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Distribution of *Cronartium x flexili,* an interspecific hybrid of two fungal tree rust pathogens, in subalpine forest ecosystems of western USA

Olga Kozhar [a,*], Kelly S. Burns [b], Anna W. Schoettle [c], Jane E. Stewart [a,**]

[a] Department of Agricultural Biology, Colorado State University, Fort Collins, CO, USA
[b] Forest Health Protection, Rocky Mountain Region, USDA Forest Service, Golden, CO, USA
[c] Rocky Mountain Research Station, USDA Forest Service, Fort Collins, CO, USA

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ABSTRACT

Interspecific hybridization plays a key role in the evolution of novel fungal pathogens, and when it occurs between native and invasive species, can lead to potentially serious consequences. In this study, we examined the temporal and spatial distribution of a recently detected hybrid (*Cronartium x flexili*) of two tree pathogens, invasive to North America *Cronartium ribicola* and native *Cronartium comandrae*. In total, 726 and 1452 aecia from 178 *Pinus contorta* ssp. *latifolia* and 357 *Pinus flexilis* trees were collected from 26 sites in four national forests in 2019–2021. Using morphological and molecular analyses, 71 aecia collected from 25 *P. flexilis* trees had intermediate morphology and contained heterozygous SNPs in two genomic regions. Population analyses revealed the presence of multiple hybrid genotypes randomly distributed among sites and years. No aecia from *P. contorta* ssp. *latifolia* were identified as hybrids suggesting unidirectional gene flow from native *C. comandrae* to invasive *C. ribicola*. Aeciospores from 2 hybrid aecia produced urediniospores on *Ribes nigrum*. Overall, these results suggest that, even though low in frequency, *C. x flexili* is persistent in the region and has pathogenic potential. Hybrid expansion into the large range of susceptible pines could have cascading impacts on forest health.

1. Introduction

Once thought to be extremely rare events, natural interspecific hybridizations and their role in organismal evolution are well recognized to d (Brasier, 2000a; Steensels et al., 2021). With the development of genetic tools, the signs of ancient and recent interspecific hybridizations have been extensively documented in plants (Alix et al., 2017), animals (Gabryś et al., 2021), and fungi (Marcet-Houben and Gabaldón, 2015; Mixão and Gabaldón, 2020). While gene flow between different species occurs in nature, anthropogenic activity provides more opportunities for species to hybridize. Similarly, globalization is hypothesized to be an important driver of increasing emergence of interspecific hybrids of various organisms, including important plant and animal fungal pathogens (Fisher et al., 2012). Species living in sympatry often have strong genetic and/or ecological barriers that prevent hybridization with other species. On the other hand, allopatric species are expected to more readily hybridize, as the reproductive barriers between species that did not coexist in the same ecosystems are likely to be weaker (Stukenbrock, 2016). Studies with *Neurospora crassa* show that hybrid fruiting bodies of sympatric crosses were aborted at a significantly higher rate compared to fruiting bodies of allopatric crosses (Turner et al., 2011). Continuous movement of organisms out of their native ranges into new ecological niches increases chances of their hybridization with close relatives. Within the last several decades multiple cases of interspecific hybridization events between introduced forest fungal and oomycete pathogenic species and native species have been documented. Some fungal examples include *Heterobasidion irregulare x Heterobasidion annosum* (Gonthier and Garbelotto, 2011), *Melampsora x columbiana* (Newcombe et al., 2000), *Ophiostoma ulmi x Ophiostoma novo-ulmi* (Konrad et al., 2002). This has also occurred with Oomycete pathogens, including but not limited to, *Phytophthora x alni* (Ioos et al., 2006), *Plasmopora x viticola* (Rouxel et al., 2013), as well as hybrids of *Phytophthora cryptogea, P. erythroseptica,* and *P. sansomeana* (Safaiefarahani et al., 2016). A more recent example of allopatric hybridization of forest pathogens is the hybridization between native *Cronartium comandrae* and invasive *Cronartium ribicola*, two fungal species that did not coexist

* Corresponding author.
** Corresponding author.
E-mail addresses: Olga.Kozhar@colostate.edu (O. Kozhar), Jane.Stewart@colostate.edu (J.E. Stewart).

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in forest ecosystems of North America until early 1900s when *C. ribicola* was accidently introduced to the continent (Stewart, 1906). A hundred y later the hybrid of these two rust species, *Cronartium x flexili*, was first reported in Canada in 2006 (Joly et al., 2006).

The genus *Cronartium* comprises several species of forest tree pathogens. Members of this genus are biotrophs and have narrow specific host ranges that often do not overlap (Wijesinghe, 2019). For example, *C. comandrae* causes comandra blister rust on 2- and 3-needle pine species while *C. ribicola* infects five-needle pines. In western USA, primary hosts of *C. comandrae* and *C. ribicola* are *Pinus contorta* spp. *Latifolia* Engelmann 1871 (common name Rocky Mountain lodgepole pine) and *Pinus flexilis* E. James (common name limber pine), respectively. Both rust species are heteroecious and require two plant host species to complete their life cycle. Alternate host for *C. comandrae* is *Comandra umbellata* (L.) Nutt (Zentz and Jacobi, 1989), while species of genera *Ribes*, *Pedicularis*, and *Castilleja* are alternate hosts for *C. ribicola* (McDonald et al., 2006). Despite having different hosts, the life cycles of these two fungal pathogens are similar (Mielke, 1943; Bergdahl and French, 1976). Both rust species infect their primary hosts (pines) by haploid basidiospores via needles or young shoots, initiating mycelial growth within branch tissue and establishing perennial cankers on tree stems (Mielke, 1943; Bergdahl and French, 1976). One to five y post infection, haploid cankers produce haploid structures called pycnia. Pycniospores, released from pycnia, consist of spermatia and receptive hyphae that act as gametes and are fertilized by pycniospore-carrying insects, forming dikaryotic mycelium in the fall (Anikster et al., 1999). The following spring, dikaryotic mycelium produces dikaryotic aecia with aeciospores (Mielke, 1943). The airborne aeciospores are extremely hardy and can travel long distances (Frank et al., 2008) to infect the alternate hosts, on which the next three spore stages are produced. After leaf infection, the rusts produce dikaryotic uredinia with urediniospores that travel short distances and reinfect alternate hosts (Mielke, 1943; Bergdahl and French, 1976). Later in the season, as the result of karyogamy, telia with teliospores are produced, that eventually undergo meiosis and produce basidia with haploid basidiospores that are infectious on primary pine hosts in the late summer-fall. Basidiospores are short lived and capable of traveling short distances (Mielke, 1943; Bergdahl and French, 1976). After initial discovery of hybrid aeciospores on *P. flexilis*, Joly et al. (2006) hypothesized that the receptive hyphae of *C. ribicola* was fertilized by *C. comandrae* pycniospores in sites where both pine species (*P. flexilis* and *P. contorta* spp. *latifolia*) coexist.

While the discovery of *C. x flexili* hybrid on *P. flexilis* is interesting from taxonomic and evolutionary perspectives, it also raises ecological and epidemiological concerns, as both rust species are serious pathogens of several pine species. *C. ribicola* can cause up to 90–95 % mortality of 5-needle pine populations in North America, and damage to 2- and 3-needle pines caused by *C. comandrae* has been increasing (Woods, 2014). As a mechanism of adaptive evolution, interspecific hybridization could lead to the development of novel strains with increased virulence, broader host range, and/or increased environmental capacity, etc., which poses great risks to the health of forest ecosystems in western US forests. Furthermore, hybrids may jeopardize the durability of genetic disease resistance in the five-needle pines that is the cornerstone of white pine blister rust management (King et al., 2010; Schoettle et al., 2022). While little is known about the frequency of *C. comandrae* sexual reproduction and genetic diversity of its populations, other studies have determined that *C. ribicola*, despite being an invasive species, undergoes extensive sexual recombination in North America (Hamelin et al., 2005). This could provide more potential opportunities for overcoming genetic reproductive barriers between two species. To date *C. x flexili* hybrid was reported in two regions in Canada (Allen, 2019; Joly et al., 2006), and it remains unknown whether the interspecific hybridization between *C. ribicola* and *C. comandrae* is localized to the sites of its initial discovery. These events may be occurring in other regions where both pathogens and their hosts coexist, including in Central and Southern Rocky Mountains alpine ecosystems of the western USA. The objectives of this study were to 1) determine whether *Cronartium x flexili* hybrids are present in forest ecosystems of Colorado and Wyoming, USA; 2) evaluate whether the hybrids consistently occur in the studied ecosystems; 3) evaluate genetic diversity of hybrid samples and its parental species; and 4) test whether aeciospores of *C. x flexili* hybrids are infectious and can produce urediniospores on an alternate host.

