1B	Minnesota, USA	*P. strobus*	-95°33	44°40
NH1-1B	New Hampshire, USA	*P. strobus*	71°57	44°02
RN32	Idaho, USA	*P. albicaulis*	-116°34	48°37
WI4-1B	Wisconsin, USA	*P. strobus*	-88°51	45°33
*Cronartium occidentale**	Idaho, USA	*P. monophylla*	-113°43	43°05

* = outgroup

haplotype network analysis was conducted with TCS 1.21 (Clement *et al.* 2000). The interspecific outgroup, *Cronartium occidentale*, was excluded in this analysis. Gaps were treated as a 5th character state. Maximum parsimony phylogenetic analysis was performed with PAUP* using the same parameters as above, with gaps coded as missing and using the MULPARS option. Node support was evaluated with 1,000 bootstrap replicates with random addition. Model Test 3.7 (Posada and Crandall 1998) was used to determine the most suitable nucleotide substitution model using Akaike information criterion (AIC) for the combined dataset. The best-fit model was the general time reversal (GTR) model. Bayesian phylogenetic analysis was performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) using a Markov chain Monte Carlo search of 100,000 generations. Maximum likelihood was performed with PAUP using the same search parameters as parsimony analysis. Node support was evaluated with 100 bootstrap replicates using the full heuristic search.

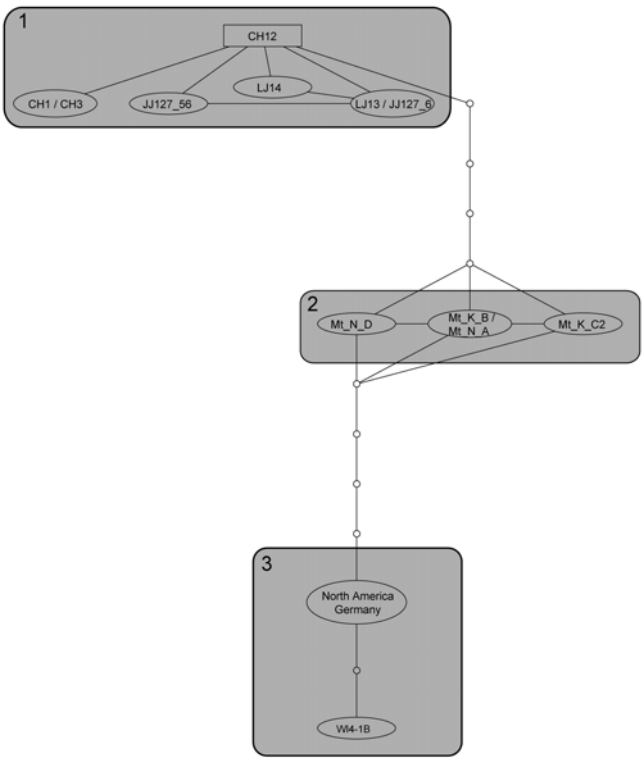

Figure 1. A statistical parsimony haplotype network analysis of 17 *Cronartium ribicola* isolates. The lines connecting isolates represent one mutation event and the small circles are unsampled or extinct haplotypes. The gray boxes signify the major groups in the network: 1) eastern Asia (Korea/China), 2) Japan and 3) North America/Germany*. Isolates of *C. ribicola* are described in Table 1. *The North America/Germany node is represented by the following isolates: Ger2, Ger6, NH1-1B, RN32 and MN2-1B.

Results and Discussion

DNA sequencing produced 395 characters for Dcon03, 441 characters for Dcon10, and 271 characters for Dcon57 with three, seven, and five intraspecific, single-nucleotide polymorphisms (SNPs), respectively. A portion of the LSU totaled 1,039 characters with nine SNPs. The partition homogeneity test results showed that all four loci could be combined (p = 0.35). The combined DNA sequence data totaled 2,146 base pairs, with 24 polymorphic sites.

The network analysis recovered 10 haplotypes from 17 isolates of *C. ribicola*. Three distinct clusters of isolates were found within the network that included 1) Korea/China, 2) Japan, and 3) Germany/North America Isolates from Japan were intermediate to Korea/China and Germany separated by five mutation steps from both clusters (Fig. 1). Five and three haplotypes, separated by one mutation, were found within Korea/China and Japan, respectively. Only two isolates were found within the Germany cluster. The Wisconsin isolate was separated by two mutation events from the other Germany isolates.