2. Materials and methods

2.1. Sampling sites and collections

Study sites were selected based on the presence of *P. flexilis* and *P. contorta* growing intermixed within a single site. Aecial sampling on both species occurred over three seasons from 2019 to 2021. Samples were collected by first identifying cankers with developed, but not yet opened fruiting bodies. Fruiting bodies were sprayed with 70 % ethanol to clean the surface, then an intact peridial membrane of aecium was ruptured with a sterile nail, and spores were collected into a clean 1.5 ml Eppendorf tube. Each aecium was collected into a separate tube and tools and gloves were thoroughly washed in 70 % ethanol between samples. When multiple aecia were collected from the same tree, the collection was conducted by first sampling lower aecial and moving upwards to avoid cross contamination of samples. At each site up to 10 trees of each host species were sampled, and GPS coordinates of each tree were recorded. Tubes with spores were immediately put into a container with ice and then frozen at −20 °C for a long-term storage. In total, 1523 and 753 aecia from *P. flexilis* (PIFL) and *P. contorta* (PICO), respectively, were collected across four forests in three y. In June and July of 2019, aecial samples were collected from 200 trees [279 aecia from 139 PIFL and 120 aecia from 61 PICO] at 16 sites in 4 national forests (NF) (Shoshone NF, Bighorn NF, Medicine Bow NF, and Roosevelt NF) in Wyoming and Colorado, USA (Fig. 1a, Table S1). In 2020, 1118 individual aecial samples were collected from a total of 257 trees (714 aecia from 168 PIFL and 404 aecia from 89 PICO) at 25 sites, and in 2021, 10 sites where hybrid samples were found in either 2019 or 2020 (see results) were resampled resulting in 759 individual aecial samples (530 aecia from 48 PIFL and 229 aecia from 38 PICO).

2.2. Microscopy

The morphology of all collected aeciospores was analyzed with an Olympus BX43 light microscope (Evident Scientific Waltham, MA, USA) as described by Joly et al. (2006). Spore shape, length/width (μm), and presence of apical acumination and/or conspicuous smooth spot were recorded for each sample. In addition to the aecial samples containing aeciospores with intermediate morphology (see results), 63 and 52 aecial samples of *C. ribicola* and *C. comandrae*, respectively, were randomly picked for further comparative analyses (Table S2). A total of 20 aeciospores per each aecial sample were measured, and these measurements were used to calculate mean and standard deviation of spore length and width for each aecium.

2.3. DNA extraction, molecular identification, and data analyses

The samples from Table S2 were further subjected to DNA extraction using a modified CTAB protocol (Kozhar et al., 2023). PCR amplification was performed in a 25 μl volume reaction containing 2.5 μl of 10× PCR buffer, 0.5 μl dNTP, 0.5ul (10 μM) forward and reverse primers each, and 0.125 μl Taq DNA polymerase (New England Biolabs, NEB). Primer pairs used were ITS1 and ITS4 (White et al., 1990), Dcon10Fw and Dcon10Rev (Joly et al., 2006), and Dcon35_6Fw and Dcon35_6Rev designed in this study (Dcon35_6-Fw AGCTTGAGCCTTGACCTTCC and Dcon35_6-Rev TCGGTCGAGGAACGAGGAAT). Dcon35_6 primers were designed based on the Dcon35 primer pair from Joly et al. (2006) covering a larger genomic region. Thermal cycle conditions were as

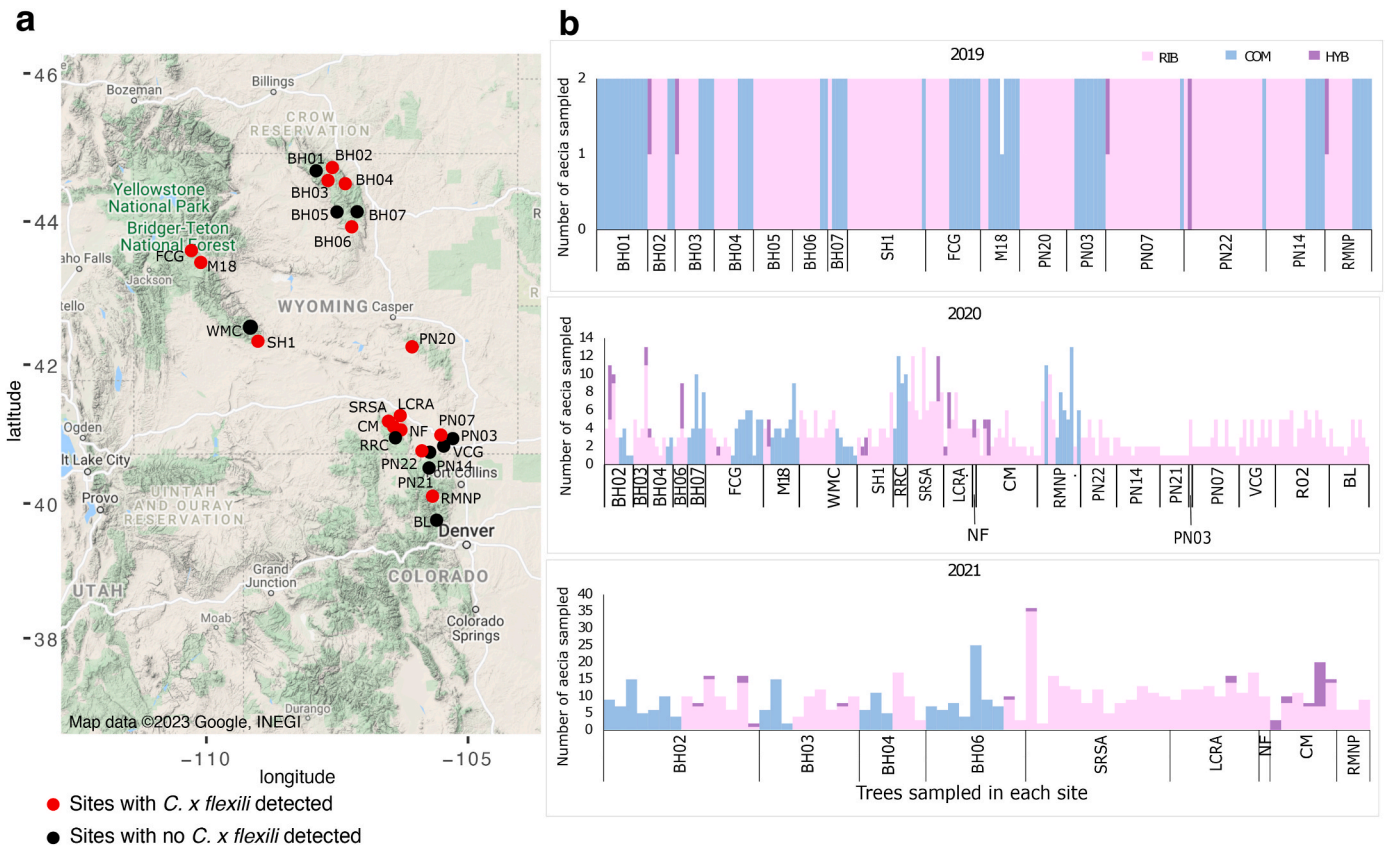

Fig. 1. A. Sites sampled in 2019–2021. Red dots indicate sites where *Cronartium x flexili* hybrids were detected, while black dots indicate sampled sites with no *C. x flexili* detected. **b.** The number of aecia sampled per each tree in sites in 2019–2021. Each bar represents one sampled tree, the height of the bar corresponds to total number of aecia sampled. Blue bars - *Cronartium comandrae* aecia sampled from *Pinus contorta*, pink bars - *Cronartium ribicola* aecia sampled from *Pinus flexilis*. Aecia that were confirmed to contain *C. x flexili* aeciospores are highlighted in purple.

follows: 94 °C for 3 min; followed by 35 cycles of 94 °C for 30 s, 57 °C for Dcon10 and 58 °C for Dcon35_6 for 30 s, and 72 °C for 30 s; with a final extension at 72 °C for 10 min (Eppendorf Vapo. Protect Mastercycler® Pro, Hamburg, Germany). ITS amplicons were subjected to the digestion with restriction enzyme BtsCI, that identifies restriction site GGATG present in *C. ribicola,* following manufacturer protocol (New England Biolabs, Ipswich, MA, USA). The result of this digest is a single band in *C. comandrae* (cleavage site is absent), two bands in *C. ribicola* (cleavage site is present), and three bands in the *C. x flexili* hybrid representing an intact ITS sequence derived from the *C. comandrae* parent and a cleaved ITS sequence from the *C. ribicola* parent (R.C. Hamelin, personal communication). Since several rust species infect PICO in the studied region, PICO ITS amplicons were sequenced for confirmation that the spores were *C. comandrae* prior to sequencing the Dcon10 and Dcon35_6 loci. Dcon10 and Dcon35_6 amplicons were sequenced in all samples listed in Table S1 by Eurofins Genomics LLC (Louisville, KY, USA).

Sequences were aligned and compared in Geneious Primer v.9.0 (Biomatters Ltd, Auckland, New Zealand). Phased haplotypes, segregating sites, haplotype diversity, Watterson theta, and Tajima D for studied populations were determined with DNAsp v.5 (Rozas, 2009). Figures were edited in Inkscape v. 1.3 (inkscape.org).

2.4. Inoculation experiments

Before inoculations, spore viability was tested with germination assays by streaking aeciospores with a sterile glass rod on potato dextrose agar medium followed by incubation at room temperature for 48 h. Established *Ribes nigrum* plants, grown in 2-L nursery pots in a greenhouse at Colorado State University, were used for the inoculation experiments, and because of the difficulty cultivating *Comandra umbellata*, inoculations were not performed on this *C. comandrae* alternate host. Aeciospores from 71 aecia (6 from 2019, 37 from 2020, to 28 from 2021), that were confirmed hybrids based on morphological and molecular analyses (see results), were used for inoculations. An individual *R. nigrum* plant was used for each of the 71 *C. x flexili* samples. One d before inoculation, plants were put in a growth chamber and watered thoroughly. The next d, three fully developed leaves on each plant were sprayed with deionized water and aeciospores of an individual *C. x flexili* sample were dusted onto the abaxial side using a sterile toothpick. Plants were then covered with plastic bags which were then sealed to maintain high humidity and kept at 18 °C in the dark for 48 h. After 48 h, plastic bags were removed, and plants were incubated at 23 °C during d and 18 °C at night at 14 h photoperiod and 60 % humidity for 14 d. Inoculated plants were kept in a growth chamber each surrounded by an inhouse-made plastic chamber to avoid cross contamination. Each chamber was a cylinder made of 20-gauge clear vinyl, the top of which was covered and sealed with 37 × 42cm Kimwipes (two wipes sealed together with a tape for each cylinder; Kimtech Science®, Kimberly–-Clark Professional, Roswell, GA, USA) to allow airflow. For each experiment, one plant was used as a negative control (only water sprayed) and one plant was inoculated with aeciospores of *C. ribicola* sample BL-3-5 as a positive control. Inoculation experiments were repeated once. To fulfill Koch's postulates, urediniospores from hybrid samples that successfully infected *R. nigrum* leaves were collected, put into clean PCR tubes, DNA extracted and used as template DNA for PCR and sequencing of Dcon10 and Dcon35_6 regions, as described above, to confirm them as *C. x flexili* hybrids, fulfilling Koch's postulates.

3. Results

3.1. Morphological and molecular identification of cronartium x flexili hybrids

Based on morphological identification, 71 aecia collected from PIFL [6 aecia (2.15 % of total sampled from PIFL) in 2019, 37 aecia (5.2 % of total sampled from PIFL) in 2020, and 28 aecia (5.3 % of total sampled from PIFL) in 2021], contained aeciospores of intermediate morphology (Fig. 1b). These aeciospores were 23–38 × 16–23 μm in size (SD = 2 to 7 × 1–4 μm) and mixed pyriform/fusiform/ovoid in shape, but without true apical acumination as in *C. comandrae* and conspicuous smooth spot as in *C. ribicola* (Fig. 2a–d, Table S2). *C. ribicola* aeciospores were 21–27 × 14–21 μm in size, whereas *C. comandrae* aeciospores were 33–59 × 18–28 μm in size (SD = 2 to 5 × 1–3 μm and 1 to 12 × 1–5 μm) (Fig. 2a–d, Table S2). According to these measurements, *C. comandrae* aeciospores had the broadest range of mean spore lengths (maximum of 26 μm difference), while *C. ribicola* aeciospore length range was the smallest (maximum of 4 μm difference). *Cronartium x flexili* aeciospore mean length difference was of 15 μm (Fig. 2d). Mean widths of aeciospores were narrower than their lengths for all three sample types – with maximum ranges of 11, 7, and 7 μm for *C. comandrae*, *C. x flexili* hybrids, and *C. ribicola*, respectively. No aeciospores of intermediate morphology were found on PICO.

The restriction digestion of the ITS region of these 71 *C. x flexili* aecial samples confirmed their hybrid origin with 3 bands, carrying both an intact ITS sequence derived from the *C. comandrae* parent and a cleaved ITS sequence from the *C. ribicola* parent, indicating genetic material from both parental species was present in these hybrid samples (Fig. 3a, Table S3).

Across all *C. ribicola* and *C. comandrae* samples, 17–22 SNPs were detected in Dcon10 (437 basepairs), and 19–25 SNPs were identified in the Dcon35 (447 basepairs) region. Sequencing results indicated the presence of interspecific heterozygous sites and alleles derived from each of the parental species in all 71 *C. x flexili* hybrid samples (Fig. 3b–c).

3.2. Frequency distribution of cronartium x flexili hybrids in four national forests in 2019–2021

Over the three sampling seasons, each of the 26 sites were sampled at least once. *Cronartium x flexili* aecia were found in five of 16 sampled sites in 2019, 11 of 25 in 2020, and seven of 10 in 2021 (Table 1, Table S1). The frequency of *C. x flexili* aecia on PIFL varied between 2.2 and 5.3 % among forests and 0.9 and 6.6 % among y (Table 1). Among three seasons of sampling, the highest frequency of *C. x flexili* was detected in the Bighorn NF in 2020 (16.1 %). The total number of sampled aecia per PIFL varied from one to 37. Among trees where hybrids were detected, the frequency of *C. x flexili* aecia within a tree ranged between 2.7 and 100 % (Table 2).

3.3. Genetic diversity of C. comandrae, C. ribicola, and C. X flexili populations

Among all sequenced samples (Table S3), a native *C. comandrae* population harbored more diversity in both genomic regions compared to a non-native *C. ribicola* population, and the Dcon35 region was more diverse than Dcon10 in both species (haplotype diversity 0.114 and 0.0 (Dcon10) and 0.610 and 0.432 (Dcon35) for *C. comandrae* and *C. ribicola*, respectively; Table 3, Table S3). Nucleotide diversity (π) for Dcon10 was 0.00031 for *C. comandrae* and 0.0 for *C. ribicola*, and Dcon35 π was 0.0028 for *C. comandrae* and 0.0018 for *C. ribicola* (Table 3). The Bighorn NF *C. comandrae* population was the most diverse (Hd = 0.1530 for Dcon10 and 0.6780 for Dcon35) and in 2021 (Hd = 0.1800 for Dcon10 and 0.6640 for Dcon35), which was likely influenced by population size as it was the largest among all *C. comandrae*