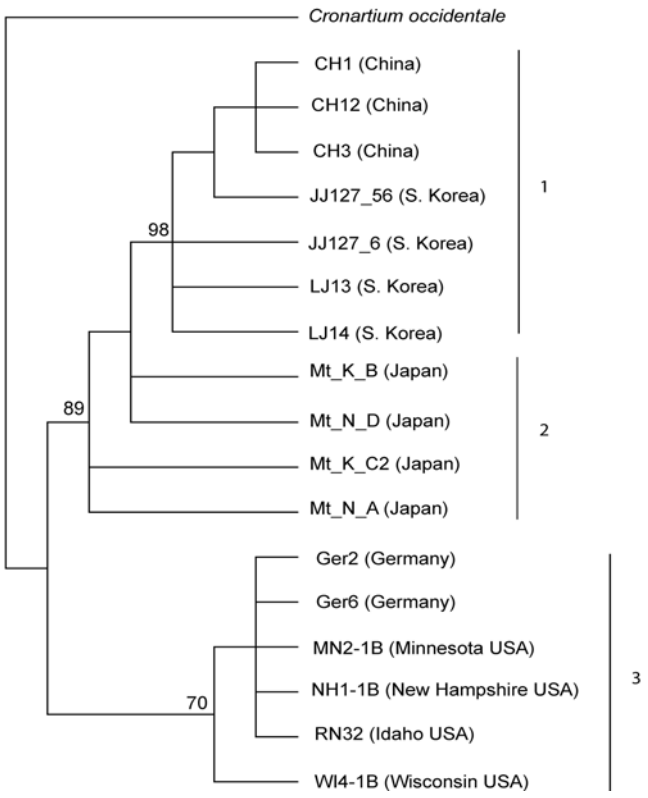

Figure 2. Maximum-likelihood topology generated from DNA sequences of four nuclear loci implementing a general time reversal model. Isolates of *Cronatirum ribicola* are described in Table 1. Bootstrap replicate values of 70 or greater are shown. Three major clades (1, 2 and 3) representing geographical regions (China/Korea, Japan, North America and Europe) are apparent with high bootstrap support.

All three phylogenetic analyses, maximum likelihood, Bayesian, and maximum parsimony, produced similar topologies. Given the redundancy among phylogenetic analyses, we present the results from maximum likelihood. Results from phylogenetic analyses resembled the results from the network analysis, with three well-supported clades. Similar relationships were also observed, with the Korea/China clade most distant and the Japan clade intermediate in relation to the Germany/North America clade (Figure 2). All three clades were supported with a high bootstrap support (>70). *Cronartium occidentale* was selected as a outgroup. *C. occidentale* had 20 SNPs and 10 gaps among the four DNA sequences when compared to *C. ribicola*.

The phylogeographic patterns of *C. ribicola* show that at least three distinct lineages exist: 1) Korea/China, 2) Japan, and 3) Germany/North America. The similarity of Germany isolates corroborates the historical records of white pine blister rust spread in western Europe in the late 1800's with subsequent introductions into North America from *P. strobus* nursery stock grown in France and Germany (reviewed in McDonald and Hoff 2001). Based on the current samples, the source(s) of the European and North American epidemic remains unclear. Our preliminary results suggest that our sampled locations in Japan, Korea, and China do not represent the source of *C. ribicola* that spread through Eurasia and North America; however, it cannot be ruled out that this genetic source could be present at low frequency at the sampled locations or from other geographic areas within one of these countries. Furthermore, these data and varying infection of different telial host species by artificial inoculation (e.g., Stephan and Hyun 1983, Yokota and Hama 1981) suggest that this pathogen has ecological races in Asia.

Further study is needed to determine the genetic relationships among populations of *C. ribicola* that occur in northeastern Siberia/Kamchatka, northern India and Pakistan, and other regions of Eurasia. Further sampling of eco-regions occupied by five-needled, white pines and *C. ribicola* should help elucidate the phylogenetic relationships and origins of the western European and North American epidemic. In addition to the intraspecific relationships in this taxon, related microcyclic species, such as *Endocronartium sahoanum*, extant in Japan and the Kamchatka region of Russia (Imazu *et al.* 2000), and another potential pine-stem rust that infects *P. armandii* in central China (Hei *et al.* 2003) warrants further phylogenetic characterization.

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