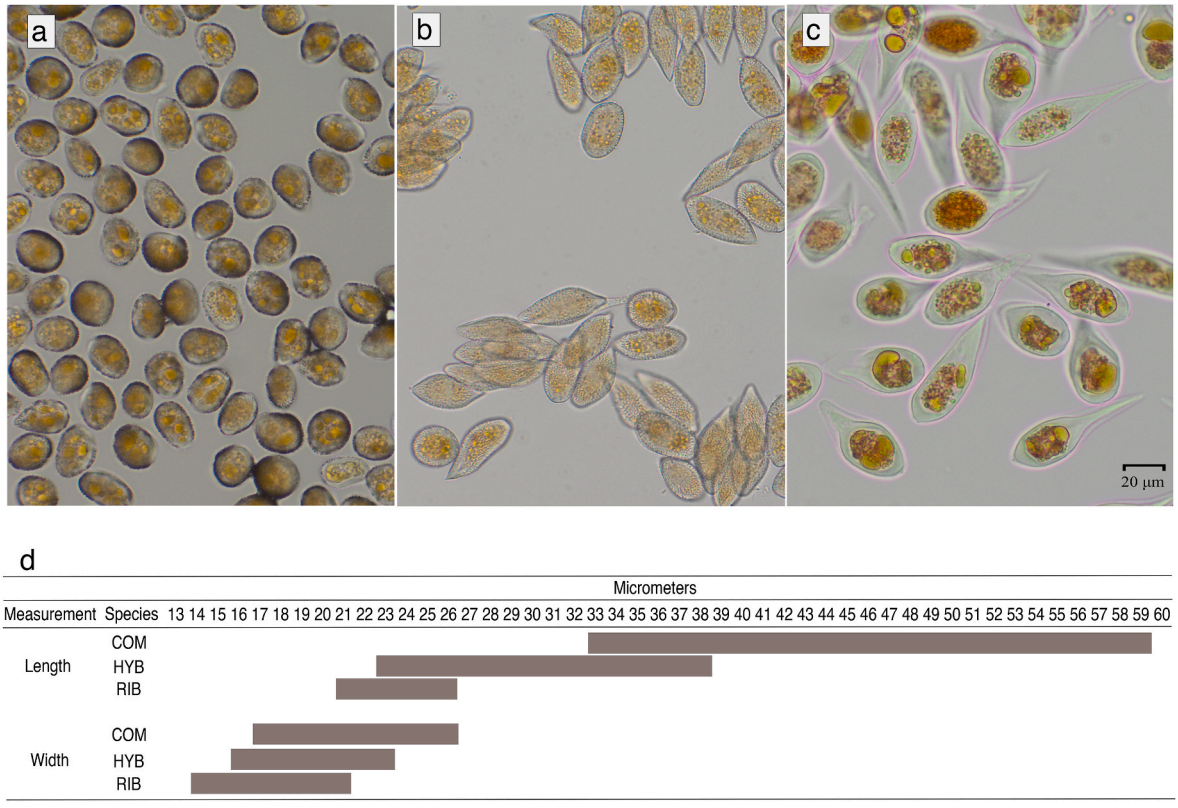

Fig. 2. Aeciospores of *Cronartium ribicola* **(a)**, *C. x flexili* **(b)**, and *C. comandrae* **(c)** and their size ranges **(d).** *COM - *C. comandrae*, HYB–*C. x flexili*, and RIB–*C. ribicola*. Details on measurements are in Table S3.

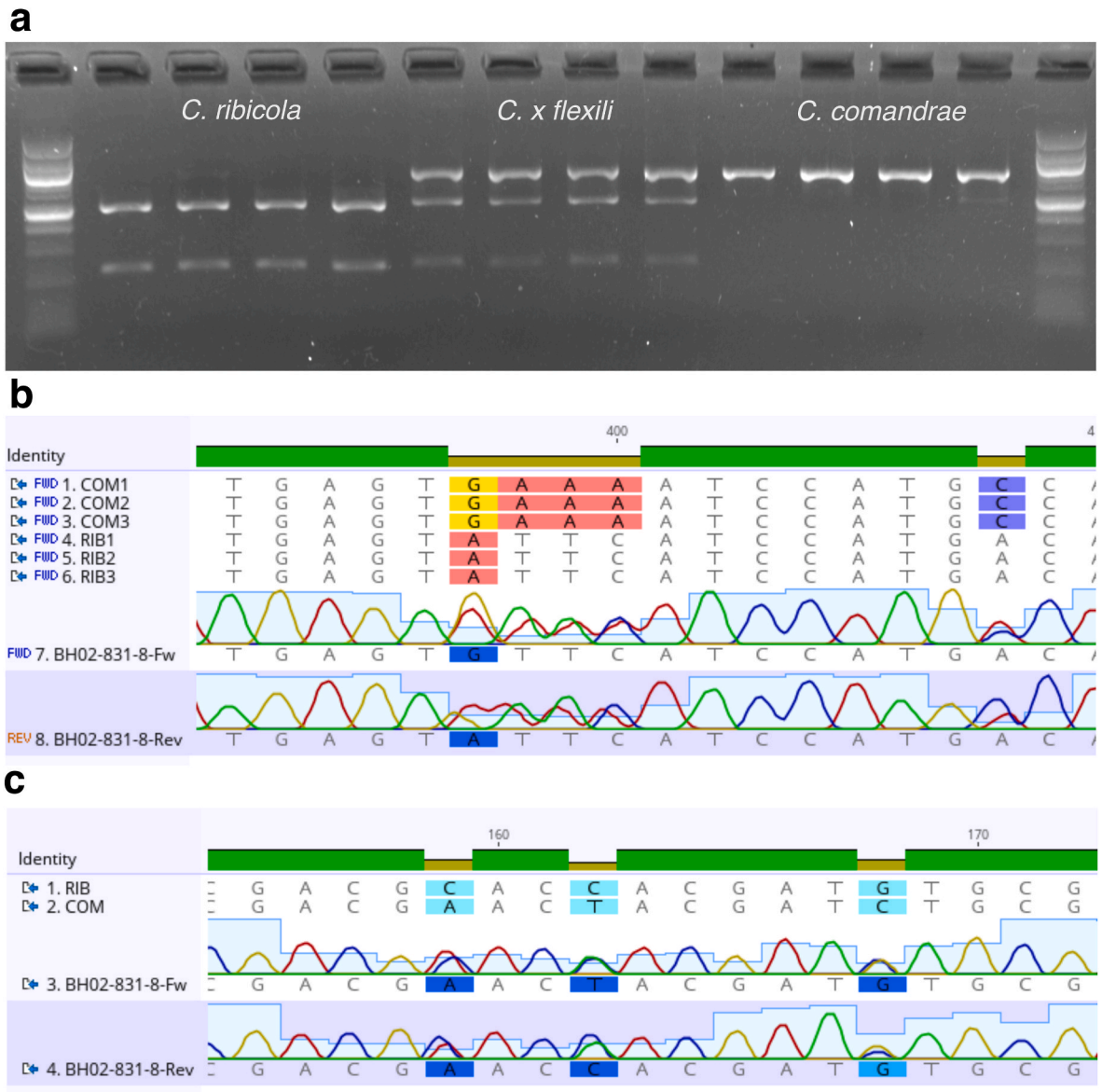

Fig. 3. A. Results of restrictive enzyme digest of ITS region using BtsCI enzyme. Cleavage site is present in ITS sequence of *Cronartium ribicola*, but not *C. comandrae*. Lanes 2–5 contain *C. ribicola* with cleaved ITS sequences. Lanes 6–9 contain *C. x flexili* hybrid samples with both intact and cleaved ITS sequences, representing genetic contribution from both *C. comandrae* and *C. ribicola* nuclei. Lanes 10–13 contain *C. comandrae* with intact ITS sequences. Sequence alignment chromatographs of Dcon35 **(b)** and Dcon10 **(c)** alleles in *C. ribicola* (RIB), *C. comandrae* (COM) and *C. x flexili* hybrid (BH02-831-8). Interspecific single nucleotide polymorphisms are highlighted in color. *Cronartium x flexili* sequences clearly show peaks from both nucleotides simultaneously on the chromatograph.

populations analyzed (Table 3). The population of *C. ribicola* was monomorphic in Dcon10 and most diverse in the Shoshone NF and in the 2020 season in Dcon35 (Table 3). Interestingly, despite Roosevelt NF having the smallest sample size (N = 4) for the *C. x flexili* population, this population appeared to be most diverse in Dcon10 (*Hd* = 0.6670). The *C.* x *flexili* population from Big Horn NF was the most diverse in Dcon35, despite a small sample size compared to the largest C. x *flexili* population that was collected from Medicine Bow NF (Table 3).

3.4. Allele and genotype frequency of cronartium x flexili hybrids

In the Dcon10 region, one *C. ribicola* and six *C. comandrae* alleles were detected, while the Dcon35 region harbored four (*C. ribicola*) and seven (*C. comandrae*) alleles among analyzed samples. The Dcon10 allele 10_R1 found in *C. ribicola* corresponded to the sequence of Dcon10-CriA allele reported by Joly et al. (2006). Two Dcon10 alleles, found in *C. comandrae* (10_C1 and 10_C2) corresponded to the sequences of Dcon10-CcmA and Dcon10-CcmB alleles reported by Joly et al. (2006), while another four were novel alleles (Table S4). In the Dcon35 region, *C. ribicola* alleles 35_R1, 35_R2, and 35_R3 corresponded to Dcon35-CriA and Dcon35-CriB sequences from Joly et al. (2006). The 35_R1 differed from 35_R3 allele in 1 SNPs at the position 416 which is outside of the Dcon35_CriA sequence. The 35_R4 allele was a novel allele. Three Dcon35 alleles, detected in *C. comandrae* (35_C1, 35_C2, and 35_C3), carried regions corresponding to Dcon35-CcmB, Dcon35-CcmC, and Dcon35-CcmD alleles from Joly et al. (2006), while four other alleles were novel. Allele details and accession numbers are listed in Table S4. In total, there were four Dcon10 and nine Dcon35 hybrid genotypes detected. In Dcon10, 95 and 100 % of *C. x flexili* samples carried 10_C1 and 10_R1 alleles, respectively, with the 10_C1R1 genotype constituting 91 % of *C.* x *flexili* population (Fig. 4a–b). These alleles were also the most frequent alleles in parental populations of *C. comandrae* and *C. ribicola* (Fig. 4a). In the Dcon35 region, alleles 35_C1, 35_C2, 35_C3, 35_R1 and 35_R2 were the most frequent in *C. x flexili* samples, alleles

Table 1
Frequency of *Cronartium x flexili* hybrid aecia in each national forest surveyed in 2019–2021.

Year	National Forest	# Aecia[a]	#*C. x flexili* [b]	%
2019				
	Bighorn	71	2	2.8
	Shoshone	54	0	0.0
	Medicine Bow	–	–	–
	Roosevelt	154	4	2.6
2020				
	Bighorn	87	14	16.1
	Shoshone	110	7	6.4
	Medicine Bow	261	16	6.1
	Roosevelt	256	0	0.0
2021				
	Bighorn	173	6	3.5
	Shoshone	–	–	–
	Medicine Bow	336	21	6.3
	Roosevelt	21	0	0.0
All y				
	Bighorn	331	22	6.7
	Shoshone	164	7	4.3
	Medicine Bow	597	37	6.2
	Roosevelt	431	4	0.9
All forests				
	2019	279	6	2.2
	2020	714	37	5.2
	2021	530	28	5.3

[a] Total number of aecia sampled from *Pinus flexilis* in each forest each y.
[b] Number of hybrid *C. x flexili* aecia detected in each forest each y.

35_C1 and 35_R1 were the most frequent in the parental populations of *C. comandrae,* and 35_C3 and 35_R2 were the most frequent alleles in the parental populations of *C. ribicola* (Fig. 4c). Hybrid genotypes 35_C1R2 and 35_C3R2 constituted 34 and 29 % of all Dcon35 genotypes of *C. x flexili* samples, respectively (Fig. 4d). The remaining 37 % of *C. x flexili* samples carried a variation of the other seven less frequent hybrid genotypes. Interestingly, although only 2 % of analyzed *C. comandrae* samples carried the 35_C2 allele, this allele was present in 26 % of *C. x flexili* samples in either of three hybrid genotypes 35_C2R1, 35_C2R2, or 35_C2R3.

The distribution of *C. x flexili* genotypes varied within and among sampled trees, locations and y (Fig. 5a–b). Over the three sampling seasons, *C. x flexili* aecia were sampled from a total of 27 PIFL trees. While hybrid aecia were detected on trees in either of three seasons, *C. x flexili* samples were repeatedly sampled from cankers of four trees (Table 2). *Cronartium x flexili* samples were collected from a single tree BH02-832 located in Bighorn NF in both 2020 and 2021, and *C. x flexili* samples were collected from trees BH06-870, CM-747, and SRSA-773, located in Bighorn and Medicine Bow NFs, all three y. The *C. x flexili* samples collected from tree BH02-832 carried different Dcon35 genotypes each y, and the Dcon10 genotypes of these samples also differed in 2020 and 2021 (Fig. 5a–b). The *C. x flexili* samples from trees BH06-870 and CM-747 carried different Dcon35 genotypes in 2020 and 2021, and the 2021 sample from BH06-870 had a unique genotype 35_C5-R1. The Dcon10 genotypes did not differ among these samples. In contrast, *C. x flexili* samples from SRSA-773 carried the same Dcon35 genotype in both seasons, but the *C. x flexili* sample from 2021 carried a unique genotype 10_C5-R1 in the Dcon10 genomic region (Fig. 5a–b).

The frequency of *C. x flexili* Dcon35 genotypes differed among y, with some genotypes being detected only in either 2020 or 2021 (Fig. S1). Among a total of 6 *C. x flexili* samples collected in 2019, 33 % (N = 2) and 66 % (N = 4) carried 35_C1R2 and 35_C3R2 Dcon35 genotypes. These genotypes were also the most frequently sampled in 2021. In 2021 the frequency of five other genotypes (35_C1R1, 35_C2R2, 35_C3R1, 35_C4R2, and 35_C5R1) was less than 10 % each, with 35_C4R2 and 35_C5R1 being unique genotypes to 2021 season. The Dcon35 genotypic

Table 2
Frequency of *Cronartium x flexili* aecia per each *Pinus flexilis* tree in 2019–2021.

National forest	Site	Tree #	2019			2020			2021		
			[a]# Aecia	[b]#*C. x flexili*	%[c]	# Aecia	#*C. x flexili*	%	# Aecia	#*C. x flexili*	%
Bighorn	BH02	831	2	0	0.0	11	6	54.6	10	0	0.0
	BH02	832	2	1	50.0	10	1	10.0	16	2	12.5
	BH02	835	2	0	0.0	2	0	0.0	2	1	50.0
	BH02	2003	–	–	–	–	–	–	8	1	12.5
	BH02	2009	–	–	–	–	–	–	16	1	6.3
	BH03	844	2	0	0.0	13	1	7.7	10	0	0.0
	BH03	845	2	1	50.0	1	0	0.0	0	0	0.0
	BH03	2010	–	–	–	–	–	–	8	1	12.5
	BH04	1	–	–	–	4	1	25.0	0	0	0.0
	BH06	870	2	0	0.0	9	5	55.6	10	1	10.0
Medicine Bow	CM	741	–	–	–	4	4	100.0	0	0	0.0
	CM	742	–	–	–	1	0	0.00	3	2	66.7
	CM	743	–	–	–	5	0	0.00	10	2	20.0
	CM	746	–	–	–	3	0	0.00	8	1	12.5
	CM	747	–	–	–	4	1	25.0	23	12	52.2
	CM	748	–	–	–	3	0	0.00	15	1	6.7
	LCRA	776	–	–	–	8	4	50.0	0	0	0.0
	LCRA	781	–	–	–	5	0	0.00	16	2	12.5
	NF	750	–	–	–	6	2	33.3	10	0	0.0
	SRSA	773	–	–	–	14	5	35.7	37	1	2.7
Shoshone	M18	895	2	0	0.0	5	3	60.0	–	–	–
	FCG	7	–	–	–	2	1	50.0	–	–	–
	SH1	2	–	–	–	2	1	50.0	–	–	–
	SH1	3	–	–	–	3	2	66.7	–	–	–
Roosevelt	PN22	374	2	2	100.0	0	0	0.0	0	0	0.0
	RMNP	381	2	1	50.0	0	0	0.0	0	0	0.0
	PN07	345	2	1	50.0	0	0	0	–	–	–

-' - not sampled, '0' - no aecia found.
[a] Total number of aecia sampled per *P. flexilis* tree.
[b] Number of *C. x flexili* aecia sampled per tree.
[c] Percent of aecia from total sampled.

Table 3
Total number of sites, haplotypes, sequence diversity, segregating sites, and nucleotide diversity for each locus in *Cronartium ribicola*, *Cronartium comandrae*, and *Cronartium x flexili* hybrid populations.

Locus (bp)	Pop	N[a]	H[b]	Seg. sites[c]	Hd[d]	Theta W[e]	π[f]	Tajima's D[g]
Dcon10 (437)								
C. x flexili	total	71	5	22	0.5440	0.0091	0.0200	n/a
	BH	23	3	20	0.5520	0.0104	0.0205	n/a
	MB	37	4	19	0.5330	0.0089	0.0198	n/a
	SH	7	2	17	0.5330	0.0117	0.0208	n/a
	RS	4	2	20	0.6670	0.0250	0.0305	n/a
	2019	6	2	20	0.5710	0.0177	0.0262	n/a
	2020	37	2	17	0.5070	0.0079	0.0197	n/a
	2021	28	4	19	0.5440	0.0095	0.0200	n/a
C. ribicola	total	63	1	0	0	0	0	0
	BH	15	1	0	0	0	0	0
	MB	24	1	0	0	0	0	0
	SH	9	1	0	0	0	0	0
	RS	15	1	0	0	0	0	0
	2019	14	1	0	0	0	0	0
	2020	30	1	0	0	0	0	0
	2021	19	1	0	0	0	0	0
C. comandrae	total	52	4	3	0.1140	0.0013	0.0003	−1.3713
	BH	31	3	2	0.1530	0.0010	0.0004	−1.0794
	MB	9	1	0	0	0	0	0
	SH	8	2	2	0.1250	0.0014	0.0006	−1.4980
	RS	4	1	0	0	0	0	0
	2019	3	1	0	0	0	0	0
	2020	22	2	2	0.0450	0.0011	0.0002	−1.4777
	2021	27	3	2	0.1800	0.0010	0.0004	−1.0355
Dcon35 (447)								
C. x flexili	total	71	9	25	0.8420	0.0098	0.0222	n/a
	BH	23	8	24	0.8840	0.0122	0.0224	n/a
	MB	37	6	21	0.7490	0.0096	0.0222	n/a
	SH	7	4	20	0.8020	0.0141	0.0233	n/a
	RS	4	2	20	0.4030	0.0196	0.0239	n/a
	2019	6	2	19	0.5710	0.0164	0.0242	n/a
	2020	37	7	22	0.8300	0.0096	0.0219	n/a
	2021	28	7	24	0.7060	0.0117	0.0227	n/a
C. ribicola	total	63	5	4	0.4320	0.0013	0.0018	0.7081
	BH	15	4	3	0.3970	0.0018	0.0018	−0.2162
	MB	24	4	3	0.4140	0.0010	0.0072	1.3423
	SH	9	3	2	0.5000	0.0014	0.0022	1.6871
	RS	15	3	2	0.3890	0.0015	0.0017	1.0740
	2019	14	2	2	0.4090	0.0012	0.0018	1.2029
	2020	30	5	4	0.4540	0.0015	0.0019	0.5430
	2021	19	3	2	0.3710	0.0011	0.0017	1.0719
C. comandrae	total	52	11	6	0.6100	0.0022	0.0028	0.6555
	BH	31	10	6	0.6780	0.0024	0.0031	0.6494
	MB	9	2	2	0.3950	0.0013	0.0018	0.9389
	SH	8	4	4	0.6210	0.0030	0.0032	0.2245
	RS	4	2	2	0.4290	0.0017	0.0019	0.4142
	2019	3	1	0	0.0000	0.0000	0.0000	0.0000
	2020	22	6	5	0.5440	0.0021	0.0024	0.3999
	2021	27	8	6	0.6640	0.0025	0.0032	0.6957
Dcon10+Dcon35 (884)								
C. x flexili	total	71	11	46	0.8190	0.0095	0.0212	n/a
	BH	23	9	44	0.8560	0.0112	0.0214	n/a
	MB	37	9	43	0.7720	0.0099	0.0213	n/a
	SH	7	4	37	0.8020	0.0132	0.0221	n/a
	RS	4	2	39	0.6670	0.0241	0.0294	n/a
	2019	6	2	39	0.5710	0.0170	0.0252	n/a
	2020	37	7	38	0.8300	0.0088	0.0209	n/a
	2021	28	9	43	0.7490	0.0106	0.0214	n/a
C. ribicola	total	63	5	4	0.4270	0.0007	0.0010	n/a
	BH	15	4	3	0.4260	0.0010	0.0008	n/a
	MB	24	4	3	0.4390	0.0006	0.0010	n/a
	SH	9	3	2	0.5420	0.0007	0.0011	n/a
	RS	15	3	2	0.3700	0.0006	0.0010	n/a
	2019	14	3	2	0.4420	0.0006	0.0009	n/a
	2020	30	5	4	0.4610	0.0008	0.0010	n/a
	2021	19	3	2	0.3710	0.0006	0.0009	n/a

(*continued on next page*)

Table 3 (*continued*)

Locus (bp)	Pop	N[a]	H[b]	Seg. sites[c]	Hd[d]	Theta W[e]	π[f]	Tajima's D[g]
C. comandrae	total	52	11	8	0.6440	0.0017	0.0016	n/a
	BH	31	10	7	0.7180	0.0017	0.0018	n/a
	MB	9	3	3	0.4510	0.0010	0.0011	n/a
	SH	8	4	6	0.6160	0.0019	0.0016	n/a
	RS	4	2	2	0.4290	0.0009	0.0010	n/a
	2019	3	1	0	0.0000	0.0000	0.0000	n/a
	2020	22	6	7	0.5840	0.0018	0.0014	n/a
	2021	27	9	7	0.7100	0.0017	0.0018	n/a

[a] - All *Cronartium comandrae, C. ribicola, and C. x flexili* samples were phased into putative haploid genotypes in DnaSP (Rosaz et al., 2003).
[b] - Number of unique haplotypes out of total number of haploid genotypes in each population.
[c] - Number of segregating sites calculated in DnaSP.
[d] - Haplotype diversity calculated in DnaSP.
[e] - Watterson's theta calculated in DnaSP.
[f] - Nucleotide diversity calculated in DnaSP.
[g] - Tajima's D calculated in DnaSP.

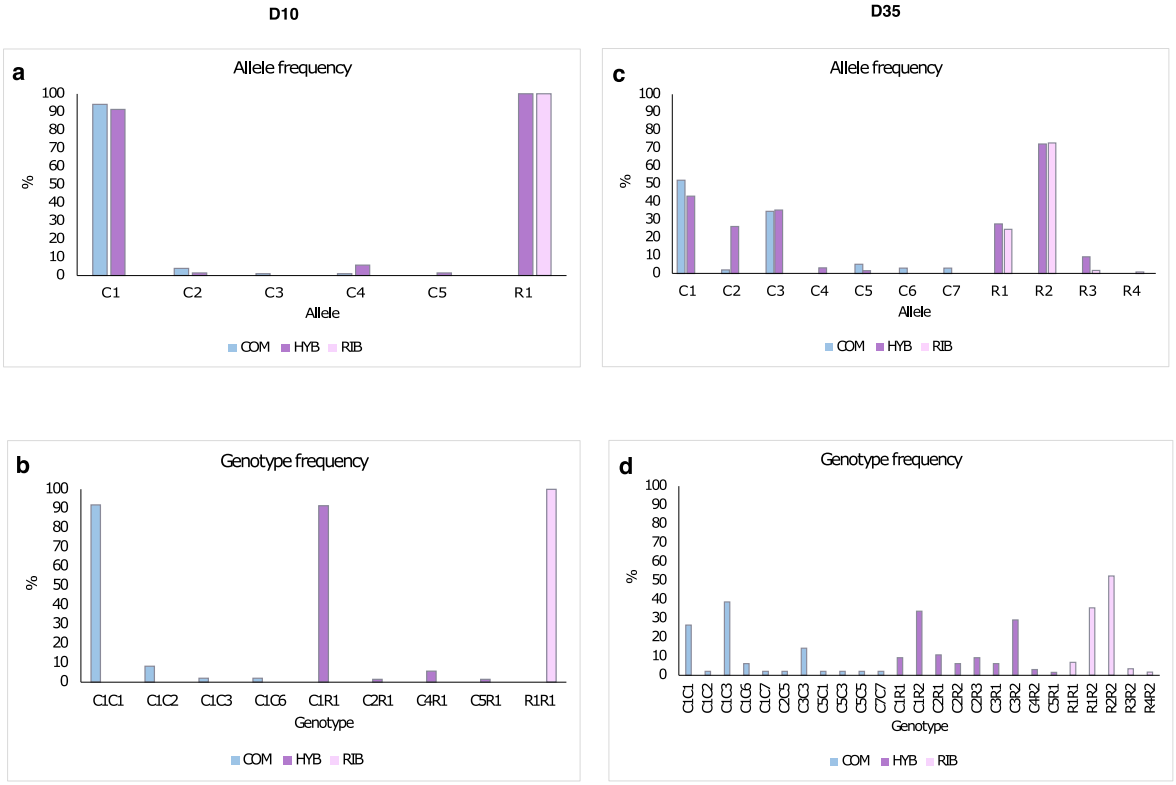

Fig. 4. Allele and genotype frequencies in Dcon10 **(a, b)** and Dcon35 **(c, d)** genomic regions of *Cronartium comandrae* (COM), *Cronartium x flexili* (HYB), and *Cronartium ribicola* (RIB). Details on alleles and genotypes are in Tables S3–S4.

diversity more evenly distributed among seven *C. x flexili* genotypes detected in 2020 and did not reach above 22 % for either of the genotypes (Fig. S1). Less frequent genotypes 35_C2R2 and 35_C3R1 each were found in 8 % of 2020 hybrids, and 35_C2R1 and 35_C2R3 were unique to 2020 season genotypes. The distribution of *C. x flexili* Dcon35 genotypes also varied among forests (Fig. S2). Eight, four, two, and six out of total nine *C. x flexili* Dcon35 genotypes were detected in Bighorn, Shoshone, Roosevelt, and Medicine Bow NFs, respectively. In the Bighorn NF, the frequency of either of eight detected *C. x flexili* Dcon35 genotypes did not exceed 22 %, while in Medicine Bow NF three *C. x flexili* Dcon35 genotypes were sampled more frequently than the other three (frequency 16–46 % for C1R1, C1R2, and C3R2; and 2.7–5.4 % for C2R2, C2R1, and C2R3). Seven *C. x flexili* samples collected in Shoshone NF comprised four *C. x flexili* Dcon35 genotypes ranging in frequencies between 14.3 and 42.9 %, and four *C. x flexili* hybrid samples collected in Roosevelt NF comprised two *C. x flexili* Dcon35 genotypes, 50 % each in frequency (Fig. S2).

3.5. Pathogenicity of cronartium x flexili aeciospores

Of 71 detected *C. x flexili* hybrid aecia, two successfully produced urediniospores on *R. nigrum* leaves (Fig. 6). Pathogenic *C. x flexili* samples SRSA-773-6 and LCRA-781-12 were collected in Medicine Bow NF in 2020 and 2021. The *C. x flexili* urediniospores of these two samples were slightly larger than urediniospores of four *C. ribicola* samples, with *C. x flexili* mean sizes of 26.17–27.27 × 16.15–17.59 μm compared to *C. ribicola* mean sizes 20.86 to 22.99 × 14.48–15.32 μm (Fig. S3, Table S5). Urediniospores of *C. comandrae* were not available for this assay, as its alternate host *Comandra umbellata* is difficult to grow outside of its natural environment (Sorensen and Holden, 1974). The consequent sequencing of Dcon10 and Dcon35 genomic regions in the uredinial samples confirmed the presence of the same genotypes as in

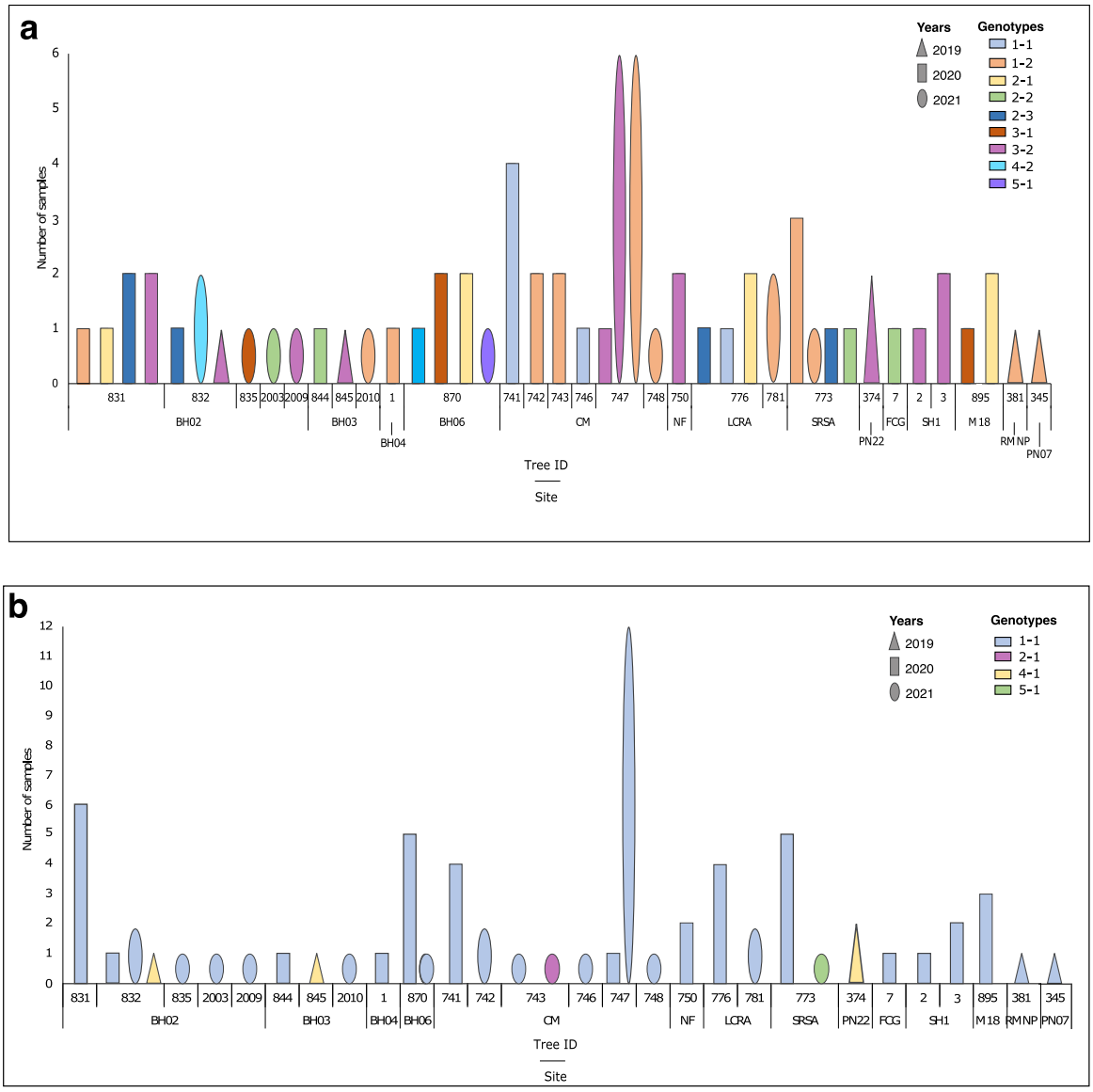

Fig. 5. Distribution of Dcon35 **(a)** and Dcon10 **(b)** genotypes of *Cronartium x flexili* among y, sites, and *Pinus flexilis* trees they were collected from. Each color corresponds to a unique genotype, bar shape corresponds to a y of collection, the height of each bar corresponds to the number of *C. x flexili* samples of a particular genotype from each tree. Details on samples and genotypes are in Table S3.

Fig. 6. *Cronartium x flexili* urediniospores on *Ribes nigrum* leaf that was infected with *Cronaritum x flexili* aeciospores collected from *Pinus flexilis*.

aecial samples of SRSA-773-6 (10_C1R1 and 35_C2R2) and LCRA-781-12 (10_C1R1 and 35_C1R2).

4. Discussion

Interspecific hybridization is considered an important driver of genome evolution within eukaryotes. The genomes of newly formed interspecific hybrids are unstable, which gives them advantage to readily adapt to changing environments or invade new niches (Steensels et al., 2021). This is particularly important for pathogens, as interspecific hybridization provides opportunities to acquire adaptive traits that increase their fitness and aggressiveness towards hosts. In this study we investigated temporal and geographical distribution of *Cronartium x flexili*, a recently identified hybrid of two fungal tree pathogens, native to North America *C. comandrae* and invasive *C. ribicola*. Here, we show that *C. x flexili*, which was previously found in Canada, is also present and occupies a broad range of forest ecosystems in the Central and Southern Rocky Mountains of western USA. *Cronartium x flexili* aeciospores were detected 3 consecutive y, suggesting that hybridization

between *C. comandrae* and *C. ribicola* is persistent within Central and Southern Rocky Mountain forests. Moreover, *C. x flexili* hybrids were found exclusively on *P. flexilis*, a primary host of *C. ribicola*, suggesting likely unidirectional gene flow from the residential species to its invasive relative. Based on the sequence data of two genomic regions, we conclude that each *C. x flexili* aecium is a product of independent hybridization events, as the *C. x flexili* population was more genetically diverse than populations of either of the parental species. Moreover, two *C. x flexili* samples were confirmed to be infectious on alternate host of *C. ribicola*, demonstrating that some hybrids carry over their pathogenic potential.

Morphologically, hybrids are often characterized by intermediate phenotypes of the parental species (Greig et al., 2002). For example, *Melampsora x columbiana*, a natural hybrid of two rust species *Melampsora medusae* and *Melampsora occidentalis*, harbors intermediate morphology of urediniospores and teliospores (Newcombe et al., 2000). Based on the aeciospore measurements conducted in this study, the length of aeciospores of *C. ribicola* and *C. comandrae* did not overlap and concur with ranges previously reported [20–34 × 13–25 μm for *C. ribicola* (Imazu and Kakishima, 1995) and 32–66 × 19–24 μm for *C. comandrae* (Mordue and Gibson, 1978)]. The aeciospore length for *C. x flexili* was in between that of the parental species, confirming intermediate phenotypes. The aeciospore sizes of *C. x flexili* hybrids identified in this study were similar to those reported by Joly et al. (2006) from Alberta, Canada (25–40 × 16–20 μm). Consistent with Joly et al. (2006), molecular analyses, including restriction digestion of internal transcribed spacer and sequences of genomic regions Dcon10 and Dcon35, further confirmed the hybrid origin of the aeciospores with intermediate morphology.

All samples with intermediate morphology carried alleles from each of the parental species in all three genomic regions analyzed. Based on sequence results, the Dcon35 region was more diverse than the Dcon10 region in all species analyzed, and the native *C. comandrae* population was more diverse than the population of invasive *C. ribicola*. The Dcon35 genomic region is homologous to the eukaryotic gene coding for ribosomal 60 S subunit protein L13A involved in translation (Planta and Mager, 1998), while Dcon10 genomic region is homologous to the gene coding for glutamine synthetase – a protein involved in the metabolism of nitrogen and secondary metabolism in fungi (Wang et al., 2022; Zhu et al., 2021). Experimental studies showed that protein L13A was not essential for viability in *Saccharomyces cerevisiae* (López et al., 1998), while disruption of glutamine synthetase gene expression led to complete glutamine deficiency and inability to grow in *Aspergillus flavus* (Wang et al., 2022). Differences in function likely affect gene diversities, as each gene is likely influenced by different selection processes (e.g., Dcon35 gene may be under balancing selection, whereas Dcon10 gene may be under directional selection).

The amount of genetic variation in hybrids can shed light on the frequency of hybridization events occurring in ecosystems. For example, the grass pathogen hybrid *Zymoseptoria pseudotritici* was characterized by substantial loss of genetic variation compared to its parental populations (Stukenbrock et al., 2012). These results suggest that *Z. pseudotritici* was formed by a single or a few crosses, and its population resembled a founder population characterized by low genetic diversity and strong effects of genetic drift. In the current study, the population of *C. x flexili* hybrids harbored higher genetic diversity compared to either of the parental populations. Also, multiple *C. x flexili* genotypes were detected in both sequenced genomic regions, with different combinations of parental alleles. Several genotypes were more common in both genomic regions than others, suggesting that chances of the interspecific hybridization between *C. ribicola* and *C. comandrae* are influenced, at least to some extent, by the genetic backgrounds of mating strains, as observed earlier in other interspecific fungal hybrid systems (Dettman et al., 2007; Turner et al., 2011). The distribution of alleles and genotypes had no association with either a forest or a y the samples were collected in, suggesting multiple independent hybridization events are likely taking place. Moreover, both common and rare parental alleles were detected in *C. x flexili* genotypes, further demonstrating that independent hybridizations are likely taking place. In this study, several trees carried *C. x flexili* aecia of different genotypes within and among sampling y. Earlier population genetics studies showed evidence that each white pine blister rust canker was a result of an infection by a distinct genotype of *C. ribicola*, sometimes with different *C. ribicola* genets present within the same canker (Hamelin, 1996; Hamelin et al., 1995, 2000). It is possible that the availability of several *C. ribicola* genotypes within a canker/tree provides more opportunities for interspecific hybridization, as more genetic combinations are possible in a given space, and that these events will be represented by progenies with distinct genetic signatures.

The first study reporting the presence of hybrids between *C. ribicola* and *C. comandrae* on *P. flexilis* in forest ecosystems of North America (Joly et al., 2006) indicated that *C. x flexili* frequencies ranged between 3 and 29 % of total sampled aecia in southwestern Alberta, Canada. According to Allen (2019) one more hybrid sample was found in British Columbia in 2012. However, the authors did not find any *C. x flexili* hybrid aecia in either of the regions in a subsequent study (Allen, 2019). In our study, *C. x flexili* hybrids were found in all three sampled seasons (2019–2021), and in all surveyed forests. Overall, *C. x flexili* aecial samples were present at low frequencies in all three y (2.2 %, 5.2 %, and 5.3 % in 2019, 2020, and 2021, respectively), and their frequency also varied by forest, being most frequently (6.6 %) and the least frequently (0.9 %) detected in Bighorn and Roosevelt NFs, respectively. These results suggest that, even though at low frequency, *C. x flexili* hybrids occur consistently in Colorado and Wyoming forest ecosystems, and that the hybridization process is likely affected by local environmental conditions that influence fungal life cycles, presence and abundance of alternate host species, as well as genetic backgrounds of mating strains.

Most progenies of interspecific hybridizations are thought to be transient, as many of them are infertile and/or have decreased fitness compared to their parents. However, even rare events of interspecific hybridization with fertile/fit progeny and/or episodic backcrossing of transient hybrids with parental species may be enough for the establishment of novel lineages and/or species (Steensels et al., 2021). This is especially important for the evolution of pathogens, including fungi. For example, a natural hybrid of two fungal pathogen species of elm, residential *O. ulmi* and invasive *O. novo-ulmi*, was reported to have the same fitness characteristics as its parental strains and quickly spread across forest ecosystems around the globe, replacing residential parental species *O. ulmi* (Brasier, 1986, 2000b; Brasier and Kirk, 2010). Rust fungi, including genus *Cronartium*, have complex life cycles consisting of the production of five types of spores and requiring infection of two plant host species (Hamelin et al., 1995). In this study, *C. x flexili* aeciospores from two individual aecia were able to infect *C. ribicola* alternate host, *R. nigrum,* and produce urediniospores in a controlled environment. Sequencing of the same two genomic regions showed that urediniospores carried the same hybrid genetic profiles as aeciospores that were used for initial inoculations. It remains to be determined whether these *C. x flexili* hybrids can complete their sexual cycle and infect the primary host *P. flexilis*, and whether they can infect hosts of *C. comandrae*.

Presence of alleles from both parents in two genomic regions of all *C. x flexili* samples identified in this study suggest that these hybrids are likely the F1 progeny of the interspecific hybridizations between *C. ribicola* and *C. comandrae*, and it remains to be determined whether they can produce F2 progeny and/or are capable of backcrossing with parental species. Studies with other organisms, such as plants, animals, and oomycetes, showed that introgressive hybridization (e.g., backcrossing with parental species) can serve as a mechanism that speeds up adaptive evolution of organisms by transferring adaptive traits between species (Arnold, 2004), and therefore can play an important epidemiological role in forest ecosystems. For example, a natural hybrid of host specific poplar rusts *M. medusae* (host *Populus deltoides*) and

M. occidentalis (host *P. trichocarpa*), *M. x columbiana*, has an extended host range that includes host species of both parents (Newcombe et al., 2000). Given the severity of impact that *C. ribicola* has had on the forest ecosystems in North America after its accidental introduction, this potential host range expansion of the hybrid could further threaten forest health. Simulation and experimental studies demonstrate that introgression between local and invasive species is asymmetrical, with genes from local species being transferred to its invasive relative (Currat et al., 2008; Et-Touil et al., 1999). Due to limited genetic data analyzed in this study, we do not currently have evidence of introgression in *C. x flexili* hybrids, but the fact that *C. x flexili* hybrids were found exclusively on *P. flexilis*, the host of invasive *C. ribicola*, concurs with the above-mentioned theory. Whole genome analyses of *C. x flexili* hybrids and parental populations are necessary to determine whether hybridization stops on the F1 progeny or serves as a bridge for interspecific genetic exchange.

Author contributions

JES, KSB, and OK designed the study, JES, KSB, and AWS received funds, OK, JES and KSB collected samples, OK conducted experiments and wrote the manuscript, JES, KSB, and AWS provided edits.

Declaration of competing interest

All authors have edited the manuscript and agreed to publishing the research findings in *Fungal Biology*. This research and it's findings have not been previously published and there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2023.11.005.

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